Identifying signal transduction components acting downstream of reactive oxygen species (ROS) in Arabidopsis thaliana

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Abstract

Traditionally, reactive oxygen species (ROS) have been regarded as toxic by-products of aerobic metabolism. However, in recent years it has become apparent that plants actively produce ROS as signalling molecules. ROS are able to mediate adaptive responses to various environmental stresses as well as processes such as stomatal closure and development. Downstream signalling events that are modulated by ROS include calcium mobilisation, protein phosphorylation and gene expression.

This study investigated signalling proteins acting downstream of ROS, in order to understand how ROS are perceived and transduced to elicit such a wide range of responses. To establish a molecular profile provoked by ROS, a microarray experiment of Arabidopsis plants exposed to exogenous H_2O_2 was analysed. Of the 895 differentially expressed transcripts, a substantial proportion had predicted functions in cell rescue and defence, including heat shock, disease resistance and antioxidant genes. Genes encoding candidate H_2O_2 signalling components were identified from this microarray experiment and their H_2O_2-induced expression was verified by northern RNA-blot analysis. Two transcription factors of the ethylene response factor (ERF) family (AtERF5 [At5g47230] and AtERF6 [At4g17490]) and an ankyrin protein kinase (APK [At4g18950]) were selected for further study.

Northern blot analysis and comparison with publicly available transcriptome data sets demonstrated that the expression of these three genes was induced by various stress treatments, such as UV-B irradiation, cold and elicitor challenge. To unravel the potential *in vivo* function of these proteins, loss- and gain-of-function lines were generated and analysed. No abnormal plant phenotypes were observed during development or in response to the stress and hormone treatments tested. A high level of functional redundancy may exist between AtERF5 and AtERF6. Microarray analyses were performed on the over-expression lines. Genes that were differentially regulated in APK over-expressor lines gave no indication of its function. However, the microarray analyses revealed that AtERF5 and AtERF6 have roles in the plant pathogen defence response, since their over-expression induced defence gene expression. Analysis of *cis* elements in the promoters of the ERF-differentially regulated genes revealed that both transcription factors displayed GCC box binding activity. However, the GCC box was not over-represented in the promoters of H_2O_2-differentially regulated genes, which suggests that this element has a ROS independent regulation.

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Abbreviations

Standard scientific abbreviations are used for units of length (mm, cm), weight (ng, µg, mg), volume (µl, ml), amount (µmol), molarity (µM, mM, M), temperature (°C), pressure (Pa) and time (s, min, h).

Standard chemical element symbols, amino acid codes and nucleic acid abbreviations are used.

The standard convention for gene and protein naming is adhered to: gene names are italicised, protein names are not. Wild-type gene and protein names are both capitalised. Mutations are denoted by lower-case type.

Chapter 1

The role of reactive oxygen species in plant signal transduction

1.1 Introduction

Throughout their life cycle, plants are continually bombarded with endogenous developmental cues and external stimuli. For example, sudden and often dramatic changes in the environmental conditions can adversely affect plant development, growth and/or productivity. Imperative for survival is the plant's ability to respond to such stimuli through physiological alterations. Since the sessile nature of plants prevents them from simply being able to move away from adverse conditions, their capacity to react successfully to numerous stimuli reflects the presence of a complex system for signal recognition and transduction. Thus, the stimulus must first be perceived by the plant before an appropriate response can be mounted. One early event in this perception can be a rapid and transient increase in the concentration of second messengers: molecules involved in conveying information from an extracellular source to within the cell. For example, calcium (Ca^{2+}) is a ubiquitous eukaryotic second messenger and transduces many signals to initiate diverse responses. More recently it has emerged that increases in the generation of partially reduced forms of molecular oxygen, termed reactive oxygen species (ROS), can also be used by eukaryotes during signal transduction. In plants, such ROS accumulations have been linked to multiple downstream responses including environmental stress tolerance, cell function and growth.

This thesis seeks to identify novel plant protein signalling components acting downstream of ROS. Using Arabidopsis as the primary example (since this was the species used experimentally within this work), this chapter introduces relevant topics as follows: Firstly, the various types of ROS and the cellular damage that they can inflict are described. After a brief review on the mechanisms of ROS production, the chapter then focuses on the various roles that ROS have been demonstrated to play in plant signal transduction cascades. The roles of ROS in response to abiotic stress and pathogen invasion, as well as during normal cell function and growth are reviewed. The interaction of ROS with other signalling molecules and hormones is then considered. Finally, known and putative ROS signal transduction components are presented.

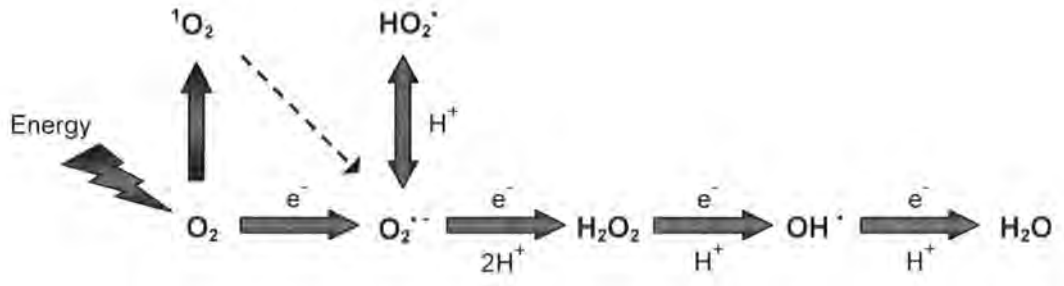
1.2 Living with oxygen

The evolution of oxygenic photosynthesis by early cyanobacteria in the proterozoic era, led to the accumulation of tonnes of the by-product, dioxygen (O_2) in the atmosphere (Falkowski *et al.*, 2006). Primitive organisms subsequently evolved to incorporate cytochrome oxidase in their electron transport complexes, enabling O_2 to act as a terminal electron acceptor during its reduction to water (H_2O; [Babcock, 1999]). This switch to aerobic metabolism increased the yield of ATP that could be produced from glucose, by over 15-fold compared to anaerobic glycolysis, thus providing the energy needed for the development of complex multicellular organisms.

However, a by-product of this beneficial utilisation of oxygen was the generation of oxygen radicals, which possess one or more unpaired electrons (denoted by a superscript $^{\bullet}$). These radicals and related (non-radical) derivatives of O_2 are collectively termed reactive oxygen species (ROS). The most stable oxygen state is the form that exists in the air around us (dioxygen) and is termed the ground state. Ground state O_2 may be converted to ROS either by energy transfer or through successive steps of one-electron reduction, as shown below in Figure 1.1.

Figure 1.1

Inter-conversion of ROS derived from O_2. Adapted from Vranova *et al.* (2002).



An input of energy rearranges the electrons of ground state molecular oxygen (O_2) to form singlet oxygen ($^1O_2$). Alternatively, one electron reduction of O_2 leads to the formation of superoxide radical ($O_2^{\bullet -}$), which exists in equilibrium with its conjugate acid, hydroperoxyl radical ($HO_2^{\bullet}$). Subsequent reduction steps then form hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and lastly water (H_2O).

1.2.1 Singlet oxygen

The acceptance of excess energy by ground state O_2 can reverse the spin of one of its unpaired electrons (O_2 has two unpaired electrons in parallel spins) and results in the formation of singlet oxygen ($^1O_2$; [Foote *et al.*, 1985]). $^1O_2$ generation is particularly associated with the illuminated chloroplast, since insufficient energy dissipation during photosynthesis can lead to the formation of a chlorophyll triplet state which can subsequently transfer its excitation energy onto ground state O_2 (Holt *et al.*, 2005). $^1O_2$ is able to directly oxidise proteins, DNA and lipids and react with them to form endoperoxides and hydroperoxides (Foote *et al.*, 1985; Halliwell and Gutteridge, 2007). However, carotenoid antioxidants exist which can quench both $^1O_2$ and triplet state chlorophyll (Holt *et al.*, 2005).

1.2.2 Superoxide radical

The first one-electron reduction step of O_2 gives rise to superoxide radical ($O_2^{\bullet -}$) which posesses one unpaired electron (Fridovich, 1997). $O_2^{\bullet -}$ exists in equilibrium with its conjugate acid, the hydroperoxyl radical ($HO_2^{\bullet}$) that forms via protonation of $O_2^{\bullet -}$ (Figure 1.1). $O_2^{\bullet -}$ is able to oxidise amino acids and NADPH, as well as being able to reduce cytochrome *c* and quinones (e.g. ubiquinones and plastoquinones; [Halliwell and Gutteridge, 2007]). In addition, transition metal ions that are mainly present in cells in the oxidised form (e.g. Fe^{3+} and Cu^{2+}) are reduced in the presence of $O_2^{\bullet -}$. Consequently $O_2^{\bullet -}$ may affect the activity of metal-containing enzymes. Moreover, it can facilitate the conversion of hydrogen peroxide (H_2O_2) to the highly reactive hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction, whereby $O_2^{\bullet -}$ is able to act as a reducing agent to maintain the metal ion catalyst in a reduced state, and thus sustain ongoing Fenton reactions:

- Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$
- Reduction of oxidised metal ion catalyst: $O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

$O_2^{\bullet -}$ is also able to react fast with the nitric oxide radical ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$), which rapidly protonates to peroxynitrous acid (ONOOH; [Beckman and Koppenol, 1996]). ONOOH is a powerful oxidising and nitrating agent and can directly damage proteins, lipids and DNA as well as undergoing homolytic fission to give the noxious products $OH^{\bullet}$ and $NO^{\bullet}$:

- Formation of peroxynitrite: $NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$
- Homolytic fission of peroxynitrous acid: $ONOOH \rightarrow NO^{\bullet} + OH^{\bullet}$

However, with a half-life of 2 to 4 μs, $O_2^{\bullet -}$ is a relatively short-lived molecule and is readily dismutated to H_2O_2.

1.2.3 Hydrogen peroxide

Further reduction of $O_2^{\bullet -}$ generates H_2O_2, a relatively long lived (half-life of 1 ms) non-radical molecule. H_2O_2 results from the dismutation of $O_2^{\bullet -}$ either spontaneously, or via superoxide dismutase (SOD) enzymes. One of the most common plant SODs is CuZnSOD, which catalyses the dismutation of $O_2^{\bullet -}$ via the alternate oxidation and reduction of copper ions (Halliwell and Gutteridge, 2007):

- Net dismutation reaction: $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$
  - Half-reaction 1: $SOD\text{-}Cu^{2+} + O_2^{\bullet -} \rightarrow SOD\text{-}Cu^{+} + O_2$
  - Half-reaction 2: $SOD\text{-}Cu^{+} + O_2^{\bullet -} + 2H^+ \rightarrow SOD\text{-}Cu^{2+} + H_2O_2$

Compared with other ROS, H_2O_2 has relatively low reactivity, although it is able to oxidise the thiol groups of proteins (Halliwell and Gutteridge, 2007). It can however diffuse some distance from its production site and permeate membranes (typically through aquaporins; [Bienert *et al.*, 2007]).

1.2.4 Hydroxyl radical

The last species to be reduced in the four-step reduction pathway of Figure 1.1, and the most reactive of all ROS, is the hydroxyl radical ($OH^{\bullet}$). It is formed from H_2O_2 by one electron donation from a reduced metal ion in the Fenton reaction (as previously described in Section 1.2.2). $OH^{\bullet}$ has a half life of <1 μs and will react within diffusion distance with any biological molecule by initiating radical chain reactions (see next Section 1.3; [Halliwell, 2006]). Severe damage can result from relatively low $OH^{\bullet}$ concentrations due to the self-propagating nature of the reaction chain. Thus organisms carefully control Fenton chemistry by limiting the availability of both Fe^{2+} and H_2O_2 (e.g. through metal ion binding proteins and H_2O_2-scavenging antioxidants; [Halliwell and Gutteridge, 2007]).

1.3 Oxidative damage

As already mentioned, ROS are capable of unrestricted oxidation of various key biological molecules such as nucleic acids, amino acids and proteins, leading to cellular dysfunction and can ultimately cause cellular death. As shown below in Table 1.1, ROS can initiate lipid peroxidation chains (primarily of cell membrane phospholipids) that give rise to chemically reactive cleavage products and further exacerbate cellular damage (Halliwell and Gutteridge, 2007). Effects of lipid peroxidation include the loss of membrane integrity via decreased membrane fluidity, increased membrane leakiness and damage to membrane proteins.

Table 1.1
Lipid peroxidation reactions.

a) Initial abstraction reaction:	$\rangle CH + OH^{\cdot} \rightarrow \rangle C^{\cdot} + H_2O$
b) Peroxyl radical formation:	$\rangle C^{\cdot} + O_2 \rightarrow \rangle C-OO^{\cdot}$
c) Propagation reaction:	$\rangle CH + \rangle C-OO^{\cdot} \rightarrow \rangle C^{\cdot} + \rangle C-OOH$

a) Lipid peroxidation begins by abstraction of a hydrogen atom from a –CH– bond (e.g. from a polyunsaturated fatty acid residue in a membrane) leaving behind an unpaired electron on the carbon ($C^{\cdot}$). **b)** The carbon radical is then able to react fast with O_2 to yield a peroxyl radical ($COO^{\cdot}$). **c)** $COO^{\cdot}$ is in turn capable of abstracting hydrogen from another lipid molecule yielding a lipid hydroperoxide (COOH) and forming a new carbon radical, propagating a lipid peroxidation chain reaction (Halliwell, 2006).

Moderate ROS intracellular levels may halt the cell cycle at specific checkpoints or drive cells into senescence resulting in reduced growth (Paulovich *et al.*, 1997). For example, treatment of tobacco suspension cell cultures with the $O_2^{\cdot -}$-generator menadione, impaired the GI-to-S cell cycle transition, delayed entry into mitosis and slowed down DNA replication (Reichheld *et al.*, 1999). Cells may adapt to such ROS levels by up-regulation of defence and/or repair systems, such as increases in chaperone and/or antioxidant activities (see Section 1.6).

1.4 The dualism of ROS

The term "oxidative stress" has been widely used in the literature to describe situations which trigger enhanced ROS production. However, this term negatively implies a harmful process, when actually, in many cases, the situation is quite the opposite. Although oxidation of biological molecules might contribute directly to a lowering of overall plant vigour, it is becoming increasingly evident that increased oxidation is an important component of the repertoire of signals that the plant uses to make appropriate physiological adjustments. Over the last 20 years, a large body of evidence has demonstrated unequivocally that plant cells produce ROS, particularly $O_2^{\bullet -}$ and H_2O_2, for a beneficial purpose: as second messengers to modulate cellular activities. Such ROS generation is induced during the course of development and by environmental fluctuations, and used to control processes as diverse as defence gene expression, programmed cell death (PCD), stomatal closure and root growth (discussed in more detail in Section 1.7).

1.5 Production of ROS in plant cells

ROS arise in plant cells via a number of routes, and most cellular compartments have the potential to become a source of ROS (see Table 1.2 below).

Table 1.2

Examples of ROS production mechanisms in plants.

Production mechanism	Localisation	Main ROS product	Reference
Metabolic:			
Photosynthetic electron transport	Chloroplast	$O_2^{\bullet -}$, $^1O_2$	Asada (1999)
Photorespiration (glycolate oxidase)	Peroxisome	H_2O_2	Corpas *et al.* (2001)
Respiratory electron transport	Mitochondria	$O_2^{\bullet -}$	Moller (2001)
Fatty acid β-oxidation (lipid catabolism)	Peroxisome	H_2O_2	Corpas *et al.* (2001)
Enzymatic:			
NADPH oxidase	Plasma membrane	$O_2^{\bullet -}$	Torres *et al.* (1998)
Peroxidase	Cell wall	H_2O_2	Bolwell *et al.* (2002)
Amine oxidase	Apoplast	H_2O_2	Bolwell and Wojtaszek (1997)
Oxalate oxidase	Apoplast	H_2O_2	Woo *et al.* (2000)
Xanthine oxidase	Peroxisome	$O_2^{\bullet -}$	Del Rio *et al.* (2002)

1.5.1 Metabolic sources of ROS

In plants ROS are continually produced as by-products of normal metabolic processes. This predominantly occurs in organelles with highly oxidising metabolic activities or with intense rates of electron flow (e.g. chloroplasts, mitochondria and peroxisomes; [Asada, 1999; Mittler *et al.*, 2004; Moller, 2001]). Under normal growth conditions these ROS are scavenged by various antioxidative components (discussed later in Section 1.6). However, the equilibrium between production and scavenging of ROS may be perturbed by the imposition of

environmental stresses that disrupt the cellular homeostasis (Malan *et al.*, 1990; Prasad, *et al.*, 1994; Tsugane *et al.*, 1999; Polle, 2001). As a result of these disturbances, intracellular levels of ROS may rapidly rise.

The light-dependent reactions associated with photosynthesis are major sources of ROS within plant cells (Karpinski *et al.*, 1999; Foyer and Noctor, 2003). Uncoupling or inhibition of the photosystem machinery as well as photorespiration associated with chloroplast and peroxisome function (e.g. Rubisco and glycolate oxidase respectively) can lead to high levels of ROS formation. The final electron acceptor during photosynthesis is usually CO_2. However, abiotic stress conditions which limit CO_2 fixation will inevitably lead to more O_2 molecules being used as electron acceptors (Aro, *et al.*, 1993). Direct photoreduction of O_2 by reduced electron transport components associated with photosystem I (PSI) leads to $O_2^{\bullet -}$ accumulation in the chloroplasts (the Mehler reaction; [Mehler, 1951; Asada, 1999; Mittler *et al.*, 2004]). As previously mentioned, $^1O_2$ is also produced in the chloroplasts as a by-product of over-excitation of chlorophylls in the reaction centre of PSII or in the antenna system (Asada, 1999; Mittler *et al.*, 2004).

Additionally, the mitochondrial electron-transport chain may become over-reduced under stress conditions (especially at the level of Complex I and Complex III; [Sweetlove *et al.*, 2002; Moller and Kristensen, 2004; Kristensen *et al.*, 2004]). This favours the generation of $O_2^{\bullet -}$, mainly via NADH dehydrogenase and the ubiquinone radical (Purvis, 2001).

1.5.2 Programmed production of ROS

Of particular relevance to signalling is the active and tightly-regulated ROS production via various oxidase and peroxidase enzymes (summarised previously in Table 1.2). NADPH oxidases, also known as respiratory burst oxidases (RBO), were originally described in mammalian neutrophils and generate the respiratory burst (Lambeth, 2004). These enzymes are able to generate $O_2^{\bullet -}$ by electron transfer from NADPH to O_2:

- NADPH oxidase: $2O_2 + NADPH \rightarrow NADP^+ + 2O_2^{\bullet -} + H^+$

Many studies have documented different *respiratory burst oxidase homologs* (*Rboh*) genes in various plant species (Groom *et al.*, 1996, Torres *et al.*, 1998; Keller *et al.*, 1998; Amicucci *et*

al., 1999; Yoshioka *et al*, 2001; Simon-Plas *et al.*, 2002; Yoshioka *et al.*, 2003). For example Arabidopsis encodes 10 *Atrboh* genes which share homology to the mammalian NADPH oxidase enzymatic subunit ($gp91^{phox}$; [Torres *et al.*, 1998; Dangl and Jones, 2001]).

Analyses of Arabidopsis *Rboh* mutants have implicated involvement of NADPH oxidases in various processes. For example AtrbohC appears to have a specific function in root hair development, whereas AtrbohD and AtrbohF are involved in pathogen responses and stomatal ABA signalling (Torres *et al.*, 2002; Foreman *et al.*, 2003; Kwak *et al.*, 2003). These will be discussed in more detail in Section 1.7.

Although much attention has been given to NADPH oxidases, another important enzymatic means of ROS production are the pH-dependent cell wall peroxidases. These enzymes can trigger rapid production of H_2O_2 following extracellular alkalinisation resulting from pathogen/elicitor recognition (Chittoor *et al.*, 1997; Sasaki *et al.*, 2004). For example, Arabidopsis plants transformed with an antisense French bean peroxidase were highly susceptible to bacterial and fungal pathogens (Bindschedler *et al.*, 2006).

- Peroxidase: $Fe^{2+}\text{-}O\text{-} + RH_2 \rightarrow Fe^{3+} + R + H_2O_2$

Other enzymes capable of ROS production include apoplastic amine oxidases (which generate H_2O_2 by oxidising various amines to their corresponding aldehydes), xanthine oxidases (which oxidise xanthine to uric acid and generate $O_2^{\bullet -}$) and oxalate oxidases (that catalyse the conversion of oxalate to CO_2 and H_2O_2; [Bolwell and Wojtaszek, 1997; Woo *et al.*, 2000; Del Rio *et al.*, 2002, Mitter *et al.*, 2004]).

1.6 Scavenging ROS: the antioxidant system of plants

Since ROS are constantly produced during normal cell metabolism it is important that their basal levels are tightly controlled. Furthermore, plant cells need to be able to scavenge cytotoxic levels of ROS during stress, but also finely modulate lower levels of ROS for signalling purposes. This is achieved by a complex battery of antioxidants, both enzymatic and non-enzymatic (summarised below in Table 1.3).

Table 1.3
Examples of antioxidants in plants.

Scavenging mechanism	Localisation	Main ROS scavenged	Reference
Enzymatic:			
Superoxide dismutase (SOD)	Chloroplast, cytosol, mitochondria, peroxisome, apoplast	$O_2^{\bullet -}$	Bowler *et al.* (1992)
Catalase (CAT)	Peroxisome	H_2O_2	Willekens *et al.* (1997)
Glutathione peroxidase (GPX)	Cytosol	H_2O_2	Rodriguez Milla *et al.* (2003)
Ascorbate peroxidase (APX)	Chloroplast, cytosol, mitochondria, peroxisome, apoplast	H_2O_2	Asada (1999)
Non-enzymatic:			
Ascorbate	Chloroplast, cytosol, mitochondria, peroxisome, apoplast	H_2O_2, $O_2^{\bullet -}$	Noctor and Foyer (1998), Asada (1999)
Glutathione	Chloroplast, cytosol, mitochondria, peroxisome, apoplast	H_2O_2	Noctor and Foyer (1998), Asada (1999)
α-Tocopherol	Thylakoid membranes	$^1O_2$	Noctor and Foyer (1998)
Carotenoids	Chloroplast	$^1O_2$	Holt (2005)

Antioxidants are able to quench ROS without themselves undergoing conversion to destructive radicals, thus enabling ROS to perform useful biological functions without too much cellular damage (Halliwell and Gutteridge, 2007). The distinct subcellular localisation and biochemical properties of the various antioxidants, allows ROS accumulation to be controlled both temporally and spatially.

1.6.1 Antioxidant enzymes

The superoxide dismutase enzymes (SODs) act as the first line of defence against ROS by catalysing dismutation of $O_2^{\bullet -}$ to H_2O_2. SODs must therefore work together with enzymes that subsequently detoxify H_2O_2, such as catalase (CAT), ascorbate peroxidase (APX) or glutathione peroxidase (GPX). Both APX and GPX require a reducing substrate for activity (ascorbate or glutathione respectively):

- Catalase (CAT): $2H_2O_2 \rightarrow 2H_2O + O_2$
- Peroxidases (APX, GPX): $SH_2 + H_2O_2 \rightarrow S + 2H_2O$

Multiple genes encode APX and SOD with different isoforms specifically targeted to various cellular compartments (Table 1.3). In contrast, CAT is located mainly in peroxisomes, whilst GPX is cytosolic. Compared to APX, CAT has a higher reaction rate but a lower affinity for H_2O_2. Therefore CAT might be responsible for removal of excess H_2O_2 during stress whilst APX may play a role in the modulation of small amounts of H_2O_2 for signalling (Willekens *et al.*, 1997).

The ability of antioxidant enzymes to compensate for one another demonstrates the flexibility of this scavenging system. For example, antisense APX tobacco plants induced expression of genes encoding SOD, CAT and GR, whilst antisense plants deficient in CAT induced APX, GPX and mitochondrial AOX gene expression (Rizhsky *et al.*, 2002).

1.6.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants include ascorbate and glutathione which provide a store for reducing power and thus serve as major cellular redox (Noctor and Foyer, 1998). An array of enzymes is needed to maintain cellular pools of their reduced forms as demonstrated in the ascorbate-glutathione cycle (shown below in Figure 1.2; [Asada, 1999]). Levels of ascorbate and glutathione have been demonstrated to increase in response to numerous stresses (e.g. chilling, heat shock, pathogen attack and drought) and mutants with decreased ascorbate levels or altered glutathione content are hypersensitive to stress Conklin *et al.*, 1996; Grant and Loake, 2000; Vanacker *et al.*, 2000; Noctor *et al.*, 2002).

Figure 1.2

The ascorbate-glutathione cycle in chloroplasts. Adapted from Noctor and Foyer (1998).

H_2O_2 is reduced to water via APX-catalysed oxidation of ascorbate to dehydroascorbate (DHA). DHA is reduced back to ascorbate by the action of DHA reductase (DHAR), using glutathione (GSH) as the reducing substrate. The oxidised dimer form of glutathione (GSSG) is in turn is reduced back to GSH by use of NADPH in a reaction catalysed by GSH reductase (GR).

Another family of non-enzymatic antioxidants are the carotenoids (e.g. carotenes and xanthophylls) which can rapidly scavenge $^1O_2$ as well as absorb energy from the triplet state of chlorophyll (Holt *et al.*, 2005). Arabidopsis plants with enhanced levels of xanthophyll exhibited increased tolerance towards high-light-induced oxidative stress (Davison *et al.*, 2002). $^1O_2$ and peroxyl radicals can also be scavenged by α-tocopherols which help to protect thylakoid membranes against lipid peroxidation (Noctor and Foyer, 1998; Halliwell and Gutteridge, 2007). Since there are no known scavengers of $OH^{\cdot}$, the only way to avoid oxidative damage through this radical would be to control the reactions that lead to its generation. Thus, cells possess proteins that bind metal ions (such as transferrin, ferritins and metallothioneins) to protect against Fenton chemistry (Halliwell and Gutteridge, 1990).

ROS production can also be avoided by the alternative channelling of electrons in the electron-transport chains. Alternative oxidase (AOX) enzymes compete for electrons with the cytochrome complex, and use them to reduce O_2 to water via ubiquinone. Thus they help to decrease ROS production in the mitochondria by two mechanisms: they prevent electrons from reducing O_2 to $O_2^{\bullet -}$, and they reduce the overall level of O_2 (the substrate for ROS production). For example, antisense plant cells with reduced levels of AOX accumulated 5 times more ROS than control cells and increased the sensitivity of cells to oxidative damage (Maxwell *et al.*, 1999).

1.6.3 Antioxidants and stress

Plant antioxidant activity generally increases in response to abiotic stress (Jiang and Zhang, 2002; Vaidyanathan *et al.*, 2003). For example, several studies have shown that under salt stress, salt-tolerant cultivars exhibit higher antioxidant activity than their sensitive counterparts (Vaidyanathan *et al.*, 2003; Neto *et al.*, 2006). Studies with mutants have also revealed a link between abiotic stress tolerance and antioxidant activity. For example, an Arabidopsis mutant with suppressed ascorbate levels (*vitamin c-1*) was hypersensitive to UV-B irradiation and ozone, whilst antisense CAT tobacco plants were more susceptible to high-light intensities, salinity and ozone (Conklin *et al.*, 1996; Willekens *et al.*, 1997). The salinity and high-light tolerant Arabidopsis mutant *photoautotrophic salt tolerance 1*, exhibited enhanced APX and SOD activities, whilst enhanced antioxidant enzyme activity was found to increase in stress-tolerant plant cultivars compared to their sensitive counterparts (Tsugane *et al.*, 1999). For example, enzymes assays revealed enhanced APX and GR activity in a drought-resistant maize cultivar (Pastori and Trippi, 1992).

However, the suppression of ROS detoxifying mechanisms and consequent accumulation of ROS appears to be important for the onset of PCD following pathogen recognition (De Pinto *et al.*, 2002). Tobacco plants with reduced CAT or APX expression levels show enhanced PCD upon exposure to a bacterial pathogen (Mittler *et al.*, 1999). ROS production at the apoplast alone without suppression of ROS detoxification does not result in the induction of PCD (Mittler *et al.*, 1999; Delledonne *et al.*, 2001).

1.7 A signalling role for ROS

It is necessary that ROS signals possess a certain degree of specificity and selectivity, so as to allow them to act efficiently in a variety of environmental responses and cellular processes. Thus the subcellular localisation, source and/or the chemical nature of the ROS, coupled with the antioxidant scavenging activities may be critical for specificity. The relative contribution of each species of ROS may vary depending on the nature of the stress. For example, spinach leaves exposed to UV-B light produced mainly $O_2^{\bullet -}$, whilst the dominant ROS in these leaves at high-light stress was $^1O_2$ (Hideg *et al.*, 2002). In addition, heat shock proteins in tomato could be induced by H_2O_2 but not by $O_2^{\bullet -}$ (Banzet *et al.*, 1998).

1.7.1 H_2O_2 as a long distance signal

ROS are ideally suited to act as signalling molecules as they are small, rapidly produced and able to diffuse over short distances (Table 1.4).

Table 1.4
The half-life and diffusion distances for the different ROS. Reproduced from Pitzschke *et al.* (2006).

ROS	Half-life	Diffusion distance
$^1O_2$	1.4 μs	0.8 μm
$O_2^{\bullet -}$	1s	8 mm
H_2O_2	∞	-
$OH^{\bullet}$	1-0.01 μs	0.5 μm

However, of all the ROS, H_2O_2 is the most stable and has a relatively low reaction rate with most biological molecules (Foyer *et al.*, 1997). It is able to diffuse some distance from its production site and is the only ROS that can cross membranes to reach neighbouring cells (Biernet *et al.*, 2007). This intercellular movement has been demonstrated in tobacco epidermal peels across 5 to 6 cell layers, thus H_2O_2 may directly function as a local cell-to-cell

signalling molecule (Allan and Fluhr, 1997). Yet given its rapid metabolism, it is unlikely that H_2O_2 diffusion from a localised site of production could function as a long distance signal, equivalent to those implicated in the induction of systemic acquired resistance (SAR) or systemic induction of wound-induced proteinase inhibitors (McGurl *et al.* 1992; Ryals *et al.*, 1994). However, this may be overcome by a relay of H_2O_2-generating microbursts, involving NADPH oxidase (Alvarez *et al.*, 1998). Such a model was proposed based on the observation of microscopic hypersensitive response lesions that appeared throughout distal parts of Arabidopsis upon infection of the leaves with avirulent bacteria, and correlated with systemic immunity and expression of defence-related genes (Alvarez *et al.*, 1998).

Additionally, the reaction products of even the relatively short-lived ROS could potentially relay signals via interaction with cellular components. For example, lipid peroxides (produced as a result of $^1O_2$ production) act as signals in mammals (Polte and Tyrrell, 2004). Similarly, in plants, the activation of lipoxygenases leads to the formation of oxylipins, which are biologically active and have diverse roles in signalling in biotic and abiotic stresses (Porta and Rocha-Sosa, 2002). More conceivably, cross-talk with other signalling molecules and hormones is a likely method in which the ROS signal can emanate as a long distance signal (discussed later in Section1.8).

1.7.2 Abiotic stresses

Abiotic environmental stresses can arise from an excess or deficit in the physical or chemical environment. ROS are implicated in most, if not all abiotic stress responses across numerous plant species, such as drought, heat, cold, UV-B, ozone, salinity and high-light stress (Prasad *et al.*, 1994; Dat *et al.*, 1998; Schraudner *et al.*, 1998; Lee *et al.*, 2000). For example, maize seedlings pre-treated with H_2O_2 or the $O_2^{\bullet -}$-generator menadione, resulted in increased chilling tolerance and increased expression of chilling-responsive genes (Prasad *et al.*, 1994). Pre-treatment with H_2O_2 or menadione also led to increased tolerance of heat stress and expression of heat shock protein genes (Banzet *et al.*, 1998; Lee *et al.*, 2000; Larkindale and Huang, 2004). Similarly, potato nodal explants sub-cultured from H_2O_2-treated microplants were significantly more thermo-tolerant than control plants and could resist a 15 h heat shock at 42 °C (Foyer *et al.*, 1997). Additionally, defects in heat tolerance were observed in the *atrbohB and atrbohD* mutants implying that a heat-induced ROS burst is an early signalling event leading to protection against heat-induced damage (Larkindale *et al.*, 2005). As well as

increasing heat tolerance, pre-treatment of rice seedlings with H_2O_2 improved tolerance to salt stress (Uchida *et al.*, 2002). Injection of H_2O_2 into Arabidopsis leaves was also able to increase protection against high-light-induced photo-bleaching (Karpinski *et al.*, 1999). Taken together, these observations strongly imply that ROS are a common factor regulating various abiotic stress signalling pathways. Therefore the identification of genes and proteins regulated by ROS is an important step towards treatments that might confer tolerance to multiple environmental stresses.

Overlap exists between the ozone and pathogen defence signalling pathways. In sensitive plants ozone (O_3) induces an oxidative burst and PCD that is highly similar reminiscent of biotic defence programs, and *AtrbohD* and *AtrbohF* have been implicated in the intercellular signalling that arises from ozone exposure (Sandermann, 2000; Overmyer *et al.*, 2003; Joo *et al.*, 2005).

1.7.3 Response to pathogens

The field of plant pathogenesis has been the most studied in relation to ROS signalling. Pathogens termed "avirulent" are unable to establish an infection and are successfully recognised by the plant via interaction between disease resistance gene products from the plant with the matching *avr* gene product from the pathogen, in a "gene-for-gene" recognition event (Flohr, 1971). Disease resistance ensues only if the corresponding *R* and *avr* genes are present in both host and pathogen (an incompatible reaction). If either is absent or inactive (as is the case with "virulent" pathogens), the pathogen avoids host recognition and the plant is susceptible to infection (a compatible reaction; [Flohr, 1971]).

The production of ROS is one of the earliest cellular responses following successful pathogen recognition (biotic stress) that is often followed by the hypersensitive response (HR): a rapid and localised programmed cell death (PCD) at the site of infection, thought to limit the spread of disease (Doke, 1983; Auh and Murphy, 1995; Grant *et al.*, 2000). A transient and enzymatically-mediated ROS increase, termed the "oxidative burst" has been well characterised in mammalian phagocytes (Baboir, 1984) and a similar biphasic oxidative burst exists in plant cells exposed to pathogens (Doke,1983; Lamb and Dixon, 1997; Grant *et al.*, 2000; Nurnberger *et al.*, 2004). This oxidative burst comprises of a low amplitude, transient first phase followed by a sustained phase of greater magnitude that correlates with disease

resistance (Lamb and Dixon, 1997). During compatible reactions only the first peak of H_2O_2 accumulation occurs (Baker and Orlandi, 1995). The second phase is triggered only by avirulent pathogens which do not infect the plant, but instead evoke the HR response (Lamb and Dixon, 1997).

The first evidence that ROS act as signal molecules to trigger pathogen defence responses, came from experiments in soybean cell cultures: demonstration of CAT-sensitive signal transmission across dialysis membranes from infected to adjacent uninfected cells, indicated that H_2O_2 functions as a mobile signal for activation of defence gene induction and HR-like cell death (Levine *et al.*, 1994). Since then, ROS have repeatedly been detected in plant pathogen responses (Auh and Murphy, 1995; Grant *et al.*, 2000). For example, a strong H_2O_2 accumulation is observed in tobacco following infiltration with fungal elicitors (Dorey *et al.*, 1998) and the bacterial elicitor harpin has been shown to induce H_2O_2 production in Arabidopsis suspension cultures (Desikan *et al.*, 1998).

ROS also accumulate after mechanical wounding of plant tissue (Orozco-Cardenas *et al.*, 2001; Watanabe *et al.*, 2001; Sagi *et al.* 2004). For instance, in tomato plants H_2O_2 levels increased at wound sites within 1 h following wounding and H_2O_2 accumulation was observed in the cell walls of non-wounded leaves after 4 h, prior to the systemic expression of various pathogen defence-related genes (Orozco-Cardenas *et al.* 2001).

NADPH oxidase enzymes are an important source of pathogen-mediated ROS generation. The down-regulation or elimination of *Rboh* gene expression leads to variable effects on pathogen growth and HR. For example, silencing of *NbrbohA* and *NbrbohB* in tobacco led to less ROS production and reduced resistance to normally avirulent *Phytophthora infestans* (Yoshioka *et al.*, 2003). Additionally, infection of Arabidopsis *atrbohD* and *atrbohF* mutants demonstrated that AtrbohD is responsible for nearly all of the ROS produced in response to avirulent bacteria or oomycete pathogens, whereas AtrbohF is important in the regulation of HR (Torres *et al.*, 2002). By contrast, the Arabidopsis *atrbohF* mutant is more resistant to a weakly virulent strain of the oomycete *Peronospora parasitica*, and actually expressed enhanced HR (Torres *et al.*, 2002). Thus although the Rboh proteins were required for pathogen-induced ROS production, these ROS might serve different signalling functions in disease resistance and HR. This suggests that ROS production alone is not sufficient to induce pathogen defence responses and may be accounted for by the effect of ROS

depletion on the levels of other signalling components of the defence cell death response (e.g. NO).

1.7.4 ROS and stomatal closure

ROS are able to mediate the activation of Ca^{2+} channels during abscisic acid (ABA)-induced stomatal closure. Micromolar concentrations of ABA were found to induce the synthesis of H_2O_2 in Arabidopsis guard cells, which in turn induced the activation of Ca^{2+} channels (Pei *et al.*, 2000). Thus, facilitating an increase in guard cell cytosolic Ca^{2+} concentrations is necessary for stomatal closure (Pei *et al.*, 2000). Furthermore, *AtrbohD* and *AtrbohF* were shown to be highly expressed in guard cells and were transcriptionally induced in response to ABA. The *atrbohD/atrbohF* double mutant was impaired in ABA-induced induction of ROS, activation of Ca^{2+} channels and stomatal closure (Kwak *et al.*, 2003). However, exogenous application of H_2O_2 was able to partially restore Ca^{2+} channel activation and stomatal closure in *atrbohD/atrbohF* mutant plants (Kwak *et al.*, 2003).

Evidence also points to the involvement of other signalling agents in the stomatal closure response (for example, NO, ethylene and JA) indicating the complexity of signalling within this system (Desikan *et al.*, 2002; Suhita *et al.*, 2004; Desikan *et al.*, 2006).

1.7.5 Cell division and growth

Predominantly evidence for the role of ROS in the control of cellular expansion comes from studies of root hair growth. The Arabidopsis NADPH oxidase *atrbohC* mutant (also called *root hair defective 2* [*rhd2*]) exhibits root hair bulges instead of elongated root hairs (Foreman *et al.*, 2003). Unlike wild-type root hairs where ROS were found to be localised in the growing tips, no ROS localisation was detected in the *atrbohC* root hair bulges. The mutant phenotype could be partly suppressed by ROS treatment ($OH^{\cdot}$ via the Fenton reaction), although it resulted in spherical (non-polar) root hair outgrowths. This report suggests that polarised ROS production is required for root hair outgrowth. Furthermore, the *atrbohC* mutant was also impaired in the hyperpolarisation-activation of plasma membrane Ca^{2+} channels that are responsible for localised cell expansion of epidermal cells in the root elongation zone (Foreman *et al.*, 2003).

ROS are also present in the expansion zone of maize leaves (Rodriguez *et al.*, 2002; Schopfer, 2001). Auxin promoted the release of $O_2^{\bullet -}$ and subsequent generation of $OH^{\bullet}$ in the growth-controlling outer epidermis of maize coleoptiles, whilst scavengers of $O_2^{\bullet -}$, H_2O_2 and $OH^{\bullet}$ inhibited auxin-induced growth (Schopfer *et al.*, 2001; 2002). In cotton fibres, exogenous H_2O_2 application was able to prematurely promote secondary wall formation, whilst treatment with diphenyl iodonium (DPI; an inhibitor of NADPH oxidases and peroxidases) or antioxidants prevented secondary wall differentiation, suggesting that H_2O_2 may function as a developmental signal in the differentiation of cotton fibre secondary walls (Potikha *et al.*, 1999).

ROS have a complex effect on mitotic activity. ROS application can promote somatic embryogenesis in callus cultures by inducing autonomous cell division. For example, direct addition of H_2O_2 or inhibition of CAT activity, stimulated somatic embryogenesis in goji berry (*Lycium barbarum*) callus cultures (Cui *et al.*, 1999), whilst an H_2O_2 scavenger (dimethylthiourea) impeded embryogenesis in callus cultures of milk vetch (*Astragalus adsurgens*) (Luo *et al.*, 2001). Treatment of Arabidopsis with the ROS-generating agents methyl viologen (a PSI electron acceptor) and alloxan (a H_2O_2-generating compound) induced localised cell proliferation in whole seedlings, isolated root segments and single cells (Pasternak *et al.*, 2005). The authors suggest that this ROS-induced cell division was due to ROS-enhanced auxin-responsiveness which might underlie the ROS-induced reorientation of growth. However, ROS can also have an inhibitory effect on the cell cycle (Reichheld *et al.*, 1999). For instance, Arabidopsis protoplasts exposed to alloxan had decreased expression of the auxin efflux carrier *PIN-FORMED* genes (*PIN1* and *PIN3*), which regulate cell division in Arabidopsis roots by controlling auxin distribution (Blilou *et al.*, 2005; Pasternak *et al.*, 2005). Therefore, ROS and auxin may potentially work together as a control system for the progression of the cell cycle to facilitate differential reorientation of growth.

1.7.6 Root gravitropism

A study on maize root gravitropism has indicated that ROS may function as downstream components in auxin-mediated gravitropic responses (Joo *et al.*, 2001). A transient increase in the intracellular concentration of ROS in the maize root endodermis resulted from either gravistimulation (placing a vertically-grown root horizontally), or asymmetric application of auxin to vertical roots (Joo *et al.*, 2001). Root curvature was brought about by application of

H_2O_2 to vertical roots pre-treated with an auxin transport inhibitor. Furthermore, the scavenging of ROS by antioxidants (e.g. ascorbate) inhibited root gravitropism, indicating that the generation of ROS plays a central role in root gravitropism. The observed up-regulation of oxidative stress-related genes during Arabidopsis gravitropism adds weight to a role for ROS in this process (Moseyko *et al.*, 2002).

1.7.7 Root nodulation

Treatment of legumes with specific rhizobial nodulation (Nod) factors can also stimulate ROS production. For example, in alfalfa the recognition by the plant of compatible Nod factors triggered a rapid production of $O_2^{\bullet -}$ close to the root tip (Ramu *et al.*, 2002). Moreover, exogenous H_2O_2 was sufficient to activate transcription of the nodulin gene *Rip1*, suggesting that ROS production is a mediator of nodulin expression (Ramu *et al.*, 2002). The synthesis of both ROS and ethylene are required for root nodulation in a variety of legumes, and it has been postulated that they act together to promote cell death associated with the formation of infection pockets (D'haeze *et al.*, 2003).

1.8 Part of a signalling network

Plants are complex organisms. At any one time there is a vast array of intricate and diverse signalling networks in motion. Therefore it is important not to isolate ROS, but to place them within the wider context of plant hormones and other second messengers. Interaction with other signalling molecules and/or hormone pathways may account for the divergent responses mediated by ROS and explain why ROS produced by the same mechanism exert variable effects in different contexts.

1.8.1 Calcium

Calcium fluxes are intimately related to ROS signalling, and appear to function both upstream and downstream of ROS production. For example, a Ca^{2+} influx was required for ROS production both after pathogen infection/elicitation and following ABA treatment (Chandra *et al.*, 1996; Blume et *al.*, 2001; Grant *et al.*, 2000; Jiang and Zhang, 2003). Arabidopsis plants challenged with avirulent bacteria exhibited a sustained increase in cytosolic Ca^{2+} that was

not affected by treatment with DPI which blocked H_2O_2 accumulation and the HR (Grant *et al.* 2000). In the same report, the Ca^{2+}-channel blocker lanthanum, was shown to suppress H_2O_2 accumulation and the HR as well as cytosolic Ca^{2+} levels (Grant *et al.* 2000). On the other hand, the oxidative burst has been implicated in activating Ca^{2+} influx following elicitation (Levine *et al.*, 1996). For example, a biphasic cytosolic Ca^{2+} signature was observed in Arabidopsis seedlings and tobacco cell cultures in response to H_2O_2 challenge (Lecourieux, *et al.*, 2002; Rental and Knight, 2004).

ROS generation and activation of Ca^{2+} channels represent a common signalling link in many plant responses: ROS function through the activation of Ca^{2+} channels during ABA-mediated stomatal closure and during root hair growth and defence (Pei *et al.*, 2000; Foreman *et al.*, 2003). Furthermore, all plant Rboh proteins contain two EF-hands in their N-terminal region that bind Ca^{2+} (Keller *et al.*, 1998). This may account for the direct regulation of these oxidases by Ca^{2+}, and plant Rboh proteins have been shown *in vitro* to be stimulated directly by Ca^{2+} (Sagi and Fluhr, 2001).

1.8.2 Salicylic acid

ROS have been proposed to act synergistically with salicylic acid (SA) in a signal amplification loop to drive the HR and establish systemic acquired resistance [SAR] (Draper, 1997; Shirasu *et al.*, 1997; Durrant and Dong, 2004). This model was based on experiments using both exogenous H_2O_2 and pathogens to induce SA accumulation (Leon *et al.*, 1995; Shirasu *et al.*, 1997). SA accumulation and enhanced ROS production also down-regulated ROS-scavenging systems and so may contribute further to increased ROS levels following pathogen recognition (Klessig *et al.*, 2000).

However, work with the Arabidopsis *lesion stimulating disease 1* (*lsd1*) mutant has shown that ROS and SA can also antagonise each other's action in the regulation of cell death expansion at the margins of pathogen-triggered HR lesions (Torres *et al.*, 2005). Mutant *lsd1* plants failed to contain the initial HR following pathogen recognition, and exhibited spontaneous leaf lesion formation accompanied by drastic $O_2^{\bullet -}$ accumulation in front of the spreading zone of cell death (Jabs *et al.*, 1996; Dietrich *et al.*, 1997). ROS produced via AtRbohD and AtRbohF antagonised SA to stop the spread of cell death beyond the site of HR. Hence these two proteins are negative regulators of the unrestricted cell death

expanding from the margins of an initial HR site in *lsd1*, whereas SA appears to be a positive regulator if this cell death (Torres *et al.*, 2005).

1.8.3 Nitric oxide and abscisic acid

ROS signalling has also been linked to nitric oxide (NO). For example, both ROS and NO can mediate ABA-induced stomata closure (Desikan *et al.*, 2004). In the NADPH oxidase double mutant *atrbohD/atrbohF* NO synthesis and stomatal closure were severely reduced in response to ABA, suggesting that endogenous ROS production elicited by ABA is required for NO synthesis (Bright *et al.*, 2006).

Additionally, NO seems to work in conjunction with ROS to potentiate PCD and defence gene expression following pathogen challenge, and both signals have been shown to modulate each other's accumulation during HR (Durner *et al.*, 1998; Delledonne *et al.*, 2001; Tada *et al.*, 2004; Zeier *et al.*, 2004).

1.8.4 Ethylene

ROS may also interact with ethylene, a hormone involved in induction of PCD and senescence (De Jong *et al.*, 2002). For example, both ROS and ethylene have been implicated in signalling in response to viral infection (Love *et al.*, 2005). Furthermore the ethylene receptor AtETR1 has been demonstrated to function as a ROS sensor, mediating stomatal closure in response to H_2O_2 (Desikan *et al.*, 2005; see Section 1.9.1). Thus, ETR1 may constitute a node mediating cross-talk between ethylene and H_2O_2, although whether such shared responses occur in cells other than guard cells has yet to be established.

1.9 Protein signalling components

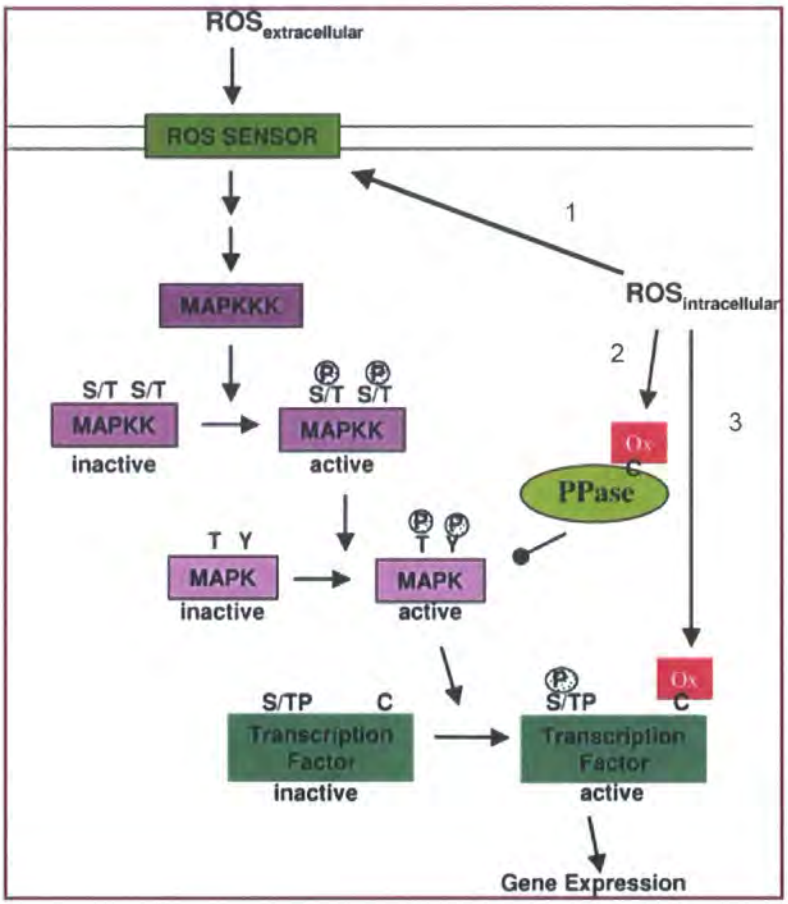
While the importance of ROS for cellular signalling has been established, relatively little is known about how the ROS stimuli are perceived, transduced and finally result in specific end responses. Since ROS generated in cellular compartments are able to result in changes to the nuclear transcriptome, information must be transmitted from organelles to the nucleus. Therefore, the ROS signal must be converted into longer lasting signalling events that can sustain the signal and relay it downstream (see Figure 1.3 overleaf). The ROS signal must first be sensed, either directly or indirectly, and then transduced and amplified, typically by way of kinase- and phosphatase- mediated reversible protein phosphorylation. Protein activation may also occur directly (without the need for phosphorylation pathways), or via other post-translational modifications (e.g. nitrosylation). Finally, changes in gene expression patterns can occur via the activation of transcription factors. Although many aspects of ROS signalling are still unclear, some ROS signalling components have been identified in different processes and will be discussed here.

1.9.1 ROS sensors

ROS could potentially be sensed directly via histidine two-component signalling systems, which are well-known redox sensors in prokaryotes and fungi (Whistler *et al.*, 1998; Quinn *et al.*, 2002). Two-component systems usually consist of a histidine kinase that senses the signal and a response regulator that functions as a transcription factor (Hwang *et al.*, 2002). A recent study has revealed that the Arabidopsis Ethylene Receptor 1 (AtETR1) histidine kinase that functions in ethylene signalling, is also a potential ROS sensor and mediator of H_2O_2 signalling in stomata (Desikan *et al.*, 2005). AtETR1 can functionally replace yeast double mutants lacking both the ROS-sensing histidine kinase SLN1 and the downstream response regulator SSK1 (Desikan *et al.*, 2005).

Figure 1.3

Schematic diagram of potential ROS signalling mechanisms. Image reproduced from Apel and Hirt (2004).



ROS may activate gene expression via three main ways: (1) ROS sensors could be activated to induce signalling cascades that ultimately impinge on gene expression. (2) Components of signalling pathways could be directly oxidised by ROS (e.g. ROS may influence mitogen-activated protein kinase (MAPK) signalling pathway through inhibition of MAPK phosphatases [PPases]). (3) ROS might directly modify the activity of transcription factors (e.g. via oxidation of cysteine residues).

ROS could also be sensed indirectly via the redox poise of the cell. For example, it has been suggested that the reduced/oxidised ratio of glutathione may be involved in ROS perception in plants (Foyer *et al.*, 1997). Changes in the redox status of chloroplasts during a light-dark cycle are known to modulate organellar enzyme activities and to influence the transcription of a variety of genes (Foyer and Noctor, 2000).

Redox groups within proteins can undergo reversible oxidation/reduction which may alter protein structure and activity, thus protein activity may potentially be switched 'on' or 'off' depending on the cellular redox state. One mechanism of redox-sensitive regulation of protein function is via the oxidation of thiol (-SH) groups of proteins, thereby affecting protein conformation and/or protein-protein interactions (Halliwell and Gutteridge, 2007). H_2O_2 can directly oxidise such thiol residues (e.g. cysteine or methionine), and if a protein contains two cysteine thiol groups a disulphide bridge (S-S) forms and the resulting conformational change may result in altered function (Halliwell and Gutteridge, 2007). For example, point mutation and deletion analyses revealed that a change of cysteine to tyrosine in the N-terminal region of AtETR1 completely abolished ROS signalling in both Arabidopsis stomata and AtETR1-transformed *sln1/ssk1* yeast double mutants (Desikan *et al.*, 2005). This observation strongly suggesting that the thiol of this particular cysteine residue is important for ROS sensing by AtETR1.

ROS may also be indirectly perceived, by detection of ROS-inflicted cell damage. Indeed, one model for ozone perception is that plants sense the products of oxidative breakdown of the cell wall (Wiese and Pell, 2003). Several lipid oxidation products can change gene expression in human cells, and various oxygenic products of lipid polyunsaturated fatty acids have been shown to be biologically active and may change the expression of specific genes (Grether-Beck *et al.*, 2000).

Redox-sensitive thioredoxins also may represent a method of indirect ROS sensing. In mammalian cells ROS can activate a signalling cascade mediated by a thioredoxin (Trx). Upon oxidation, Trx dissociates from a MAPKKK (ASK1) and the subsequent activation of a MAPK pathway follows (Saitoh *et al.*, 1998). In plants, the CITRX thioredoxin of tomato plays a role in regulating pathogen defence against the fungal pathogen *Cladosporium flavum*. Although the exact mechanism is unclear, silencing of CITRX was shown to enhance pathogen-induced ROS accumulation and defence gene expression. This study

demonstrated that CITRX acts as a negative regulator of defence responses, although it remains to be shown whether this function of CITRX actually requires its redox-regulatory activity (Rivas *et al.*, 2004).

1.9.2 Kinases

Several lines of evidence show that ROS are able to activate mitogen-activated protein kinase (MAPK) pathways in plants. The basic MAPK module consists of a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK), which in turn phosphorylates a MAPK that phosphorylates a range of target proteins including transcription factors and other protein kinases.

In Arabidopsis, there seem to exist multiple ways to activate the MAPKs AtMPK3 and AtMPK6 in response to ROS. H_2O_2 activates the AtMPK3 and AtMPK6 via the MAPKKK ANP1 (Kovtun *et al.*, 2000). Over-expression of ANP1 in transgenic plants resulted in increased tolerance to heat shock, freezing and salt stress (Kovtun *et al.*, 2000). H_2O_2 also increased expression of the Arabidopsis nucleotide diphosphate (NDP) kinase 2 (AtNDPK2), which when over-expressed reduced accumulation of H_2O_2 and enhanced tolerance to multiple stresses including cold, salt and oxidative stress (Moon *et al.*, 2003). The effect of AtNDPK2 may be mediated by AtMPK3 and AtMPK6, because AtNDPK2 can interact and activate these two MAPKs (Moon *et al.*, 2003).

Another upstream mediator of AtMPK3 and AtMPK6 is the serine/threonine oxidative signal-inducible 1 kinase (OXI1; Rentel *et al.*, 2004). *OXI1* expression is induced *in vivo* by H_2O_2 and in response to a wide range of stimuli that produce ROS including cold, heat, wounding and pathogen attack (Rentel *et al.*, 2004). Mutant *oxi1* plants were hypersensitive to infection by the virulent fungal pathogen *Peronospora parasitica*, showed a strong reduction in the number and length of root hairs and were compromised in ROS- and elicitor-induced activation of AtMPK3 and AtMPK6 (Rentel *et al.*, 2004). Therefore, OXI1 is a central part of the signal transduction pathway linking ROS to diverse downstream responses.

The stress-induced activation of MAPKs could be explained in most studies by the notion that ROS act upstream of MAPK pathways. However, an investigation of *Phythophthora infestans* infection of tobacco showed that the MEK2 pathway might be part of an amplification cascade

upstream of the NADPH oxidase genes, which produce ROS in response to fungal infection (Yoshioka *et al.*, 2003). Congruent with these studies, expression of constitutively active Arabidopsis MKK4 or MKK5, the orthologs of tobacco MEK2 (and activators of MPK3/6), resulted in generation of H_2O_2 and cell death (Ren *et al.*, 2002). Furthermore protein kinase inhibitors blocked elicitin-induced cell death, oxidative burst and expression of defence genes in tobacco (Sasabe *et al.*, 2000).

1.9.3 Phosphatases

Phosphatases are responsible for the removal of phosphate groups from proteins. The dephosphorylation of components of MAPK cascades provides a means to regulate the magnitude and duration of the kinase activity. ROS are able to control the activity of several protein phosphatases. For example, the Arabidopsis protein phosphatase 2C (PP2C) ABI2 was rapidly inactivated upon H_2O_2 challenge, via the oxidation of cysteine residues (Meinhard *et al.*, 2002). ABI1 and ABI2 encode protein phosphatase 2C enzymes that are both involved in stomatal closing. Using the ABA insensitive mutants, it was shown that ABA is unable to generate ROS in *abi1* mutants but ABA still induces ROS production in *abi2* mutants (Murata *et al.*, 2001). These data indicate that ABI1 may act upstream and ABI2 downstream of ROS signalling.

Tyrosine phosphatases have been shown to be inactivated by H_2O_2. For example human protein tyrosine phosphatase PTP1B was reversibly inactivated by H_2O_2, oxidising a cysteine residue in the catalytic site (Lee *et al.*, 1998; Van Montford *et al.*, 2003). Mammalian tyrosine phosphatases have also been shown to inactivate eukaryotic MAPK cascades (Van Montford *et al.*, 2003). A similar regulation is likely to occur in plants because Arabidopsis PTP1, which can inactivate AtMPK6, can be inactivated by H_2O_2 (Gupta and Luan, 2003).

1.9.4 Transcription factors and promoter elements

NPR1 was identified as a redox-sensitive transcription factor in plants, and is an essential regulator of plant systemic acquired resistance (SAR; Cao *et al.*, 1997; Mou *et al.*, 2003). The expression of various zinc finger proteins can be induced by H_2O_2 treatment and these proteins have wide-ranging functions (Desikan *et al.*, 2001). For example, ZAT12 has been

demonstrated to play a role in cold acclimation and tolerance to osmotic, oxidative and salinity stresses (Rizhsky *et al.*, 2004; Davletova *et al.*, 2005; Vogel *et al.*, 2005).

There are several promoter elements which may act in a ROS- or redox- responsive manner to control gene expression. For example, the W-box promoter element is present in the promoters of the 26 genes that make up the Arabidopsis "pathogen regulon" and in the *PR* genes of parsley (Rushton, 1996; Maleck *et al.*, 2000). These W-boxes are responsible for pathogen-triggered gene expression via the binding of WRKY transcription factors, which are induced by wounding, pathogen infection and/or abiotic stresses (Eulgem *et al.*, 2000). WRKYs possess a redox-sensitive zinc finger DNA-binding domain, making them strong candidates for redox regulation (Arrigo, 1999). Heat shock elements can also participate in redox-regulated gene expression. For example, a mutation of the heat shock element in the promoter of the Arabidopsis *APX1* gene delayed its inducibility by ROS (Storozhenko *et al.*, 1998).

1.10 Summary

Aside from exerting oxidative damage, ROS can act as second messengers in plants and be utilised for various tasks. For example, ROS production is a central aspect of how plants defend themselves against pathogens and abiotic stress, and more recent work has revealed that ROS can function as intrinsic signals during growth and stomatal closure.

Specificity of ROS signalling may potentially be achieved via the temporal and spatial control of both ROS production and scavenging, as well as by the chemical nature of the ROS and magnitude of the ROS increase. Communication with hormones and other second messengers is also a central part of ROS signalling. However, still relatively little is known about how ROS signals are perceived and transduced in order to orchestrate such downstream responses.

1.11 Thesis outline

The aim of this study was to:

- Identify candidate protein signalling components acting downstream of H_2O_2 in Arabidopsis (Chapter 3)
- Examine the expression patterns of these candidate genes in response to a range of environmental stresses (Chapter 5)
- Construct and identify loss-and gain-of-function plant lines of these candidate H_2O_2-signalling components (Chapter 4)
- Investigate the loss- and gain-of-function lines for altered phenotypes (enhanced sensitivity or tolerance) in response to a range of environmental stress treatments (Chapter 5)
- Monitor the loss- and gain-of-function lines for abnormal developmental phenotypes (Chapter 5)
- Analyse the genome wide transcript abundance within the gain-of-function lines in order to identify potential downstream target genes (Chapter 6).

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals and media used were obtained either from BDH Laboratory Supplies Ltd. (Lutterworth, Leicestershire, UK), Bioline Ltd. (London, UK) or Sigma-Aldrich Company Ltd. (Gillingham, Dorset, UK), unless stated otherwise.

2.1.2 Plant material

Arabidopsis thaliana (*A. thaliana*) seeds of ecotypes Columbia (Col-0) and Wassilewskija (WS-2) seeds were obtained from Lehle Seeds (Round Rock, Texas, USA).

2.1.3 Bacterial material

Escherichia coli (*E. coli*) strains DH5α and DB3.1 (Bernard and Couturier, 1992) were obtained from Invitrogen Ltd. (Paisley, Renfrewshire, UK).

Agrobacterium tumefaciens (*A. tumefaciens*) strain C58C1 (Deblaere *et al.*, 1985) was propagated in-house.

Pseudomonas syringae pv. tomato (*Pst*) isolates DC3000 (virulent) and Avr13 (avirulent) (Grant *et al.*, 1995) were kindly donated by Dr Haruko Okamoto (Department of Plant Sciences, University of Oxford, Oxford, UK).

2.1.4 Modifying enzymes

All DNA and RNA modifying enzymes were obtained either from Bioline, Invitrogen, or New England Biolabs Ltd. (NEB) (Hitchin, Hertfordshire, UK), unless stated otherwise.

2.1.5 Nucleotides

Nucleotides were obtained from NEB.

Radionucleotide [α–^{32}P]-dCTP (deoxycytidine 5'-[α-^{32}P] triphosphate, triethylammonium salt; 10 mCi/ml, 3000 Ci/mmol) was obtained from Amersham Plc (Little Chalfont, Buckinghamshire, UK).

2.2 Sterilisation

2.2.1 Solution sterilisation

All growth and other heat-stable solutions were sterilised by autoclaving at 121 °C and 10^5 Pa for 20 min. Heat-sensitive solutions were filter-sterilised using 0.2 µm filters (Fisher Scientific Ltd., Loughborough, Leicestershire, UK) attached to syringes (Terumo Ltd., Egham, Surrey, UK).

2.2.2 Seed sterilisation

2.2.2.1 Ethanol surface-sterilisation

Commercial seed was sterilised with 70 % (v/v) ethanol by shaking in 1.5 ml microtubes for 5 to10 min. The seed was then air-dried on filter paper (Whatman International Ltd, Maidstone, Kent, UK) in a sterile laminar flow hood.

2.2.2.2 Bleach surface-sterilisation

Seed obtained from in-house *A. tumefaciens*-dipped plants (see Section 2.19.2) was first surface-sterilised with ethanol (as described above in Section 2.2.2.1). Seed was subsequently shaken in a solution of 10 % (v/v) sodium hypochlorite (NaOCl) and 0.25 % (w/v) sodium dodecyl sulphate (SDS) for 10 min. Following this, the seed was then washed 6 times in sterile water, then pipetted directly onto solid agar germination medium (see next Section 2.3.1) and left to dry in a sterile laminar flow hood.

2.3 Growth media

2.3.1 Plant growth media

Sterilised *A. thaliana* seed was sown onto solid agar germination medium consisting of plant tissue culture grade agar (Sigma-Aldrich) supplemented with 1 x Murashige and Skoog salts including vitamins (MS; Duchefa Biochemie BV, Haarlem, Netherlands; Murashige and Skoog, 1962). For horizontal plates (9 cm diameter Petri dishes; Greiner Bio-One Ltd, Stonehouse, Gloucestershire, UK) 0.8 % (w/v) agar was used, whilst vertical plates (12 cm x 12 cm square plates; Greiner Bio-One) were made using 1.2 % (w/v) agar. Prior to autoclaving, the pH was adjusted to 5.8 with 0.1 M KOH.

Where mature plants were required, 10- to 14-day old seedlings were carefully transferred from MS agar plates onto re-hydrated peat plugs (Jiffy Products Ltd, Winchester, Hampshire, UK) using hooked forceps. Individual plants were grown on 38 mm (diameter) peat plugs, whilst 42 mm plugs were used to grow up to 3 plants per plug for large scale seed-bulking or for *A. tumefaciens*-dipping (see Section 2.19.2).

2.3.2 Bacterial growth media

E. coli and *A. tumefaciens* were grown either on solid agar plates consisting of 1.5 % (w/v) micro agar (Duchefa Biochemie) and 2 % (w/v) Luria-Bertani (LB) medium (Sigma-Aldrich), or in liquid media made from 2 % (w/v) LB. For *P. syringae*, King's medium B (KB; Sigma-Aldrich; King *et al.*, 1954) was used instead of LB.

2.3.3 Antibiotics

All antibiotics used are listed overleaf in Table 2.1 and were obtained from either Duchefa Biochemie, Sigma-Aldrich or Melford Laboratories Ltd (Ipswich, Suffolk, UK). Antibiotics were added (as required) to autoclaved MS, LB or KB agar once it had cooled to approximately 50 °C.

Table 2.1

Details of antibiotics added to MS (plant) and LB or KB (bacteria) media.

Organism	Antibiotic	Working concentration (µg / ml)	Stock concentration (mg / ml)	Stock solvent
Plant	Kanamycin	50	100	Water
	Phosphinothricin (Basta)	10	10	Water
	Timentin	200	200	Water
Bacteria	Ampicillin	100	100	Water
	Kanamycin	100	100	Water
	Spectinomycin	100	50	Water
	Rifampicin	100	50	DMSO

2.4 Growth conditions

2.4.1 Plant growth conditions

To ensure germination was uniform, sterilised seed (on MS agar plates) was stratified at 5 °C and in darkness for at least 48 h. Plates were subsequently transferred to a growth chamber maintained at a constant temperature of 21 °C, with a 16 h photoperiod (16 h light, 8 h dark; at a light intensity of approximately 60 $\mu E/m^{-2}\ s^{-1}$).

If mature plants were required, seedlings (on peat plugs) were transferred to a greenhouse maintained at approximately 21 °C with a 16 h photoperiod. The Aracon system (BetaTech, Gent, Belgium) was used to isolate each individual mature plant. Compost was regularly sub-irrigated until fertilisation had occurred and siliques had developed fully. Thereafter, plants were moved to a "drying room" where they were allowed to senesce and dry out prior to seed collection.

2.4.2 Bacterial growth conditions

Bacteria were incubated either at 37 °C (*E. coli*), 28 °C (*A. tumefaciens*) or 25 °C (*P. syringae*). Solid agar plates were incubated static, whilst liquid media cultures were shaken at 200 rpm. Under the Specified Animal Pathogen Order (DEFRA; 1988) all *P. syringae* work was confined to a designated Category 2 Pathogen laboratory.

2.5 Plant treatments for transcript level analyses

In order to examine gene expression changes, 10-day old wild-type seedlings were subjected to a variety of stress, hormone and chemical treatments as summarised overleaf in Table 2.2. For each sample point, two biological replicates were performed.

Unless otherwise indicated, seedlings were carefully transferred (using hooked forceps) from horizontal MS agar plates into 6-well plates (10ml wells; Greiner Bio-One). Approximately 40 to 50 seedlings were placed in each well containing 5 ml of sterile water. Seedlings were then placed in a growth chamber with the plate lids on, and allowed 3 h to recover from the transfer. After this period, 5 ml of the chemical/elicitor was added per well (at twice the final concentration) and swirled gently to allow mixing. Unless otherwise stated, the plates were then placed back in the growth chamber. Using hooked forceps, seedlings were removed from the solution at various time points, blotted dry and quickly flash frozen with liquid nitrogen. Samples were stored at -80 °C. Northern blot analyses (see Section 2.16) were later performed on the extracted RNA (see Section 2.8.4).

Table 2.2

Summary of the stress, hormone and chemical treatments performed on wild-type seedlings prior to northern blot analyses.

Stress / Hormone / Chemical		Treatment details (final concentration)	Time points	Control
Abiotic stressor	Cold	Incubated at 5 °C (in 10 ml of water per well). The 6-well plates were wrapped in foil to control for light between the 5 °C and control growth cabinets.	1 and 3 h	20 °C
	Drought	Incubated in 0.22 M mannitol	1 and 3 h	Water
	Heat	Seedlings were treated on MS agar plates for 1 h at 40 °C in a controlled growth cabinet.	0, 1 and 3 h post-treatment	20 °C
	Salt	Incubated in 0.44 M NaCl	1 and 3 h	Water
	UV-B	Seedlings were treated on MS agar plates with 1 J/cm^2 (approximately 60 s) by removal of lids and placing in a UV cross-linker.	1 and 3 h post-treatment	Removal of plate lids
Biotic stressor	Cellulase	Incubated in 0.1 % cellulase	1 and 3 h	Water
	Flagellin	Incubated in 1 µM flagellin-22 (in 0.1 % DMSO)	1 and 3 h	0.1 % DMSO
Hormone	Abscisic acid (ABA)	Incubated in 100 µM ABA (in 0.1 % ethanol)	1 and 3 h	0.1 % ethanol
	Auxin	Incubated in 1 µg/ml 1-naphthaleneacetic acid (NAA)	1 and 3 h	Water
	Ethylene	Incubated in 100 µM 1-aminocylcopropane-1-carboxylic acid (ACC)	1 and 3 h	Water
	Jasmonic acid (JA)	Incubated in 100 µM methyl jasmonate (in 0.1 % ethanol)	1 and 3 h	0.1 % ethanol

(Table continues on the following page)

Table 2.2 *(continued from the previous page)*				
	Salicylic acid (SA)	Incubated in 100 µM sodium salicylate	1 and 3 h	Water
ROS	H_2O_2	*Concentration gradient*: Incubated in 0.1, 1, 5, 10 or 20 mM H_2O_2	3 h	Water
		Time scale: Incubated in 10 mM	0.5, 1, 2, 3 and 6 h	Water
	Menadione (superoxide generator)	*Concentration gradient*: Incubated in 0.5, 1, 10 or 50 µM menadione (stock solution of 1mM dissolved in 1 % dimethyl sulphoxide; DMSO)	3 h	DMSO (concentration as appropriate)

2.6 Plant stress tolerance screens

In order to screen for differences to wild-type plants, seedlings (age as indicated in the following subsections) of the loss- and gain-of-function lines were subjected to a variety of stress and hormone treatments. In the first instance, experiments were performed as "pilot" screens to test for stress susceptibility and tolerance. If a positive result was found, the screen was then repeated with more biological replicates in order to confirm the finding.

Wild-type plants were used as the control line for the T-DNA insertion mutants, whilst empty vector lines were used to control for the 35*S* over-expression lines (for details of lines used please refer to Chapter 4).

Plants were photographed regularly during each treatment using a digital camera (Nikon CoolPix 4500; Nikon Ltd., Kingston upon Thames, Surrey, UK), except in the case of the ethylene root experiment (Section 2.6.3.2; as the dissecting microscope was not mounted with a camera).

2.6.1 Abiotic stresses

2.6.1.1 Cold stress

Using sterile cocktail sticks, seeds were sown individually and evenly spaced on horizontal MS agar plates (17 seeds per plate and 1 plate per treatment per line). After 14 days, the seedling plates were moved to either a 5 or 20 °C (control) growth chamber. Plants were monitored daily over 2 weeks for their general health and growth as a score of chilling tolerance.

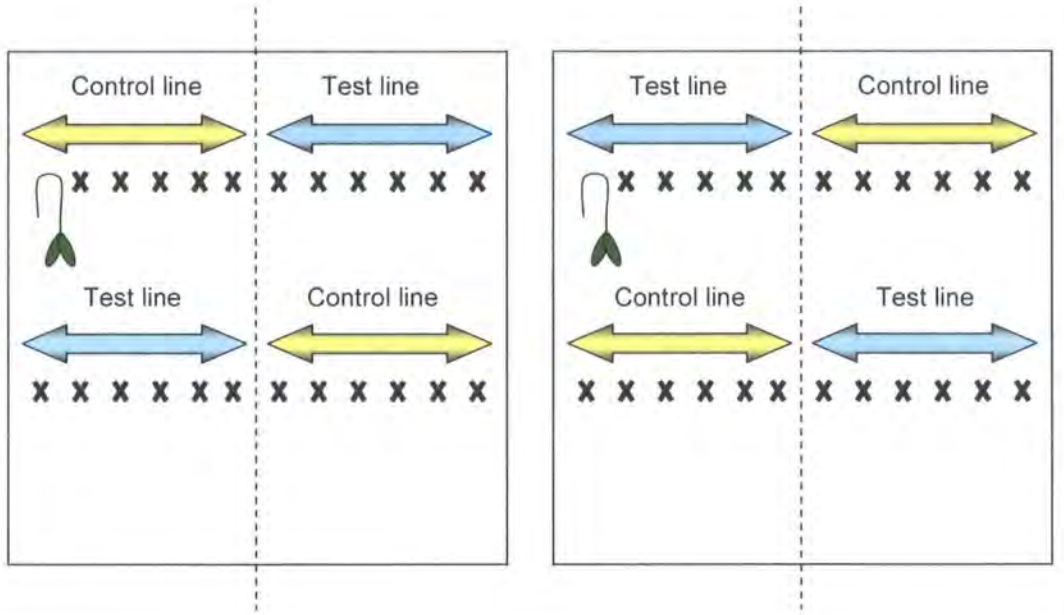
In addition (due to significant anthocyanin accumulation), seedlings were also transferred onto peat plugs (6 plants per treatment per line). On day 14 they were moved to either a 5 or 20 °C growth chamber and monitored over a 6 week period.

2.6.1.2 Drought stress

Seedlings were germinated and grown vertically on (non-supplemented) MS agar plates. At 5 days old the seedlings were carefully transferred (using hooked forceps) onto vertical plates of MS agar supplemented with either 100, 200 or 300 mM mannitol. Seedlings were transferred such that their roots pointed upwards and shoots downwards, and placed back in the growth chamber in this manner. For each line tested, a total of 24 seedlings were screened per concentration (set up out as shown overleaf in Figure 2.1). Plants were also transferred onto non-supplemented MS agar plates as a control. After a further 3 days in the growth chamber, the amount of root reorientation (past 90 degrees) and root growth was examined as a score of drought tolerance.

Figure 2.1

Schematic of the layout for a root reorientation experiment. X represents the position of the transferred seedlings.



2.6.1.3 Heat stress

As previously described for cold stress (see Section 2.6.1.1) seeds were sown individually and evenly spaced out on horizontal MS agar plates (17 per plate and 1 plate per treatment per line). On day 14 the seedlings were transferred to growth cabinets (with plate lids still on) for one of 3 treatments: (i) 38 °C for 1.5 h, (ii) 38 °C for 1.5 h followed by 45 °C for 2 h or (iii) 45 °C for 2 h. Control seedlings were maintained at 20 °C. After each treatment the seedling plates were transferred back to the 20 °C growth cabinet. Plants were monitored daily for survival up to 18 days post-treatment.

2.6.1.4 Oxidative stress

Seedlings were germinated on horizontal MS agar plates. After 10 days, the seedlings were carefully transferred (using hooked forceps) into 6-well plates containing 5 ml of sterile water (approximately 30 seedlings per well) and were allowed 3 h to recover from the transfer. Then 5 ml of the ROS reagent were added per well (at twice the final

concentration) and swirled gently to allow mixing. The following concentrations were used:

- H_2O_2: 0, 5, 10, 30, 50 or 100 mM
- Menadione: 0, 0.5, 1, 10, 50 or 100 µM

(to generate $O_2^{\bullet -}$)

- Fenton reaction: H_2O_2: 0, 0.1, 0.5, 1, 3 or 5 mM

(to generate $OH^{\bullet}$) Ascorbate: 0, 0.1, 0.5, 1, 3 or 5 mM

$CuSO_4$: 10, 50, 100, 300 or 500 µM

Control plants were treated with 5 ml water or in the case of menadione, with the appropriate concentration of DMSO. Plants were monitored over 5 days for cotyledon bleaching as a score of oxidative stress tolerance.

2.6.1.5 Salinity stress

Seedlings were grown and screened on vertical MS agar plates by the same root reorientation method as previously described for the drought screen (see Section 2.6.1.2). MS agar plates were supplemented with either 50, 100, 150 or 200 mM NaCl.

2.6.1.6 UV-B stress

Seeds were individually sown on horizontal MS agar plates using sterile cocktail sticks (15 seeds per plate and 1 plate per treatment per line). The seeds were evenly spaced such that the seedling leaves would not obscure one another. After 10 days growth, the seedlings were treated with either 0.5, 1 or 2 J/cm^2 of UV-B, by removing the plate lids and placing in a UV cross-linker set to the designated amount of energy. Although not treated with UV-B, control plant plates were placed in the UV cross-linker with their lids removed for the amount of time appropriate for each UV-B treatment (approximately 30 to 120 s). After each treatment the plates were quickly placed back in the growth chamber and their lids were resealed. The plants were then monitored daily for bleaching and growth retardation, and after 10 days their fresh weights were measured (pooled for each plate).

2.6.2 Biotic stress

2.6.2.1 *Pseudomonas syringae* inoculation (via dipping)

Plants were inoculated based on the dipping method described by Tornero and Dangl (2001). Approximately 50 seeds were sown on 42 mm peat plugs (1 plug per line per inoculation). The plugs were inverted for seed sowing so that the unbroken mesh faced upwards. After 11 days of growth, the plants were transferred to the growth chamber in the Category 2 Pathogen laboratory. The inoculations were then performed at day 14.

Approximately 24 h prior to inoculation, the *Pst* isolates (DC3000 and Avr13) were re-plated using a spreader to obtain a lawn of bacteria. After 24 h at 25 °C, 10 ml of 10 mM $MgCl_2$ was added to each plate. After 10 min, the bacterial suspension was washed out of the plates using a pipette. The OD_{600} was measured and samples were adjusted to OD_{600} = 0.05. The surfactant Silwet L-77 (Lehle Seeds) was then added to a final concentration of 200 µl/l.

To dip, the peat plugs were turned upside down and submerged for 10 s in the bacterial solution, approximately 1 cm above the soil. The leaf surfaces were checked for even coating with the bacterial suspension. A control mock inoculation was also performed with the equivalent concentration of $MgCl_2$ and Silwet L-77. The dipped plants were then placed back in the Category 2 growth chamber in transparent boxes with lids on (in order to maintain high humidity, since *P. syringae* infects the leaf via stomata; Goto, 1992). Plants were then examined for disease symptoms up to 5 days post-inoculation.

2.6.3 Hormone treatments

2.6.3.1 Auxin

Plants were screened using the method as advised by Professor Malcolm Bennett (personal communication; School of Biosciences, University of Nottingham, Loughborough, UK). Seedlings were germinated on vertical non-supplemented MS agar plates. After 4 days they were carefully transferred (using hooked forceps) onto vertical

MS agar plates supplemented with either 0.1, 1, or 10 µM 1-naphthaleneacetic acid (NAA). For each line to be tested, a total of 24 seedlings were screened per concentration (set up as previously shown in Figure 2.1). Control seedlings were also transferred onto non-supplemented vertical MS plates. The position of the root tip was marked and the seedlings were placed back in the growth chamber for a further 3 days. Root growth was then examined as a score of auxin sensitivity.

2.6.3.2 Ethylene

Seeds were sown directly onto vertical MS agar plates supplemented with 10 µM 1-aminocylcopropane-1-carboxylic acid (ACC), so as to increase root hair density via ectopic root hair production (Dolan, 2001). For each line to be tested, a total of 24 seedlings were screened (set up as previously shown in Figure 2.1). Control plants were sown onto non-supplemented MS agar plates. After 5 days the roots were examined under a dissecting microscope for altered length and root hair formation.

2.7 Plant abnormal development screen

The growth and development of the loss- and gain-of-function lines were examined throughout the plant life cycle based on the method described by Boyes *et al.* (2001).

Seeds were sown individually in a single row on vertical MS agar plates using a sterile cocktail stick (5 seeds of the test line and 5 of the control line per plate; 4 plates per line). Seedlings were photographed daily and characteristics monitored included: the size, shape and colour of cotyledons and leaves, root length and root branching. Root lengths were measured from digital photographs at days 7, 10 and 14 using the ImageJ image analysis software tool developed by Wayne Rasband (National Institute of Health, USA; *http://rsb.info.nih.gov/ij/*).

At day 14, seedlings were transferred onto peat plugs, and moved into the greenhouse. Characteristics monitored for differences to control plants included: the size, shape, colour and number of leaves, time of flowering, flower morphology and time to senescence.

2.7.1 Dark-induced senescence screen

Five-day old seedlings (20 per line) were cut at the hypocotyl base and placed in 0.2 ml microfuge tubes (Greiner Bio-One) filled to the top with water. The microfuge tubes were then placed in a box container, wrapped in foil and placed in the growth chamber. The extent and speed of seedling senescence was monitored up to 10 days later.

2.8 Nucleic acid extraction

2.8.1 Plant genomic DNA extraction

Approximately 30 (7-day old) seedlings or the unopened flower buds from two vigorously flowering shoots were collected in 1.5 ml microtubes and flash frozen in liquid nitrogen. The tissue was ground with a micropestle in 200 µl of Edwards' extraction buffer (see Appendix A1.1 for recipe) and briefly vortexed. The microtubes were then spun at 14,000 *g* for 3 min, and 150 µl of the resulting supernatant was added to 150 µl of 100 % (v/v) isoproanol in a fresh microfuge tube, and gently mixed by inversion. The microtubes were then left at room temperature for 5 min to allow the DNA to precipitate, and then spun at 14, 000 *g* for 5 min. The resulting supernatant was removed and the pellet left to air-dry for 10 min. Finally, the DNA was resuspended in 50 µl of TE buffer (see Appendix A1.2 for recipe).

2.8.2 Bacterial plasmid DNA purification

2.8.2.1 STET prep method

Crude bacterial plasmid DNA was obtained by the Sucrose-Tris-EDTA-Triton (STET) prep method. A single colony was used to inoculate 5 ml of liquid LB. The culture was left shaking overnight and the next day 1.5 ml was spun down at 10,000 *g* for or 30 s. The supernatant was discarded and the cell pellet was resuspended in 250 µl of STET buffer (pre-chilled on ice; see Appendix A1.3 for recipe). Then 20 µl of lysozyme (10mg/ml in STET buffer) was added and gently mixed by inversion. The microtube was incubated at 100 °C for 40 s. Next 270 µl of pre-chilled 5 M LiCl was added, mixed by

inversion and incubated on ice for 30 min. The microtube was then spun at 14,000 *g* for 15 min (at 4 °C), and the resulting pellet removed with a sterile cocktail stick. One ml of 100 % (v/v) ethanol (pre-chilled to -20 °C) was added to the remaining supernatant and incubated at -80 °C for 30 min, so as to allow precipitation of plasmid DNA. The microtube was then centrifuged at 14,000 *g* for 10 min (at 4 °C) and the supernatant discarded. The pellet was washed with 80 % (v/v) ethanol by gentle inversion prior to another 10 min 14,000 *g* centrifugation step at 4 °C. Finally, the supernatant was discarded and the DNA pellet left to air-dry for 10 min before resuspension in 50 µl of sterile water.

2.8.2.2 Mini-prep method

High purity, small scale bacterial plasmid DNA extraction was performed using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. In this method, overnight cultures were subjected to a modified alkaline-SDS lysis procedure, followed by adsorption of the DNA onto a silica membrane in the presence of high salts. Contaminants were then removed by a spin-wash step and the bound DNA was eluted in Tris-EDTA buffer.

2.8.2.3 Maxi-prep method

High purity, large scale bacterial plasmid DNA extraction was performed using the GenElute High Performance Plasmid Maxiprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. This kit works using the same principle as that of the mini-prep method (as described previously in Section 2.8.2.2), except it can recover up to 1.2 mg of plasmid DNA from a 150 ml overnight culture.

2.8.3 DNA extraction from agarose gels

Following agarose gel electrophoresis (described overleaf in Section 2.9.1), DNA bands were excised from the agarose gel by cutting the gel using a scalpel blade on a UV trans-illuminator (Ultra-Violet Products Ltd, Cambridge, Cambridgeshire, UK).

DNA fragments were purified via the GenElute Gel Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions. In this method, the agarose gel slices were first solubilised in an isopropanol and guanidium salt buffer. DNA was then adsorbed onto a silica membrane and contaminants were removed via an ethanol-based spin-wash step. Finally, DNA was eluted in a Tris buffer.

2.8.4 Plant RNA extraction

The RNeasy Plant Total RNA kit (Qiagen Ltd, Crawley, West Sussex, UK) was used to extract total plant RNA from 7 to 10 day old seedlings according to the manufacturer's instructions. In this method, the plant tissue was lysed and homogenised in the presence of highly denaturing guanidine isothiocyanate (which inactivates RNases). RNA was then bound to a silica-gel membrane whilst contaminants were washed away. The RNA was eluted in RNase-free water.

2.9 Nucleic acid size separation

2.9.1 Agarose gel electrophoresis of DNA

Gels were prepared by melting 1 % (w/v) electrophoresis grade agarose in 0.5 x TBE buffer (see Appendix A.2.1 for recipe) in a microwave oven. After cooling to approximately 50 °C, ethidium bromide (10mg/ml) was added to a final concentration of 5 µg/ml. The molten gel was poured into the gel tank an allowed to set.

DNA samples were loaded in DNA sample loading buffer (see Appendix A.2.2 for recipe) and 0.5 x TBE was used as the running buffer. Gels were run at 35 mA (constant current) to a satisfactory resolution (approximately 1 h). Nucleic acid bands were visualised on a UV trans-illuminator (at a wavelength of 254 nm). Fragment size was approximated by comparing positions with molecular size standards run on the same gel: either a 100 bp or 1 Kb ladder (NEB).

2.9.2 Formaldehyde agarose gel electrophoresis of RNA

Gels were made by melting 1 % (w/v) electrophoresis grade agarose (in autoclaved milliQ water) in a microwave oven. Once dissolved, the melted agarose was placed in a 55 °C oven, along with 0.6 % (v/v) formaldehyde (2.2 M) and 1 x MOPS buffer in a separate vesicle (see Appendix A.3.1 for recipe). After a minimum of 45 min, the two components were mixed and the gel was poured and left to set in a fume hood for 1 h.

RNA samples of 10 µg were completely dried down using a rotary evaporator (Eppendorf Ltd, Cambridge, Cambridgeshire, UK) and resuspended on ice in 5 µl of RNase-free water and 15 µl of RNA sample loading buffer (see Appendix A.3.2 for recipe). Immediately prior to loading, the RNA samples were denatured at 65 °C for 10 min and then quickly placed on ice. The running buffer was composed of 0.6 M formaldehyde and 1 x MOPS buffer. The gel was run at a constant voltage of 45 V until satisfactory resolution had occurred (approximately 3 h). The RNA bands were then visualised on a UV trans-illuminator.

2.10 Nucleic acid quantification

2.10.1 DNA quantification (low mass ladder comparison)

Following agarose gel electrophoresis (see Section 2.9.1 on previous page), DNA concentrations were estimated via the comparison of the intensity or UV fluorescence of ethidium bromide stained DNA, to bands of a known volume of DNA low mass ladder (Invitrogen).

2.10.2 RNA quantification

2.10.2.1 Spectrophotometry

RNA samples were diluted by 1:1000 and concentrations were determined by spectrophotometry (at a wavelength of 260 nm, where OD_{260} 1 = 40 µg RNA/ml). Water was used as a zero reference for the spectrophotometer (Cecil Instruments Ltd, Cambridge, Cambridgeshire, UK).

2.10.2.2 NanoDrop

Undiluted RNA concentrations were determined using a ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) according to the manufactuer's instructions. Water was used as a zero reference.

2.11 cDNA synthesis

Total plant RNA was reverse-transcribed using the Superscript II RNase H- Reverse Transcriptase enzyme (Invitrogen) according to the manufacturer's instructions. One µg of plant RNA was added to 1 µl oligo dT primer (500 µg/ml; Invitrogen) and made up to 12 µl with RNase-free water (Sigma-Aldrich) on ice. The microtube was then incubated at 70 °C for 10 min in order to denature the RNA and oligo dT primer. After this period, the microtube was quickly transferred to ice for 2 min to allow annealing of the oligo dT with the poly-adenylated RNA tails. A "cocktail" of 4 µl of 5 x First Strand Buffer (Invitrogen supplied with enzyme; see Appendix A.4.1 for recipe), 2 µl of 0.1 M DTT (Invitrogen; supplied with enzyme), 1 µl of 10 mM dNTPs (see Appendix A.4.2 for recipe) and 1 µl of Superscript II Reverse Transcriptase (200 U/µl) was added. The PCR machine was programmed to run at 42 °C for 50 min to allow cDNA synthesis, followed by 72 °C for 15 min to inactivate the enzyme. The resulting cDNA was diluted prior to use in PCR reactions to either 1:10 or 1:100.

For cDNA for use in the PCR amplification of gene-specific probes (see Section 2.15 later), RNA was used from 7-day old wild-type seedlings treated with 10 mM H_2O_2 for 3 h.

2.12 Amplification of DNA fragments (via polymerase chain reactions [PCR])

2.12.1 DNA polymerases

The DNA polymerase BioTaq (Bioline) was used for general PCR amplifications, whilst the proof-reading DNA polymerase, Pyrobest (Takara Bio, Shiga, Japan) was used for high accuracy applications.

2.12.2 Oligonucleotide primers

Primers (see Appendix B for a full list and details of those used) of at least 20 bp were designed to consist of at least 40 % guanine (G) and cytosine (C) bases and to have similar melting temperature (T_m) values, where T_m = [2 °C x (number of adenine (A) and Thymine (T) bases)] + [4 °C x (number of C and G bases)]. All primers were ordered from MWG Biotech AG (Ebersberg, Germany).

2.12.3 DNA template

Genomic DNA from wild-type seedlings or cDNA treated for 1 h with 10 mM H_2O_2 was used as the template in the PCR reactions. The optimum concentration of template DNA varied between transcripts (dependent on the level of expression) and between primer combinations (dependent on the primer efficiency). This was established by testing 1:10 and 1:100 cDNA dilutions for each primer combination.

2.12.4 PCR reaction mixes

PCR reaction mixes were determined according the DNA polymerase manufacturer's instructions (Bioline or Takara) using the supplied buffers.

2.12.5 PCR cycles

For PCR reactions with BioTaq DNA polymerase, the PCR machine was programmed to hold each temperature (see below) for 5 min in the first cycle to allow complete denaturation of the template. This was followed by 25 to 35 cycles of 1 min at 95 °C for denaturation, 1 min per 1 Kb at 50 to 66 °C for annealing and 1 min at 72 °C for extension. After the specified number of cycles, the samples were left for 10 min at 72 °C to complete the final extension cycle.

For PCR reactions with Pyrobest DNA polymerase, the PCR machine was programmed to 25 to 35 cycles of 10 s at 98 °C, 30 s at 50 to 66 °C and 1 min at 72 °C. After the specified number of cycles the prgram finished with a final step of 10 min at 72 °C.

The annealing temperatures (T_m) of all the primers used were optimised empirically and are listed in Appendix B.

Where a PCR machine was used without a heated lid, reaction mixes were overlaid with 40 µl of Chill-out liquid wax (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) to prevent evaporation.

2.13 Restriction enzyme digestion of DNA

Restriction digests were performed according to the restriction enzyme manufacturer's instructions, using the recommended buffer (supplied) and incubation temperature. For double digestions with restriction enzymes requiring different buffers, digestions were performed sequentially, such that the enzyme with the lower salt buffer was incubated first and the buffer condition adjusted before incubation with the second enzyme. For all digestions the volume of enzyme was ≤ 10 % of the total volume.

2.14 DNA sequencing

2.14.1 DNA sequencing reaction

DNA sequencing was performed using the Big Dye reaction mix (Applied Biosystems, Warrington, Cheshire, UK). The reaction was prepared as follows: 250 ng of purified DNA, 1 µl of primer (4 pmol/µl), 4 µl of Big-Dye reaction mix, 4 µl of 2.5 x sequencing buffer (see Appendix A.5.1 for recipe) and water to a final volume of 20 µl. The reaction mixture was then placed in a PCR machine programmed to 96 °C for 1 min, then 25 cycles of: 30 s at 96 °C, 15 s at 50 °C and 4 min at 60 °C. The resulting reaction product was then precipitated by ethanol (see Section 2.14.2 below). All sequencing was performed by the DNA Sequencing Service, Sir William Dunn School of Pathology, University of Oxford, Oxford, UK.

2.14.2 Ethanol precipitation

DNA samples were precipitated by addition of 50 µl of 95 % (v/v) ethanol and 2 µl of NaOAc (pH 5.2) at room temperature and left for 15 min. Samples were then centrifuged at 15,000 *g* for 20 min and the supernatant immediately removed. Then DNA pellet was washed with 70 % (w/v) ethanol and left to air-dry at room temperature.

2.14.3 Sequence analysis

DNA sequence data was analysed using internet-based software: the Multalin multiple sequence alignment tool (*http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html*) and the BoxShade multiple alignments designer (*http//:www.ch.embnet.org/software/BOX_form.html*). Database similarity searches were carried out using the BLAST search tool available at the Arabidopsis Information Resource database (TAIR; *http//:www.arabidopsis.org/Blast/index.jspfor nucleotide*) to search for homology.

2.15 Gene-specific probes

DNA probes were designed to be gene-specific using both the sequence information and the BLAST nucleotide search system available on TAIR database (*http//:www.arabidopsis.org/Blast/index.jspfor nucleotide*). From this, gene-specific primers (see Appendix B.2) were designed and used to amplify the probes via PCR (as described previously in Section 2.12). The probes were then cloned into the vector pBlueScript II SK (+) (Stratagene, Amsterdam, The Netherlands) (for vector map see Appendix C.1) and sequenced in both directions using M13 primers (Appendix B.1) prior to use in northern blot analyses (see Section 2.16 below).

2.16 Northern blot analysis

2.16.1 Transfer of RNA to nylon membrane

RNA was transferred by capillary action from a formaldehyde agarose gel (as described previously in Section 2.9.2) to a positively charged nylon membrane (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) as follows: the RNA gel was placed on a glass plate covered in filter paper (Whatman) so as to form a wick in contact with a reservoir of 20 x SSC (sodium citrate/sodium chloride buffer; see Appendix A.6.1 for recipe). Positively charged nylon membrane (pre-wet in water first and then 1 x SSC) was laid on top of the gel and any air bubbles were expelled by rolling a glass rod over the surface. On top of the membrane were placed (in this order): two sheets of filter paper (pre-wet in 1 x SSC), a layer of 100 paper tissues, a glass plate and a weight (a 250 ml Duran bottle filled with water) and left overnight.

The following day, the blotting system was dismantled and the RNA was fixed to the membrane by UV cross-linking both sides of the membrane using a UV Stratalinker 2400 (Stratagene) on the auto cross-link setting (120 mJ/cm^2 for 30 to 45 s).

2.16.2 Synthesis of radio-labelled DNA probes

Gene-specific DNA probes (50 ng) (described previously in Section 2.15) were first denatured in a microfuge tube via a 5 min incubation at 95 °C, and then transferred to ice for 2 min. The denatured DNA probe was then added to a Ready-To-Go DNA Labelling Bead (Amersham) according to the manufacturer's instructions. In this method, each bead provides DNA polymerase and random sequences of oligomers which anneal to random sites on the DNA probe and thus serve as primers for DNA synthesis (oligolabelling). Then 2.5 µl (25 µCi) of [α-^{32}P] dCTP was added and the tube incubated for 1 h at 37 °C. DNA synthesis occurs in the presence of labelled nucleotide to generate labelled DNA. Un-incorporated nucleotides were subsequently removed by spinning the solution for 2 min at 3000 *g* through a ProbeQuant G-50 Microcolumn (Amersham). The eluted labelled probe was then denatured by a 5 min incubation at 95 °C, and then chilled on ice before being added to the pre-hybridisation solution (see Appendix A.6.2 for recipe).

For the northern blots carried out at Durham University, the DNA probes were labelled via the Rediprime II DNA random prime labelling system (Amersham) according to the manufacturer's instructions. In this method, random sequence hexanucleotides are used to prime DNA synthesis on denatured template DNA at numerous sites along its length. The primer-template complex serves as a substrate for the Klenow fragment of DNA polymerase I. By replacing a non-radioactive nucleotide with the radio-labelled equivalent in the reaction mixture, newly synthesised DNA is made radioactive. A total of 25 ng of gene-specific DNA probe in 45 µl volume of 10 mM Tris HCl (pH 8.0, 1mM EDTA) was denatured for 5 min at 95 °C. After chilling on ice for 5 min, it was added to the Rediprime II DNA labelling tube. Then 2.5 µl of [α-^{32}P] dCTP was added and incubated at 37 °C for a minimum of 10 min. To stop the reaction, 5 µl of 0.2 M EDTA was added. Un-incorporated nucleotides were removed via a ProbeQuant G-50 Microcolumn as previously described.

2.16.3 Northern hybridisation

Northern hybridisations were carried out with ^{32}P-dCTP labelled DNA probes in a rotary hybridisation oven (ThermoFisher Scientific, Reading, Berkshire, UK) using boro-silicate

hybridisation bottles (ThermoFisher Scientific). Membranes were pre-hybridised for 4 h at 42 °C, in 50 ml of pre-hybridisation solution (see Appendix A.6.2 for recipe). The denatured radioactively-labelled probe (as described above in Section 2.16.2) was added to the pre-hybridisation solution and the membranes were hybridised overnight at 42 °C at a constant rotation (5-15 rpm).

2.16.4 Post-hybridisation washes

Following overnight northern hybridisation, membranes were washed as follows: twice for 15 min in wash solution 1, twice for 15 min in wash solution 2 and twice for 15 min in wash solution 3 (see Appendix A.6.4, A.6.5 and A.6.6 for wash solutions recipes). Wash solutions were preheated to 42 °C and all washes were carried out at 42 °C in the hybridisation oven at a constant rotation (5-15 rpm). Washed membranes were then sealed in cling film.

2.16.5 Detection of hybridisation

Radioactivity intensity was initially detected on the washed membranes using a Geiger counter. Depending on the intensity, membranes were placed for 4 to 24 h on a Molecular Imager FX Imaging Screen (Bio-Rad Laboratories) which had previously been erased using a Screen Eraser-K light box (Bio-Rad Laboratories). The exposed image was detected by infra-red laser scanning of the imaging screen using a Molecular Imager FX scanner (Bio-Rad). The image was then analysed with Quantity One image analysis software (Bio-Rad).

Following this, the membrane was transferred onto Biomax XAR film (Kodak, Rochester, New York, USA) within an autoradiography cassette with intensifying screens (CAWO Photochemisches Erk GmbH, Strobenhausen, Germany). Depending on the intensity of the radioactivity the cassette was left at -80 °C for 2 h to 14 days. The film was then developed using a Curix 60 X-ray processor (Agfa Ltd, Brentford, Middlesex, UK) according to the manufacturer's instructions.

For northern blots performed at Durham University, signal was detected using a Typhoon 9400 phosphorimager and supplied screens according to the manufacturer's instructions (Amersham). The image was then analysed using the supplied Image Quant TL software (Amersham).

2.16.6 Removing radioactive probe from membrane

Membranes can be stripped of the radio-labelled probe and subsequently re-probed. Thus, when desired, probed membranes were stripped by briefly washing in water and incubating for 1 h at 68 °C in preheated strip solution (see Appendix A.6.7 for recipe). The membranes were finally rinsed in 2 x SSC and checked for remaining radioactivity using a Geiger counter and Molecular Imager FX System (as previously described above in Section 2.16.5).

2.17 Microarray analysis (via indirect labelling)

2.17.1 Preparation of microarrays

Arabidopsis 70-mer oligonucleotide microarrays printed with the Operon Arabidopsis version 3.0 AROS oligo set (*http//:www.arizona.edu/microarray*; University of Arizona, Tucson, Arizona, USA) were used. The microarray slides were baked for 40 min at 80°C and UV cross-linked twice at 300 mJ in a Stratalinker 2400 (Stratagene). Immediately prior to use, slides were pre-hybridised for 20 min at 65 °C in a coplin jar containing 3.5 x SSC, 0.1 % (w/v) SDS and 10 mg/ml bovine serum albumin (BSA). Slides were then washed for 1 min in water and for 1 min in isopropanol, and finally dried with an airbrush.

2.17.2 cDNA synthesis and labelling

RNA was isolated (as described previously in Section 2.8.4) and quantified by the NanoDrop method (as described in Section 2.10.2.2). RNA quality was determined using an Agilent 2100 Bioanalyser (Agilent Technologies Ltd, Wokingham, Berkshire, UK) according to the manufacturer's instructions. Two µg of total RNA was labelled using the

Genisphere 3DNA 900 indirect labelling kit (Genisphere, Hatfield, Pennsylvania, USA) according to the manufacturer's instructions (*http//:www.genisphere.com/pdf/array900_manual_05_16_05.pdf*). In this method the fluorescent dye is part of the 3DNA capture reagent (dendrimer), so it does not have to be incorporated during cDNA preparation. The resulting signal is largely independent of base composition or transcript length as each 3DNA dendrimer contains approximately 850 fluorescent dyes.

2.17.3 Microarray hybridisation

cDNA was hybridised to the microarray slide for 16 h at 55 °C under a 22 x 60 Lifterslip (Erie Scientific Company, Portsmouth, New Hampshire, USA) using an Advalytix SlideBooster SB400 (Advalytix AG, Munich, Germany) set to a power of 27 and a pulse/pause of 3/7. After washing (see Section 2.17.4 below), the slide was then hybridised for 4 h with the 3DNA dendrimer capture reagents (Genisphere).

2.17.4 Microarray washing

After each hybridisation the arrays were washed for 10 min at 55 °C in 2 x SSC and 0.2 % SDS buffer, followed by room temperature washes of 2 x SSC followed by 0.2 x SSC. Slides were dried with an airbrush after each wash. All washes were performed in a shaking incubator set to 150 rpm.

2.17.5 Microarray scanning and detection

Hybridised slides were scanned using a PerkinElmer ScanArray Express HT scanner (PerkinElmer LAS Ltd, Beaconsfield, Buckinghamshire) set to 100 % laser power and a variable photomultiplier tube (PMT) setting. The PMT setting was determined by automatic sensitivity calibration with a signal target ratio of 98 % (arrays hybridised with 2 µg of RNA generally require a PMT setting of around 55 %).

The resulting image files were loaded into the analysis program BlueFuse version 3.2 (BlueGnome Ltd, Cambridge, Cambridgeshire, UK). Artefacts and missing data were "flagged out" and removed from the data sets both by manual flagging and by automatic

exclusion using an empirically determined BlueFuse pON score. According to Snyder and Saunders (2006), "the pON score non-comparatively evaluates the data from each microarray spot and uses this to report a probability of there being a hybridisation signal for each spot. Unlike ratio-metric methods, this score is not dependent upon or influenced by the signal in the other channel. A pON score of zero indicates that there is no evidence for spot hybridisation, while a score of one indicates that there is strong evidence that the spot has hybridised".

2.17.6 Microarray data normalisation and analysis

2.17.6.1 Dchip microarray data extraction

Data from the H_2O_2 microarray (that had been performed by NASC) was extracted using DChip software designed for Affymetrix microarray analysis (*http//:www.dchip.org*; developed by Cheng Li, Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA). CEL data files were imported, normalised and modelled using default settings of DChip. For details please refer to Results Chapter 3.

2.17.6.2 Indirectly labelled microarrays

Data manipulation of indirectly labelled over-expressor microarrays was performed using Microsoft Excel. If the PMT power settings are perfectly matched during scanning normalisation is not usually required. Where it was required, the global median of all the spots was calculated (excluding the top and bottom 5% by intensity, those flagged to be excluded from further analysis and blank/control spots). After this, the channel with the lower of the two intensities was corrected by multiplying all the values by the ratio of the medians of the two channels

2.17.7 Ontological data classification

The TAIR GO ontology functional characterisation of gene lists was performed using the default settings (*http//:www.arabidopsis.org/tools/bulk/go/index.jsp*). Lists were further

analysed for over- or under-representation using the "Classify Genes" function of the DChip software.

2.17.8 Promoter motif analysis

Analysis of both 1000 bp and 500 bp of promoter sequences (downloaded from the TAIR database) was performed using the oligo analysis, pattern assembly and DNA pattern matching tools available online at the Regulatory Sequence Analyses Tools website (RSAT) (*http://rsat.ulb.ac.be/rsat*) according to the developer's instructions (Van Helden, 2003).

2.18 Cloning of DNA fragments

2.18.1 Plasmids

pBlueScript II SK (Stratagene) was used to clone gene-specific probes (for vector map see Appendix C.1). Full length coding sequences were cloned using the Gateway entry vector pENTR/D-TOPO (Invitrogen; for vector map see Appendix C.2) and the 35*S* destination vector pK2GW7 (Karimi and Depicker, 2002; for vector map see Appendix C.3).

2.18.2 Ligation

DNA fragments were ligated using T4 DNA ligase (NEB) in 1 x ligase buffer (supplied) with 1 mM of added adenosine 5'-triphosphate (ATP; NEB) according to the manufacturer's instructions. Reactions were performed overnight at 16 °C. A 1:3 molar ratio of linearised plasmid (100 ng) to potential insert was used.

2.18.3 Gateway recombination

All Gateway recombination reactions were performed according to the manufacturer's instructions (Invitrogen) using the half volume stated. The pENTR/SD/D-TOPO

Directional Cloning Kit (Invitrogen) supplied with One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) was used to clone blunt-ended coding sequences (obtained from PCR or cDNA clones) into the Gateway entry vector pENTR/D-TOPO. Once in the entry vector, the coding sequence was transferred to the 35*S* over-expression destination binary vector (pK2GW7) in a recombination reaction using the LR Clonase enzyme (Invitrogen). The pK2GW7 destination vector was propagated in *E. coli* strain DB3.1 (Invitrogen) that contains a gyrase mutation which renders it resistant to the lethal effects of the CcdB protein present in the un-reacted vector (Bernard and Couturier, 1992).

2.19 Transformation

2.19.1 Transformation into competent bacterial cells

2.19.1.1 Transformation of *E. coli*

A 50 µl aliquot of DH5α chemically competent *E. coli* cells (Invitrogen) was thawed on ice before addition of 1 to 10 ng of DNA in a 1 to 5 µl volume and tapped gently to mix. After a 30 min incubation on ice, the cells were heat-shocked at 37 °C for 30 s and placed immediately onto ice for 2 min. Then 950 µl of pre-warmed liquid LB medium was added and the tube was shaken at 37 °C for 1 h at 225 rpm. Aliquots of 20 to 200 µl of cells were plated out onto LB agar plates containing the appropriate antibiotics. Transformed recombined colonies were then selected via resistance to the antibiotic in the selective medium.

For bacterial blue/white selection plates, 476 µg/ml (2 mM) of the chemical inducer isopropyl-β-D-thiogalactopyranoside (IPTG; Melford) and 40 µg/ml of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Melford) were added in addition to the appropriate antibiotic. Successful recombinants resulted in white colonies whilst non-recombinants produce blue colonies.

2.19.1.2 Transformation of *A. tumefaciens*

A 100 µl aliquot of frozen *A. tumefaciens* C58C1 competent cells (OD_{600} of 0.5 to 1.0 in 20 mM $CaCl_2$) was thawed on ice. Approximately 1 µg of DNA was added (in a volume ≤ 12 µl) and the cells heat-shocked for 5 min at 37 °C. After addition of 1 ml LB, the tubes were shaken gently for 2 to 4 h at 28 °C. Subsequently, the cells were pelleted by centrifugation and all but 200 µl of supernatant was removed. The cell pellet was re-suspended in the remaining supernatant and the suspension plated out onto LB agar plates containing rifampicin (to inhibit growth of bacteria other than the resistant *A. tumefaciens*) and other appropriate antibiotics for transformant selection.

2.19.2 Transformation of plants

2.19.2.1 *A. tumefaciens*-mediated transformation of *A. thaliana* (by floral dip)

Wild-type Arabidopsis plants were grown up on peat plugs and their primary flowering bolt was clipped, so as to cause numerous secondary flowering stems to emerge. Seven to ten days after clipping off the primary bolts, an overnight culture of *A. tumefaciens* cells transformed with the appropriate plasmid containing a gene of particular interest was used to inoculate a 200 ml flask of LB (at a 1:100 dilution) supplemented with the appropriate antibiotics. The culture was grown to an OD_{600} of 0.8 to 1.2 and cells were harvested by centrifugation at 3000 *g* at room temperature. Cells were then re-suspended in the same volume of dipping medium: 5 % (w/v) sucrose and 0.05 % (v/v) Silwet L-77, as described by Clough and Brent (1998). The aerial parts of the Arabidopsis plants were completely submerged in the cell suspension for 5 s. Subsequently, the plants were laid sideways onto a tray with moistened paper towel, covered in plastic wrap (to maintain high humidity) and returned to the greenhouse. After 24 h they were then uncovered, placed in an upright position and left to set seed.

Seeds harvested from dipped Arabidopsis plants were bleach sterilised (as described previously in Section 2.2.2.2) and plated out on to MS agar supplemented with timentin and the appropriate antibiotics. Resistant plants were then transferred to peat plugs and grown to maturity.

2.20 Suppliers

Advalytix AG, Sauerbruchstrabe 50, 81377 Munich, Germany. *http//:www.adalytix.com*

Agfa Ltd, 27 Great West Road, Brentford, Middlesex, TW8 9AX, UK. *http//:www.agfa.com*

Agilent Technologies Ltd, 710 Wharfedale Road, Winnersh Triangle, Wokingham, Berkshire, RG41 5TP, UK. *http//:www.agilent.com*

Amersham Plc, Little Chalfont, Buckinghamshire, HP7 9NA, UK. *http//:www.amersham.com*

Applied Biosystems, Lingley House, 120 Birchwood Boulevard, Warrington, Cheshire, WA3 7QH, UK. *http//:www.appliedbiosystems.com*

BetaTech bvba, New Yorkstraat 4, B-9000 Gent, Belgium. *http//:www.arasystem.com*

BDH Laboratory Supplies Ltd, VWR International Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, UK. *http//:www.bdh.com*

Bioline Ltd, 16 The Edge Business Centre, Humber Road, London, NW2 6EW, UK. *http//:www.bioline.com*

Bio-Rad Laboratories Ltd, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX, UK. *http//:www.bio-rad.com*

BlueGnome Ltd, Breaks House, Mill Court, Great Shelford, Cambridge, Cambridgeshire, CB22 5LD, UK. *http//:www.cambridgebluegnome.com*

CAWO Photochemisches Werk GmbH, P.O. Box 1129, 86521 Schrobenhausen, Steingriffer Str. 2-6, 86529 Schrobenhausen, Germany. *http//:www.cawo.com*

Cecil Instruments Ltd, Milton Technical Centre, Milton, Cambridge, Cambridgeshire, CB4 6AZ, UK. *http//:www.cecilinstruments.com*

Duchefa Biochemie BV, A. Hofmanweg 71, 2031 BH Haarlem, The Netherlands. *http//:www.duchefa.com*

Eppendorf Ltd, Endurance House, Chivers Way, Histon, Cambridge, Cambridgeshire, CB24 9ZR, UK. *http//:www.eppendorf.com*

Fisher Scientific Ltd, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, UK. *http//:www.fisher.co.uk*

Genisphere Inc, 2801 Sterling Drive, Hatfield, Pennsylvania 19440, USA. *http//:www.genisphere.com*

Greiner Bio-One Ltd, Brunel Way, Stroudwater Business Park, Stonehouse, Gloucestershire, GL10 3SX, UK. *http//:www.greinerbioone.com*

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, Renfrewshire, PA4 9RF, UK. *http//:www.invitrogen.com*

Jiffy Products Ltd, P.O. Box 329, Winchester, Hampshire, SO23 9WQ, UK. *http//:www.jiffypot.com*

Kodak Ltd, Hemel One, Boundary Way, Hemel Hempstead, Hertfordshire, HP2 7YU, UK. *http//:www.kodak.com*

Lehle Seeds, 1102 South Industrial Boulevard, Suite D, Round Rock, Texas 78681, USA. *http//:www.arabidopsis.com*

Melford Laboratories Ltd, Bildeston Road, Chelsworth, Ipswich, Suffolk, IP7 7LE, UK. *http//:www.melford.co.uk*

MWG Biotech AG, Anzingerstr. 7a, 85560 Ebersberg, Germany. *http//:www.mwg-biotech.com*

NanoDrop Technologies, 3411 Silverside Road, Bancroft Building, Wilmington, Delaware 19810, USA. *http//:www.nanodrop.com*

New England Biolabs (NEB), 75-77 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY, UK. *http//:www.neb.com*

Nikon Ltd, 380 Richmond Road, Kingston upon Thames, Surrey, KT2 5PR, UK. *http//:www.nikon.com*

PerkinElmer LAS Ltd, Chalfont Road, Seer Green, Beaconsfield, Buckinghamshire, HP9 2FX, UK. *http//:www.perkinelmer.com*

Qiagen Ltd, Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK. *http//:www.qiagen.com*

Roche Diagnostics Ltd, Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, UK. *http//:www.roche-applied-science.com*

Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK. *http//:www.sigmaaldrich.com*

Stratagene, Gebouw Califonia, Hogehilweg 15, 1101 CB Amsterdam Zuidoost, The Netherlands. *http//:www.stratagene.com*

Takara Bio, Seta 3-4-1, Otsu, Shiga, 520-2193, Japan. *http//:www.takara-bio.com*

Terumo Ltd, Tamesis, The Causeway, Egham, Surrey, TW20 9AW, UK. *http//:www.terumomedical.com*

ThermoFisher Scientific, Bath Road, Reading, Berkshire, RG5 7PR, UK. *http//:www.thermofisher.com*

Ultra-Violet Products Ltd, Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge, Cambridgeshire, CB4 1TG, UK. *http//:www.uvp.com*

Whatman International Ltd, Springfield Mill, James Whatman Way, Maidstone, Kent, ME14 2LE, UK. *http//:www.whatman.com*

Chapter 3
Regulation of Arabidopsis gene expression in response to H_2O_2 treatment

3.1 Introduction

As reviewed in Chapter 1, many stimuli can induce ROS generation in plants, and ROS in turn, can induce gene expression changes (e.g. defence genes). Therefore, ROS can potentially regulate many signalling pathways and play a key role in tolerance to multiple environmental stresses. However, relatively little is known about the responses downstream of ROS, and in particular how the ROS signal is perceived and transduced.

Components of ROS signalling have been revealed by mutagenesis and pharmacological studies. Moreover, with the recent advances in genome-wide cDNA microarrays, it has become possible to profile changes in transcript levels of thousands of genes at a given time point. Several reports have provided such inventories of ROS-regulated genes. For example, elevated ROS levels have been shown to induce a reorientation of the transcriptome of bacteria (Mostertz *et al.*, 2004), yeast (Causton *et al.*, 2001) and mammalian cell lines (Yoneda *et al.*, 2001; Suzuki *et al.*, 2002). This has also been demonstrated in plants, for example Arabidopsis cell cultures treated with exogenous H_2O_2 (Desikan *et al.*, 2001). More recently, transcriptomic profiles have been produced for mutant or transgenic plants in which the activity of a particular antioxidant enzyme is reduced or completely abolished. Examples in Arabidopsis include plants mutated in genes encoding APX (Pnueli *et al.*, 2003; Davletova *et al.*, 2005), CAT (Vandenabeele *et al.*, 2004; Vanderauwera *et al.*, 2005), Cu/ZnSOD (Rizhsky *et al.*, 2003) and AOX (Umbach *et al.*, 2005). A microarray experiment has also been performed using the conditional *fluorescent* (*flu*) mutant of Arabidopsis (op den Camp *et al.*, 2003) which accumulates the photosensitiser protochlorophyllide in the dark, and upon re-illumination, generates $^1O_2$ in plastids (Meskauskiene *et al.*, 2001). These transcriptome studies serve as a powerful tool for the investigation of ROS signalling and may also reveal specificity to different ROS species.

This chapter focuses on the analysis of a microarray experiment performed on Arabidopsis seedlings treated with exogenous H_2O_2, in order to identify candidate H_2O_2-regulated signalling genes.

The aim of this chapter was to:

- Investigate gene expression changes by analysis of a microarray experiment performed on H_2O_2-treated Arabidopsis seedlings
- Analyse upstream promoter sequences of the H_2O_2-regulated genes in order to identify potential transcription factor binding sites
- Use this microarray data to identify genes encoding potential signalling components operating downstream of H_2O_2
- Select specific candidate H_2O_2-signalling genes for further study
- Confirm the effects of H_2O_2 on the expression of the candidate genes via northern blot analyses

3.2 Results

3.2.1 Microarray analysis of H_2O_2-treated plants

Raw data generated from a microarray experiment performed by a colleague (Maike Rentel, University of Oxford, Oxford, UK) was analysed in order to investigate transcript level changes of 7-day old wild-type Arabidopsis seedlings resulting from a 3 h 10 mM H_2O_2 treatment (Materials and Methods 2.5). This microarray experiment was originally designed to investigate the role of the OXI1 protein kinase in H_2O_2 signal transduction. The original aim of the microarray was to compare gene induction in an *oxi1* homozygous knock-out line to that of the wild-type (ecotype Wassilewskija-2; WS-2) following H_2O_2 treatment (for full details please refer to NASCArray experiment 28 at *http://www.affymetrix.arabidopsis.info.narrays/experimentbrowse.pl*).

Affymetrix ATH1 Arabidopsis Genome Array slides, containing probes sets representing approximately 22,000 genes, were used. The Arabidopsis Information Resource (TAIR) (*http://www.arabidopsis.org/index.jsp*) version 6 genome release (2007) contains a total of 31,407 genes (including pseudogenes and non-coding RNA genes) of which 26,751 are protein coding genes. Thus approximately 82% of the protein coding *Arabidopsis thaliana* genome was represented on the array.

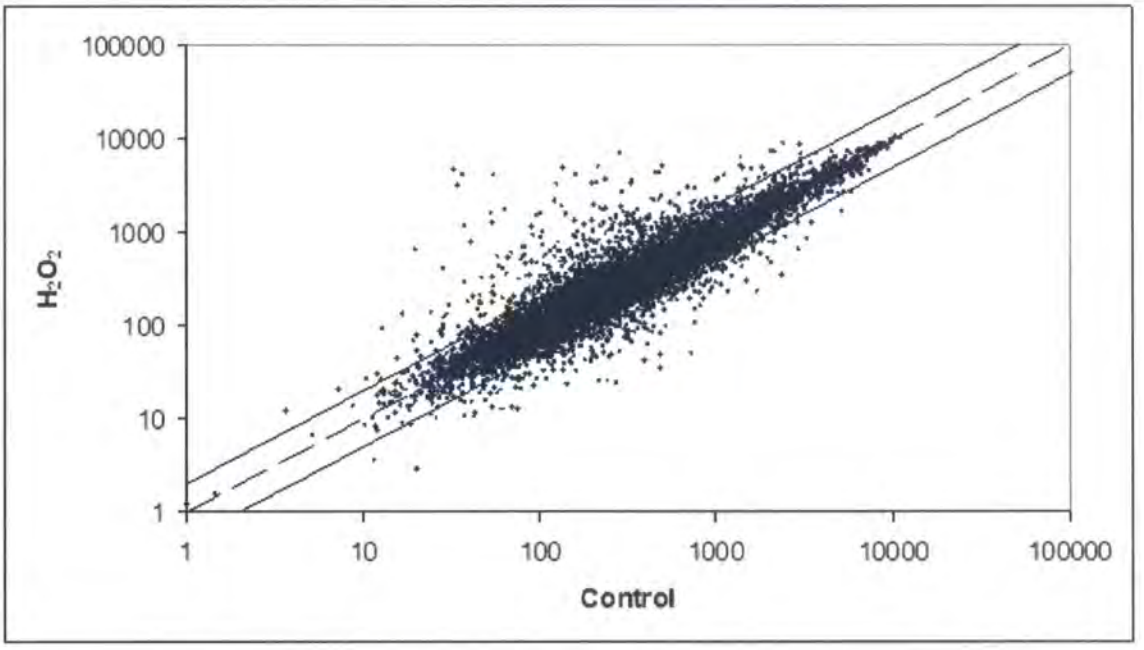
The raw data (in the form of CEL files) supplied by the Nottingham Arabidopsis Stock Centre (NASC) for H_2O_2-treated and control wild-type plants (one slide each) was normalised and modelled using DChip software (*http://www.dchip.org*; Materials and Methods 2.17.6.2). Probe sets were included or excluded from further analysis depending on their detection call. Only probes with present calls across both H_2O_2 and water control slides were used. Fold changes were calculated from signal value ratios. Although only one slide was used per treatment, similar values for the majority of genes were also obtained in the slides for the *oxi1* mutant performed in parallel (data not shown). Differentially expressed probe sets that had a fold change equal or greater than 2.00 were extracted for further analysis.

A global depiction of the changes in expression of all the probe sets on the wild-type microarray is shown overleaf in Figure 3.1. This is a comparison between only two slides, and

so the data in the Figure 3.1 appear somewhat noisy as a result. Expression appeared unchanged following H_2O_2 treatment for the majority of transcripts, when 2-fold was used as a cut-off ratio. A total of 13,165 probe sets had detection calls of present across both the wild-type H_2O_2-treated and control slides. Of these, 895 probe sets (6.81%) had a change in expression equal or greater than 2-fold in response to H_2O_2. A similar number of probe sets were up- or down-regulated in response to H_2O_2; 483 (3.68%) and 412 (3.13%) respectively. The fold change ratios reached higher values in those up-regulated (90 probe sets with ≥5-fold and of those 31 ≥10-fold) compared to those down-regulated (24 probe sets ≥5-fold and of those 3 ≥10-fold).

Figure 3.1

Scatter plot of normalised expression values for all present detected probe sets on the WS-2 wild-type microarray.



The dashed diagonal line represents no change, whilst the solid diagonal lines represent 2-fold up- and down-regulation ratio cut-offs.

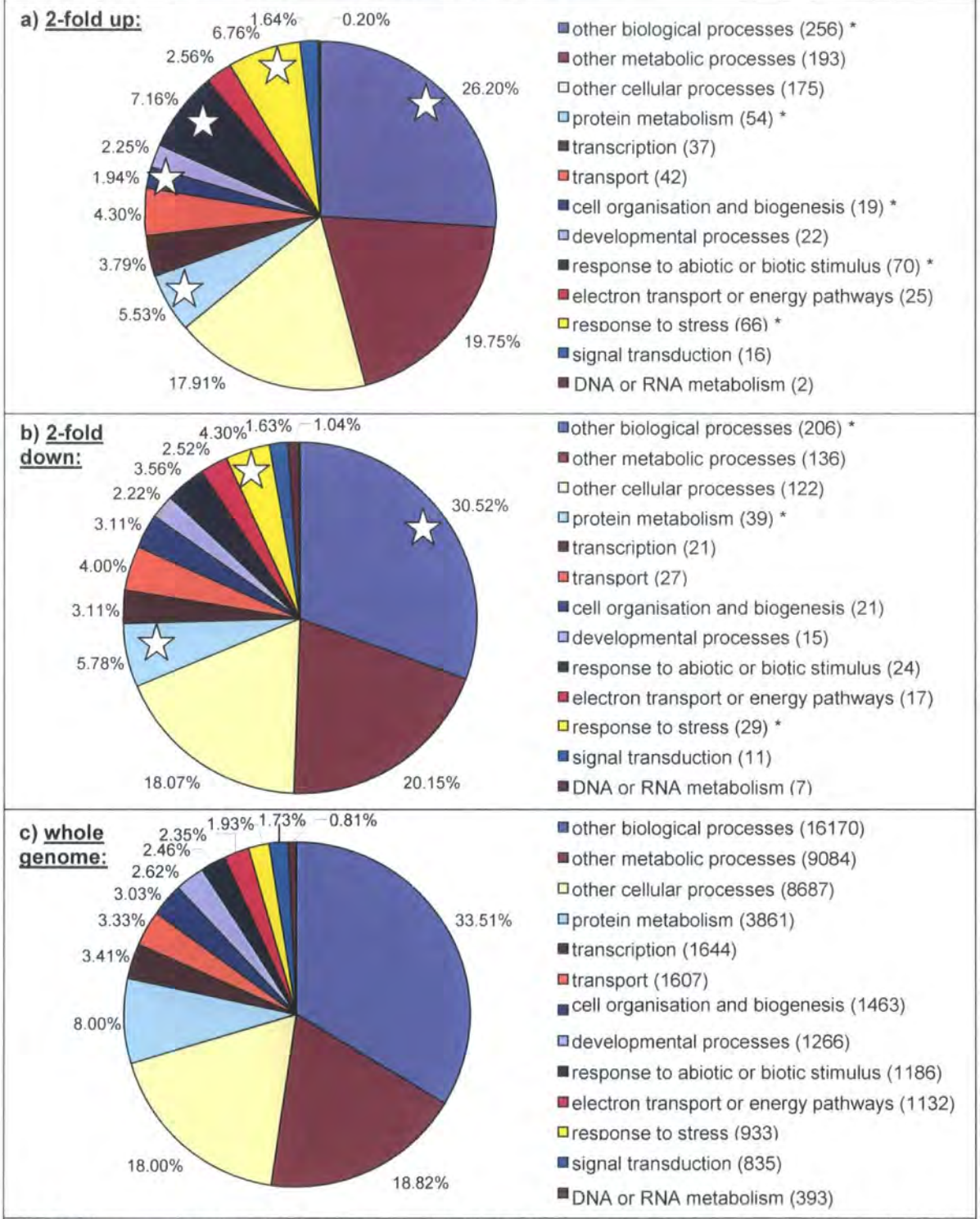
3.2.2 Functional classification of genes

To determine whether H_2O_2 regulates the differential expression of particular classes of genes, a functional classification of both the H_2O_2 up- and down-regulated probe sets was performed. The bulk Gene Ontology (GO) annotation download tool available at the TAIR 6 database was used to group genes into broad categories based upon their GO annotations (*http://www.arabidopsis.org/tools/bulk/index.jsp*). The corresponding figures are shown on the following 3 pages. Gene products were classified into three aspects; biological process (Figure 3.2), molecular function (Figure 3.3) and cellular components (Figure 3.4). For comparison, the "whole genome categorisation" function also available at the TAIR 6 database was used to retrieve GO functional categories for the entire *Arabidopsis thaliana* genome. Significantly over- and under- represented functional categories within the 2-fold up- or down-regulated genes are displayed in Table 3.1 (page 70).

As might be expected, the proportion of probe sets corresponding to genes involved in response to stress and abiotic/biotic stimuli were significantly over-represented. Transcription factor activity was also enriched indicating that the subsequent expression of further genes is likely at later time points. However, since these functional categories were automatically derived from the TAIR database, the use of this information for biological interpretation of the expression data is limited. Therefore, the lists of genes 2-fold up- or down-regulated were assigned to more detailed functional categories based on the ontological classification tool provided by MapMan (Thimm, *et al.*, 2004; Usadel *et al.*, 2005) at the Plant Proteome Database (PPDB; *http://ppdb.tc.cornell.edu/searchacc.aspx*). The full gene lists are shown in Appendices D1 and D2. Some of these genes and their roles in response to H_2O_2 are discussed later in more detail in Section 3.3.

It should be noted that due to annotation updating, these H_2O_2-regulated gene lists are considerably more comprehensive than those produced at the time of candidate signalling gene selection, which was based on The Institute for Genomic Research (TIGR) version 4 genome release (2003; http://www.tigr.org/tdb/e2k1/ath1/).

Figure 3.2
Functional categorisation of genes by GO **biological process**.



a) 2-fold up-regulated on H_2O_2 microarray **b)** 2-fold down-regulated on H_2O_2 microarray **c)** Whole *Arabidopsis thaliana* genome. Stars denote significant categories at $p < 0.01$ (hypergeometric test). Raw values are shown in brackets.

Figure 3.3

Functional categorisation of genes by GO **molecular function**. (Detail as Figure 3.2).

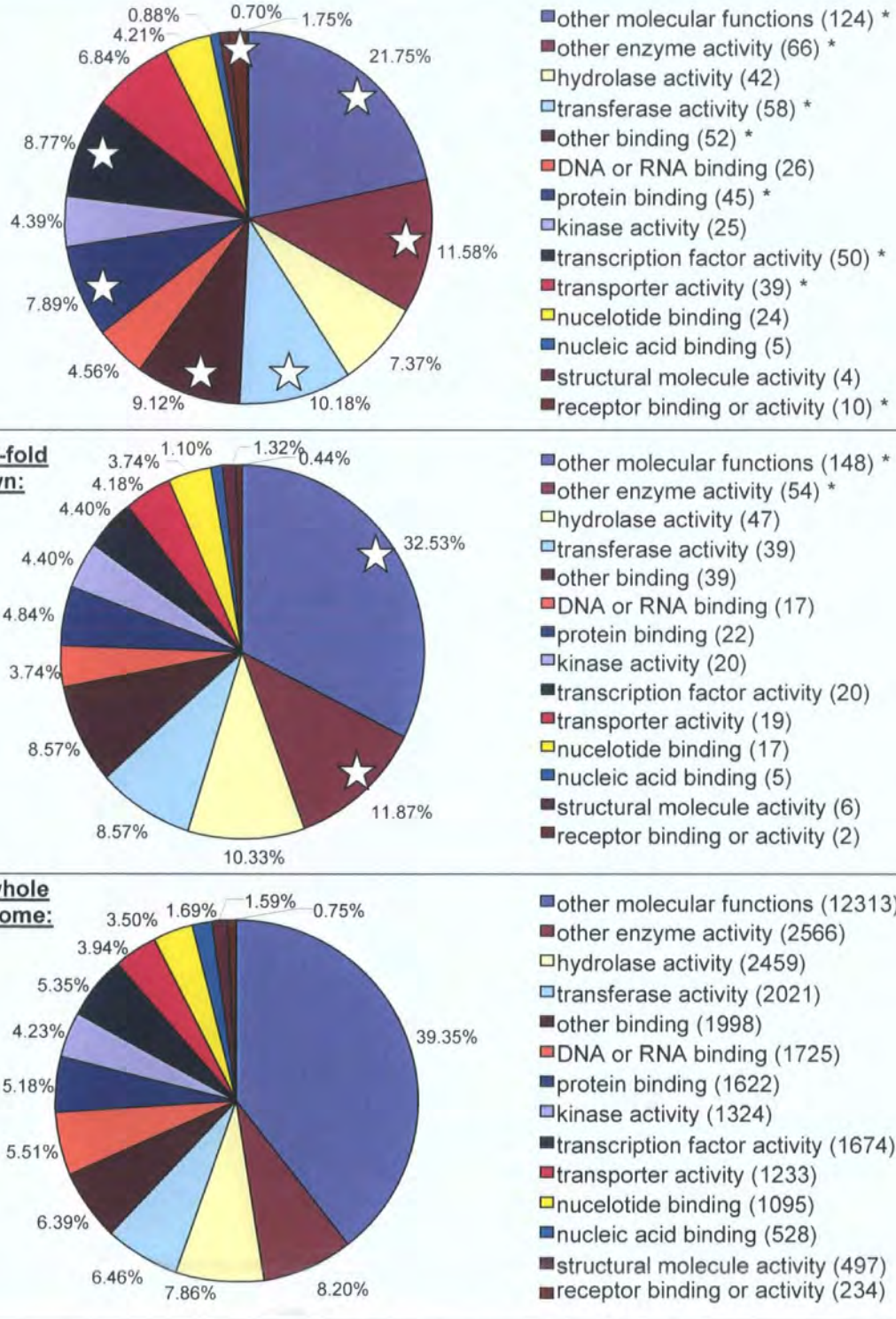
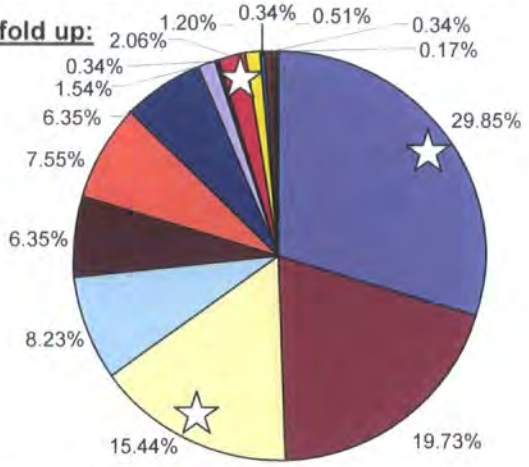


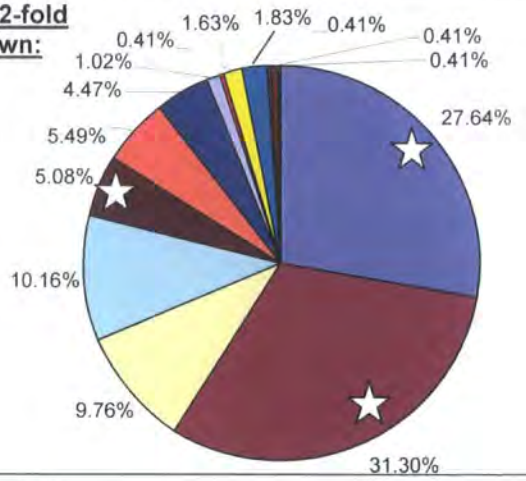
Figure 3.4
Functional categorisation of genes by GO **cellular components**. (Detail as Figure 3.2)

a) 2-fold up:



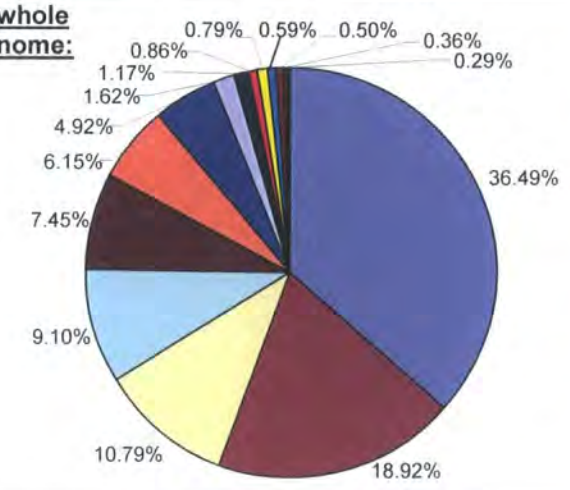
- other cellular components (174) *
- other membranes (115)
- chloroplast (90) *
- mitochondria (48)
- other intracellular components (37)
- nucleus (44)
- other cytoplasmic components (37)
- plastid (9)
- ribosome (2)
- cytosol (12) *
- plasma membrane (7)
- cell wall (2)
- extracellular (3)
- ER (2)
- Golgi apparatus (1)

b) 2-fold down:



- other cellular components (136) *
- other membranes (154) *
- chloroplast (48)
- mitochondria (50)
- other intracellular components (25) *
- nucleus (27)
- other cytoplasmic components (22)
- plastid (5)
- ribosome (0)
- cytosol (2)
- plasma membrane (8)
- cell wall (9)
- extracellular (2)
- ER (2)
- Golgi apparatus (2)

c) whole genome:



- other cellular components (12619)
- other membranes (6543)
- chloroplast (3732)
- mitochondria (3145)
- other intracellular components (2576)
- nucleus (2126)
- other cytoplasmic components (1702)
- plastid (559)
- ribosome (405)
- cytosol (298)
- plasma membrane (273)
- cell wall (205)
- extracellular (173)
- ER (123)
- Golgi apparatus (99)

Table 3.1

Over- and under-represented functional categories from probe sets that are at least 2-fold up- or down-regulated following the 10 mM H_2O_2 treatment.

TAIR Functional Category	Whole genome (%)	H_2O_2-up regulated (%)	H_2O_2-down regulated (%)
GO Biological Process:			
Cell organisation and biogenesis	3.03	1.94	(3.11)
Protein metabolism	8.00	5.53	5.46
Response to abiotic or biotic stimulus	2.46	7.16	(3.56)
Response to stress	1.93	6.76	4.06
Other biological processes	33.51	26.20	30.52
GO Molecular Function:			
Protein binding	5.18	7.89	(4.84)
Receptor binding or activity	0.75	1.75	(0.44)
Transcription factor activity	5.35	8.77	(4.40)
Transferase activity	6.46	10.18	(8.57)
Transporter activity	3.94	6.84	(4.18)
Other binding	6.39	9.12	(8.57)
Other enzyme activity	8.20	11.58	11.87
Other molecular functions	39.35	21.75	32.53
GO Cellular Component:			
Cell wall	0.59	(0.34)	1.83
Chloroplast	10.79	15.44	(9.76)
Cytosol	0.86	2.06	(0.41)
Ribosome	1.17	(0.34)	0.00
Other cellular components	36.49	29.85	27.64
Other intracellular components	7.45	(6.35)	5.08
Other membranes	18.92	(19.73)	31.30

Red and green shading denotes over- and under-represented categories respectively. All shaded categories are statistically significant at the stringent $p < 0.01$ level (hypergeometric test). Bracketed values are non-significant and are shown for reference only.

3.2.3 Promoter motif analysis

The upstream sequences of the 2-fold regulated genes were analysed in order to identify over-represented oligonucleotide motifs which may represent transcription factor binding sites or regulatory sites. Both 500 and 1000 bp of upstream promoter sequence were analysed, as the influence of the motif on gene expression has been shown to decrease the further from the start of transcription that the motif was found (Geisler *et al.*, 2006). Genuine motifs isolated in the promoters of differentially regulated gene will be enriched close to the start of transcription (indicating that this particular motif is potentially acting as a regulatory site in these genes). Promoter sequences were downloaded from the TAIR database (*http://www.arabidopsis.org/tools/bulk/sequencesindex.jsp*) and analysed using the "oligo analysis" tool available online at the Regulatory Sequence Analysis Tools (RSAT) site (*http://rsat.ulb.ac.be/rsat*; Van Helden, 2003). Only motifs with a *p* value less than 1e-04 were considered significant. All over-represented motifs were then compared to those listed in the PLACE database (plant *cis*-acting regulatory DNA elements; *http://www.dna.affrc.go.jp/PLACE/*) to check if they had been previously characterised in the literature. The results of the analyses are shown on the following pages in Tables 3.2 to 3.5.

As might be expected, stress-related motifs were over-represented in the promoter sequences of the H_2O_2 up-regulated genes. These included the ABA response element (ABRE)-like motif, W-box, G-box and the TGA1/AS1 motif (Table 3.3 [page 75]).

Table 3.2

RSAT motif analysis of 2-fold **up**-regulated genes in the H_2O_2 microarray experiment. Grey highlighting shows promoters which have previously been described in the literature. (473 of the 483 up-regulated genes were analysed, as 10 were listed as obsolete by TAIR).

Column headings are as follows: "Seq" oligomer sequence; "Identifier" oligomer identifier; "Occ" observed occurrences; "Exp Occ" expected occurrences; "Occ P" occurrence probability (binomial); "Occ E" E-value for occurrences (binomial); "Z score" Z-score (Gaussian approximation); "Ratio" observed/expected ratio.

Seq	Identifier	Occ	Exp occ	Occ P	Occ E	Z score	Ratio
500 bp of upstream sequence:							
5-mers:							
acgtg	acgtg|cacgt	357	222.47	6.7e-17	3.4e-14	9.02	1.60
gtcaa	gtcaa|ttgac	720	526.04	6.2e-16	3.2e-13	8.46	1.37
acacg	acacg|cgtgt	346	228.61	3.1e-13	1.6e-10	7.76	1.51
cgtca	cgtca|tgacg	308	202.67	3.7e-12	1.9e-09	7.40	1.52
acgtc	acgtc|gacgt	256	168.02	1.7e-10	8.8e-08	6.79	1.52
acgta	acgta|tacgt	324	240.68	1.9e-07	9.5e-05	5.37	1.35
agtca	agtca|tgact	530	422.25	2.4e-07	1.2e-04	5.24	1.26
aagtc	aagtc|gactt	537	428.99	2.8e-07	1.4e-04	5.21	1.25
ataaa	ataaa|tttat	2168	1956.48	1.2e-06	6.3e-04	4.78	1.11
aaagt	aaagt|acttt	1198	1054.16	7.3e-06	3.7e-03	4.43	1.14
ccaca	ccaca|tgtgg	419	337.96	1.1e-05	5.9e-03	4.41	1.24
acgcg	acgcg|cgcgt	122	80.91	1.2e-05	6.4e-03	4.57	1.51
accaa	accaa|ttggt	917	798.27	2.1e-05	1.1e-02	4.20	1.15
aaaaa	aaaaa|ttttt	2729	2523.19	2.5e-05	1.3e-02	4.10	1.08
gtgga	gtgga|tccac	402	326.14	2.7e-05	1.4e-02	4.20	1.23
gccca	gccca|tgggc	296	232.51	3.5e-05	1.8e-02	4.16	1.27
aaccg	aaccg|cggtt	343	275.24	4.5e-05	2.3e-02	4.08	1.25
ggtca	ggtca|tgacc	312	247.95	5e-05	2.5e-02	4.07	1.26
gccac	gccac|gtggc	217	164.93	6.1e-05	3.1e-02	4.05	1.32
ccacg	ccacg|cgtgg	201	151.95	8.3e-05	4.2e-02	3.98	1.32
6-mers:							
acacgt	acacgt|acgtgt	166	85.90	1.2e-14	2.5e-11	8.64	1.93
gtcaaa	gtcaaa|tttgac	329	215.78	4.9e-13	1.0e-09	7.71	1.52
acgtca	acgtca|tgacgt	126	63.19	2.3e-12	4.7e-09	7.90	1.99
cacgtg	cacgtg|cacgtg	101	47.64	1.2e-11	2.5e-08	7.73	2.12
agtcaa	agtcaa|ttgact	261	175.77	1.2e-09	2.4e-06	6.43	1.48
cgtgtc	cgtgtc|gacacg	94	48.80	6.1e-09	1.3e-05	6.47	1.93
aaagtc	aaagtc|gacttt	253	173.64	9.8e-09	2.0e-05	6.02	1.46
acgtgg	acgtgg|ccacgt	108	61.61	5.7e-08	1.2e-04	5.91	1.75
ggccca	ggccca|tgggcc	138	85.48	1.1e-07	2.3e-04	5.68	1.61
cgtcac	cgtcac|gtgacg	82	43.80	1.7e-07	3.4e-04	5.77	1.87
aagtca	aagtca|tgactt	232	163.29	2.4e-07	5.0e-04	5.38	1.42
ggtcaa	ggtcaa|ttgacc	161	105.10	2.5e-07	5.2e-04	5.45	1.53
aataaa	aataaa|tttatt	929	791.98	1.1e-06	2.3e-03	4.87	1.17
cacgta	cacgta|tacgtg	98	58.81	1.9e-06	3.9e-03	5.11	1.67
cacgtc	cacgtc|gacgtg	80	46.40	4.7e-06	9.9e-03	4.93	1.72
cgtggc	cgtggc|gccacg	66	36.03	4.8e-06	1.0e-02	4.99	1.83

(Table continues on the following page)

Table 3.2 *(Continued from the previous page)*

aaccaa	aaccaa|ttggtt	423	339.21	6.3e-06	1.3e-02	4.55	1.25
gacgtc	gacgtc|gacgtc	46	22.31	7.4e-06	1.5e-02	5.02	2.06
cacacg	cacacg|cgtgtg	85	51.20	9.6e-06	2.0e-02	4.72	1.66
tgacca	tgacca|tggtca	150	103.81	1.2e-05	2.5e-02	4.53	1.44
aaacca	aaacca|tggttt	387	309.63	1.2e-05	2.6e-02	4.40	1.25
cgtgta	cgtgta|tacacg	94	59.03	1.6e-05	3.4e-02	4.55	1.59
aaaaaa	aaaaaa|tttttt	1179	1042.73	1.8e-05	3.7e-02	4.22	1.13
agccac	agccac|gtggct	85	53.08	3.3e-05	6.9e-02	4.38	1.60
aaattg	aaattg|caattt	475	393.25	3.5e-05	7.2e-02	4.12	1.21
acgtaa	acgtaa|ttacgt	135	94.30	4.7e-05	9.8e-02	4.19	1.43
cacgcg	cacgcg|cgcgtg	47	24.91	5.1e-05	1.1e-01	4.42	1.89
atgacg	atgacg|cgtcat	91	58.62	5.4e-05	1.1e-01	4.23	1.55
aaataa	aaataa|ttattt	935	823.16	6.9e-05	1.4e-01	3.90	1.14
gtcaac	gtcaac|gttgac	138	97.76	7.2e-05	1.5e-01	4.07	1.41
acgcgg	acgcgg|ccgcgt	38	18.91	7.3e-05	1.5e-01	4.39	2.01
acgtat	acgtat|atacgt	117	80.49	7.9e-05	1.6e-01	4.07	1.45
cgtcag	cgtcag|ctgacg	54	30.55	7.9e-05	1.7e-01	4.24	1.77
7-mers:							
acacgtg	acacgtg|cacgtgt	74	26.83	5.5e-14	4.5e-10	9.11	2.76
acgtgtc	acgtgtc|gacacgt	65	23.23	9.4e-13	7.7e-09	8.67	2.80
agtcaaa	agtcaaa|tttgact	129	73.91	4.2e-09	3.5e-05	6.41	1.75
acgtggc	acgtggc|gccacgt	50	19.67	7.5e-09	6.1e-05	6.84	2.54
aagtcaa	aagtcaa|ttgactt	125	73.32	2.5e-08	2.1e-04	6.04	1.70
cgtgtca	cgtgtca|tgacacg	42	16.83	1.8e-07	1.5e-03	6.14	2.50
acgtcac	acgtcac|gtgacgt	37	14.08	2.7e-07	2.3e-03	6.11	2.63
aaagtca	aaagtca|tgacttt	114	69.56	6.4e-07	5.3e-03	5.33	1.64
ggtcaaa	ggtcaaa|tttgacc	79	43.65	9.6e-07	7.9e-03	5.35	1.81
tggtcaa	tggtcaa|ttgacca	81	45.42	1.2e-06	1.0e-02	5.28	1.78
acgtcat	acgtcat|atgacgt	45	20.19	1.4e-06	1.1e-02	5.52	2.23
aaaagtc	aaaagtc|gacttttt	114	70.89	1.5e-06	1.2e-02	5.12	1.61
gtcaaaa	gtcaaaa|ttttgac	127	81.96	2.4e-06	2.0e-02	4.98	1.55
aaataaa	aaataaa|tttattt	480	386.82	2.6e-06	2.2e-02	4.74	1.24
caacttg	caacttg|caagttg	66	35.88	4.2e-06	3.5e-02	5.03	1.84
cgtggaa	cgtggaa|ttccacg	38	16.64	4.9e-06	4.0e-02	5.24	2.28
aaaccaa	aaaccaa|ttggttt	212	154.08	5.7e-06	4.6e-02	4.67	1.38
accgcgt	accgcgt|acgcggt	19	5.54	5.9e-06	4.9e-02	5.72	3.43
gacgtca	gacgtca|tgacgtc	27	10.00	6.5e-06	5.3e-02	5.37	2.70
tacgtca	tacgtca|tgacgta	34	14.44	8.1e-06	6.6e-02	5.15	2.35
acgtgta	acgtgta|tacacgt	47	23.08	8.2e-06	6.7e-02	4.98	2.04
ccacgtc	ccacgtc|gacgtgg	33	14.30	1.6e-05	1.3e-01	4.94	2.31
acgtcag	acgtcag|ctgacgt	25	9.56	2.3e-05	1.9e-01	4.99	2.61
cgtcagc	cgtcagc|gctgacg	22	7.93	2.9e-05	2.4e-01	5.00	2.77
gtgtgaa	gtgtgaa|ttcacac	62	35.48	3.5e-05	2.8e-01	4.45	1.75
aggccca	aggccca|tgggcct	68	40.25	4.2e-05	3.4e-01	4.37	1.69
ctttgac	ctttgac|gtcaaag	64	37.22	4.2e-05	3.4e-01	4.39	1.72
cgtgtaa	cgtgtaa|ttacacg	40	19.93	4.9e-05	4.0e-01	4.50	2.01
cgtaagc	cgtaagc|gcttacg	24	9.48	5.4e-05	4.4e-01	4.72	2.53
cgcgtga	cgcgtga|tcacgcg	22	8.39	6.6e-05	5.4e-01	4.70	2.62
cgtggca	cgtggca|tgccacg	29	13.06	9.7e-05	7.9e-01	4.41	2.22
8-mers:							
cacgtgtc	cacgtgtc|gacacgtg	39	10.97	4.3e-11	1.4e-06	8.46	3.56
acacgtgt	acacgtgt|acacgtgt	32	7.69	5.1e-11	1.7e-06	8.76	4.16

(Table continues on the following page)

Table 3.2 *(Continued from the previous page)*							
acgtgtca	acgtgtca|tgacacgt	32	9.98	2.3e-08	7.7e-04	6.97	3.21
aaagtcaa	aaagtcaa|ttgacttt	67	33.20	1.7e-07	5.5e-03	5.87	2.02
aagtcaaa	aagtcaaa|tttgactt	63	30.52	1.8e-07	5.9e-03	5.88	2.06
gaaaagtc	gaaaagtc|gacttttc	35	13.06	3.7e-07	1.2e-02	6.07	2.68
acgtaagc	acgtaagc|gcttacgt	15	3.41	3.2e-06	1.0e-01	6.27	4.39
acgtgtaa	acgtgtaa|ttacacgt	24	7.95	3.4e-06	1.1e-01	5.69	3.02
aaccgcgt	aaccgcgt|acgcggtt	12	2.38	7.8e-06	2.6e-01	6.24	5.04
gacgtggc	gacgtggc|gccacgtc	17	4.87	1.4e-05	4.7e-01	5.50	3.49
gacgtcac	gacgtcac|gtgacgtc	12	2.56	1.6e-05	5.1e-01	5.91	4.69
acgtggct	acgtggct|agccacgt	16	4.55	2.3e-05	7.7e-01	5.36	3.51
1000 bp of upstream sequence:							
5-mers:							
gtcaa	gtcaa|ttgac	1280	1053.38	7.3e-12	3.8e-09	6.98	1.22
acgtg	acgtg|cacgt	587	445.49	8.9e-11	4.6e-08	6.70	1.32
acgtc	acgtc|gacgt	437	336.45	8.8e-08	4.5e-05	5.48	1.30
acacg	acacg|cgtgt	570	457.77	2.3e-07	1.2e-04	5.25	1.25
agtca	agtca|tgact	984	845.53	1.8e-06	9.2e-04	4.76	1.16
gtcca	gtcca|tggac	600	498.32	5.4e-06	2.8e-03	4.55	1.20
tggaa	tggaa|ttcca	1299	1150.26	8.9e-06	4.5e-03	4.39	1.13
cgtca	cgtca|tgacg	492	405.84	1.9e-05	9.5e-03	4.28	1.21
acgta	acgta|tacgt	572	481.94	3.6e-05	1.8e-02	4.10	1.19
tgaca	tgaca|tgtca	1033	915.22	7e-05	3.6e-02	3.89	1.13
6-mers:							
acacgt	acacgt|acgtgt	257	172.19	9.9e-10	2.1e-06	6.46	1.49
cacgtg	cacgtg|cacgtg	148	95.49	3.9e-07	8.1e-04	5.37	1.55
agtcaa	agtcaa|ttgact	449	352.32	4.2e-07	8.8e-04	5.15	1.27
acgtca	acgtca|tgacgt	186	126.66	4.8e-07	9.9e-04	5.27	1.47
gtcaaa	gtcaaa|tttgac	537	432.52	6.9e-07	1.4e-03	5.02	1.24
aagtca	aagtca|tgactt	407	327.31	1.2e-05	2.5e-02	4.40	1.24
tccaca	tccaca|tgtgga	288	222.17	1.3e-05	2.7e-02	4.42	1.30
ggtcca	ggtcca|tggacc	163	115.48	1.8e-05	3.7e-02	4.42	1.41
gtccac	gtccac|gtggac	146	101.67	2.1e-05	4.4e-02	4.40	1.44
cacgtc	cacgtc|gacgtg	135	93.01	2.6e-05	5.4e-02	4.35	1.45
gatgac	gatgac|gtcatc	206	153.56	3.2e-05	6.7e-02	4.23	1.34
agccac	agccac|gtggct	150	106.40	3.9e-05	8.1e-02	4.23	1.41
aataaa	aataaa|tttatt	1740	1587.48	8.2e-05	1.7e-01	3.83	1.10
aaagtc	aaagtc|gacttt	420	348.05	1e-04	2.1e-01	3.86	1.21
7-mers:							
agtcaaa	agtcaaa|tttgact	217	148.30	7.6e-08	6.2e-04	5.64	1.46
acacgtg	acacgtg|cacgtgt	96	53.84	1.4e-07	1.2e-03	5.75	1.78
acgtcat	acgtcat|atgacgt	74	40.52	1.5e-06	1.2e-02	5.26	1.83
acgtgtc	acgtgtc|gacacgt	81	46.61	3.1e-06	2.6e-02	5.04	1.74
caacttg	caacttg|caagttg	112	71.99	7.6e-06	6.3e-02	4.72	1.56
tacgtca	tacgtca|tgacgta	54	28.97	2.1e-05	1.7e-01	4.65	1.86
aagtcaa	aagtcaa|ttgactt	199	147.10	2.7e-05	2.2e-01	4.28	1.35
accgcgt	accgcgt|acgcggt	27	11.12	3.9e-05	3.2e-01	4.76	2.43
gtgtgaa	gtgtgaa|ttcacac	107	71.18	4.5e-05	3.7e-01	4.25	1.50
agtattg	agtattg|caatact	94	61.30	6.3e-05	5.2e-01	4.18	1.53
acgtggc	acgtggc|gccacgt	66	39.48	7.1e-05	5.8e-01	4.22	1.67
8-mers:							
acacgtgt	acacgtgt|acacgtgt	38	15.45	9.3e-07	3.1e-02	5.74	2.46
gaaaagtc	gaaaagtc|gacttttc	52	26.23	5.9e-06	1.9e-01	5.03	1.98

(Table continues on the following page)

Table 3.2 *(Continued from the previous page)*							
aagtcaaa	aagtcaaa|tttgactt	99	61.30	5.9e-06	1.9e-01	4.82	1.62
aaccgcgt	aaccgcgt|acgcggtt	17	4.78	1.1e-05	3.7e-01	5.59	3.56
cacgtgtc	cacgtgtc|gacacgtg	45	22.03	1.2e-05	3.8e-01	4.89	2.04
aatgacgt	aatgacgt|acgtcatt	33	14.44	2e-05	6.4e-01	4.88	2.28
actgctgc	actgctgc|gcagcagt	15	4.02	2.1e-05	7.0e-01	5.47	3.73

Table 3.3

Published promoter elements identified from the analysis of 2-fold **up**-regulated genes by H_2O_2.

Promoter	O/E Ratio	Upstream (bp)	Description	References
5-mers:				
ACGTG	1.60 1.32	500 1000	**ABRE-like sequence** ABA-responsive elements are enriched in genes involved in response to abiotic stresses e.g. dehydration and cold. E.g. required for etiolation-induced expression of Arabidopsis *ERD1* (*EARLY RESPONSE TO DEHYDRATION*).	Shinozaki and Yamaguchi-Shinozaki (2000), Simpson *et al.* (2003)
TTGAC	1.37 1.22	500 1000	**W-box** Recognised by WRKY DNA binding proteins. Found in promoters of stress-tolerance genes e.g. Arabidopsis *NPR1* (*NON EXPRESSOR OF PR GENES 1*).	Eulgem *et al.* (2000), Yu *et al.* (2001), Xu *et al.* (2006)
TGACG	1.52 1.21	500 1000	**TGA1 motif/AS1 motif** Biding site for basic domain/leucine zipper (bZIP) TGA factors e.g. Arabidopsis TGA1. Activation sequence-1 in GST genes.	Schindler *et al.* (1992), Xiang *et al.* (1997), Klinedinst *et al.* (2000)
TGACT	1.26 1.16	500 1000	**W-box related element** WRKY binding site. E.g. for the barley WRKY transcription factor SUSIBA2. Present in the barley *ISO1* (*ISOAMYLASE* 1) promoter and in a tobacco basic chitinase gene (*CHN48*).	Sun *et al.* (2003), Yamamoto *et al.* (2004)
TGTCA	1.13	1000	Binding site of a rice BELL homeodomain transcription factor (OsBIHD1) involved in the disease resistance response.	Luo *et al.* (2005)
GCCAC	1.32	500	One of the **Sequences Over-Represented in Light-Induced Promoters (SORLIPs)** in Arabidopsis. Present in phytochrome A-regulated genes.	Hudson and Quail (2003), Jiao *et al.* (2005)

(Table continues on the following page)

Table 3.3 *(Continued from the previous page)*

6-mers:				
TGACGT	1.99 1.47	500 1000	**TGACGT motif (similar to TGA1 motif)** Binding site for the rice bZIP protein OsOBF1 (involved in cold-signalling). Binding site of the wheat histone DNA binding protein-1 (HBP-1). Present in promoter of the wheat histone genes H3 and H4. Present in the *Vigna mungo* alpha-Amylase (Amy) gene promoter.	Terada *et al.* (1995), Yamauchi (2001), Shimizu *et al.* (2005)
CACGTG	2.12 1.55	500 1000	**G-box (similar to ABRE-like sequence)** Binding sites for G-box factors (GBFs) that mediate a wide variety of gene expression patterns. Essential for expression of beta-phaseolin gene during embryogenesis in bean, tobacco and Arabidopsis. Tomato Pti4 (an ERF) regulates defence-related gene expression via G-box. The rose GBFs (CrGBF1 and CrGBF2 can act as transcriptional repressors of the strictosidine synthase promoter via direct interaction with the G-box.	Menkens *et al.* (1995), Siberil *et al.* (2001), Chakravarthy *et al.* (2003), Chandrasekharan *et al.* (2003)
TTGACT	1.48 1.27	500 1000	**W-box related** Present in the *PR1* gene in parsley, and in the amylase genes of sweet potato, wheat, barley, and wild oat.	Ishiguro and Nakamura (1994), Ruston *et al.* (1995; 1996), Eulgem *et al.* (2000)
TTGACC	1.53	500	**W-box related** **EIRE (Elicitor Responsive Element) core** of parsley *PR1* genes; consensus sequence of elements W1 and W2 of parsley *PR1-1* and *PR1-2* promoters, which are the binding site of WRKY1 and WRKY2, respectively. Present in the Arabidopsis thioredoxin *h5* gene (involved in response to pathogens).	Rushton *et al.* (1996), Eulgem *et al.* (2000) Laloi *et al.* (2004)
AATAAA	1.17 1.10	500 1000	**PolyA signal** Present in rice alpha-amylase and in *legA* gene of pea. Near upstream elements (NUE) in Arabidopsis.	Joshi (1987), O'Neill *et al.* (1990), Loke *et al.* (2005)
AACCAA	1.25	500	**REalpha** Required for phytochrome regulation. Present in *Lemna gibba Lhcb21* gene promoter.	Degenhardt and Tobin (1996)
GACGTC	2.06	500	**C-box** bZIP protein DNA binding site.	Izawa *et al.* (1993), Foster *et al.* (1994)
TTATTT	1.14	500	**TATA box** Present in the 5' upstream region of pea glutamine synthetase gene.	Tjaden *et al.* (1995)

(Table continues on the following page)

Table 3.3 *(Continued from the previous page)*				
7-mers:				
ACGTGTC	2.80, 1.74	500, 1000	**Similar to ABRE-like element** Present in 24 genes in the GA-down regulated cluster during Arabidopsis seed germination.	Ogawa *et al.* (2003)
ACGTGGC	2.54, 1.67	500, 1000	**Box II/G box core (Similar to ABRE-like element)** Present in the parsley chalcone synthase (*chs*) promoter. Essential for light regulation.	Block *et al.* (1990), Terzaghi and Cashmore (1995)

Table 3.4

RSAT motif analyses of 2-fold **down**-regulated genes in the H_2O_2 microarray experiment. Those highlighted in grey have previously been described in the literature. All 412 genes were analysed. Column headings are as detailed previously in Table 3.2.

Seq	Identifier	Occ	Exp occ	Occ P	Occ E	Z score	Ratio
500 bp of upstream sequence:							
5-mers:							
aaata	aaata|tattt	2120	1819.56	2.9e-12	1.5e-09	7.04	1.17
aatat	aatat|atatt	1452	1217.13	3e-11	1.5e-08	6.73	1.19
atata	atata|tatat	1212	1016.41	1.3e-09	6.5e-07	6.14	1.19
ataat	ataat|attat	1450	1235.65	1.4e-09	7.2e-07	6.10	1.17
tataa	tataa|ttata	1220	1037.88	1.9e-08	9.5e-06	5.65	1.18
ataaa	ataaa|tttat	1940	1710.53	2.6e-08	1.3e-05	5.55	1.13
aataa	aataa|ttatt	1800	1588.14	9.1e-08	4.6e-05	5.32	1.13
taata	taata|tatta	1254	1078.94	1e-07	5.1e-05	5.33	1.16
attta	attta|taaat	1568	1396.43	3.3e-06	1.7e-03	4.59	1.12
acgtg	acgtg|cacgt	258	194.51	8e-06	4.1e-03	4.55	1.33
gataa	gataa|ttatc	756	643.63	8.4e-06	4.3e-03	4.43	1.17
caata	caata|tattg	771	659.26	1.2e-05	5.9e-03	4.35	1.17
attaa	attaa|ttaat	1279	1137.62	2e-05	1.0e-02	4.19	1.12
aatac	aatac|gtatt	646	551.49	4.6e-05	2.4e-02	4.02	1.17
catta	catta|taatg	713	613.60	4.7e-05	2.4e-02	4.01	1.16
gtata	gtata|tatac	592	503.28	6.2e-05	3.2e-02	3.95	1.18
taaaa	taaaa|tttta	2156	1984.51	7e-05	3.6e-02	3.85	1.09
ataca	ataca|tgtat	776	676.64	9.7e-05	5.0e-02	3.82	1.15
6-mers:							
atataa	atataa|ttatat	585	464.40	3.9e-08	8.2e-05	5.60	1.26
tataaa	tataaa|tttata	606	486.80	1e-07	2.1e-04	5.40	1.24
ataaat	ataaat|atttat	648	529.12	3.1e-07	6.5e-04	5.17	1.22
aaaata	aaaata|tatttt	968	826.15	7.8e-07	1.6e-03	4.94	1.17
aataat	aataat|attatt	610	502.47	1.8e-06	3.8e-03	4.80	1.21

(Table continues on the following page)

Table 3.4 *(Continued from the previous page)*							
atatag	atatag|ctatat	284	213.16	2.1e-06	4.5e-03	4.85	1.33
aatatc	aatatc|gatatt	294	223.10	3.3e-06	6.9e-03	4.75	1.32
tatata	tatata|tatata	404	320.02	3.5e-06	7.2e-03	4.69	1.26
taataa	taataa|ttatta	522	433.28	1.9e-05	4.0e-02	4.26	1.20
aatata	aatata|tatatt	559	467.15	2e-05	4.1e-02	4.25	1.20
aaatat	aaatat|atattt	711	609.36	3.1e-05	6.5e-02	4.12	1.17
tagata	tagata|tatcta	231	175.67	3.8e-05	7.8e-02	4.17	1.32
tattaa	tattaa|ttaata	455	375.75	4e-05	8.3e-02	4.09	1.21
agacta	agacta|tagtct	134	93.40	4.6e-05	9.5e-02	4.20	1.43
aattat	aattat|ataatt	547	461.10	5.3e-05	1.1e-01	4.00	1.19
taaata	taaata|tattta	558	472.26	6.6e-05	1.4e-01	3.95	1.18
cttatc	cttatc|gataag	150	107.96	7.5e-05	1.6e-01	4.05	1.39
attaaa	attaaa|tttaat	629	538.47	7.5e-05	1.6e-01	3.90	1.17
attgta	attgta|tacaat	253	197.68	8.8e-05	1.8e-01	3.93	1.28
ataata	ataata|tattat	485	406.96	9.1e-05	1.9e-01	3.87	1.19
7-mers:							
ctatata	ctatata|tatatag	148	89.61	1e-08	8.5e-05	6.17	1.65
atataga	atataga|tctatat	136	85.82	3.6e-07	2.9e-03	5.42	1.58
taaaata	taaaata|tatttta	291	214.45	3.9e-07	3.2e-03	5.23	1.36
atataaa	atataaa|tttatat	271	202.04	2.2e-06	1.8e-02	4.85	1.34
tatataa	tatataa|ttatata	231	168.33	2.7e-06	2.2e-02	4.83	1.37
atttata	atttata|tataaat	226	169.36	1.9e-05	1.6e-01	4.35	1.33
aactgta	aactgta|tacagtt	56	31.10	3.7e-05	3.0e-01	4.46	1.80
aaatatc	aaatatc|gatattt	138	98.35	9.3e-05	7.6e-01	4.00	1.40
8-mers:							
tatataga	tatataga|tctatata	73	37.49	1.8e-07	6.0e-03	5.80	1.95
atatatag	atatatag|ctatatat	84	46.25	4e-07	1.3e-02	5.55	1.82
atcaggtg	atcaggtg|cacctgat	13	2.56	3e-06	1.0e-01	6.53	5.08
gctttata	gctttata|tataaagc	25	9.22	1.3e-05	4.3e-01	5.19	2.71
1000 bp of upstream sequence:							
5-mers:							
ataat	ataat|attat	2814	2470.87	6.6e-12	3.4e-09	6.90	1.14
atata	atata|tatat	2319	2032.45	2.4e-10	1.2e-07	6.36	1.14
aatat	aatat|atatt	2744	2433.82	3.3e-10	1.7e-07	6.29	1.13
gtata	gtata|tatac	1203	1006.38	9.3e-10	4.8e-07	6.20	1.20
gataa	gataa|ttatc	1488	1287.03	2.3e-08	1.2e-05	5.60	1.16
ataca	ataca|tgtat	1558	1353.03	2.7e-08	1.4e-05	5.57	1.15
aaata	aaata|tattt	3953	3638.47	1.2e-07	6.3e-05	5.21	1.09
agata	agata|tatct	1477	1297.73	5.7e-07	2.9e-04	4.98	1.14
catta	catta|taatg	1399	1226.99	7.9e-07	4.0e-04	4.91	1.14
tataa	tataa|ttata	2288	2075.39	2.2e-06	1.1e-03	4.67	1.10
atatc	atatc|gatat	1323	1167.26	4.1e-06	2.1e-03	4.56	1.13
atatg	atatg|catat	1530	1368.65	9.4e-06	4.8e-03	4.36	1.12
tgaca	tgaca|tgtca	923	799.04	9.7e-06	5.0e-03	4.39	1.16
caata	caata|tattg	1469	1318.28	2.3e-05	1.2e-02	4.15	1.11
agtat	agtat|atact	1101	971.86	2.6e-05	1.3e-02	4.14	1.13
actat	actat|atagt	1228	1092.75	3.1e-05	1.6e-02	4.09	1.12
atgta	atgta|tacat	1407	1263.11	3.6e-05	1.8e-02	4.05	1.11
tatca	tatca|tgata	1434	1289.81	4.1e-05	2.1e-02	4.01	1.11
ggata	ggata|tatcc	668	573.79	6.6e-05	3.4e-02	3.93	1.16
ctata	ctata|tatag	1143	1019.18	7.3e-05	3.7e-02	3.88	1.12
accat	accat|atggt	921	810.60	7.6e-05	3.9e-02	3.88	1.14

(Table continues on the following page)

Table 3.4 *(Continued from the previous page)*							
6-mers:							
atatac	atatac|gtatat	561	437.79	9e-09	1.9e-05	5.89	1.28
ataatg	ataatg|cattat	509	406.56	5.5e-07	1.1e-03	5.08	1.25
attgta	attgta|tacaat	496	395.69	6.6e-07	1.4e-03	5.04	1.25
aatatc	aatatc|gatatt	551	446.56	1e-06	2.1e-03	4.94	1.23
atatag	atatag|ctatat	526	426.67	1.9e-06	3.9e-03	4.81	1.23
tataca	tataca|tgtata	548	449.77	4e-06	8.3e-03	4.63	1.22
catgca	catgca|tgcatg	229	169.38	7.7e-06	1.6e-02	4.58	1.35
tatata	tatata|tatata	750	640.56	1.3e-05	2.8e-02	4.32	1.17
cttatc	cttatc|gataag	281	216.09	1.3e-05	2.8e-02	4.42	1.30
aaatat	aaatat|atattt	1369	1219.72	1.4e-05	2.9e-02	4.27	1.12
atagta	atagta|tactat	408	329.98	1.9e-05	3.9e-02	4.29	1.24
tagata	tagata|tatcta	431	351.62	2.3e-05	4.8e-02	4.23	1.23
attatc	attatc|gataat	464	382.19	2.7e-05	5.7e-02	4.18	1.21
agataa	agataa|ttatct	560	469.75	2.8e-05	5.8e-02	4.16	1.19
agtata	agtata|tatact	393	318.99	3.4e-05	7.1e-02	4.14	1.23
tataaa	tataaa|tttata	1098	974.40	5.3e-05	1.1e-01	3.96	1.13
aattat	aattat|ataatt	1043	922.94	5.6e-05	1.2e-01	3.95	1.13
catata	catata|tatatg	611	520.15	5.6e-05	1.2e-01	3.98	1.17
7-mers:							
atacaat	atacaat|attgtat	197	136.62	7.2e-07	5.9e-03	5.17	1.44
taaaata	taaaata|tatttta	528	429.68	2.5e-06	2.0e-02	4.74	1.23
ctatata	ctatata|tatatag	240	179.55	9.9e-06	8.1e-02	4.51	1.34
agataat	agataat|attatct	184	133.10	1.7e-05	1.4e-01	4.41	1.38
atataga	atataga|tctatat	229	171.95	1.9e-05	1.6e-01	4.35	1.33
gatacaa	gatacaa|ttgtatc	122	81.78	2e-05	1.6e-01	4.45	1.49
atataca	atataca|tgtatat	261	201.55	3.4e-05	2.8e-01	4.19	1.29
atggata	atggata|tatccat	110	73.80	4.9e-05	4.0e-01	4.21	1.49
gtatata	gtatata|tatatac	235	180.42	5.7e-05	4.7e-01	4.06	1.30
atgtata	atgtata|tatacat	217	165.28	6.8e-05	5.6e-01	4.02	1.31
atccata	atccata|tatggat	111	75.49	7.7e-05	6.3e-01	4.09	1.47
catgcac	catgcac|gtgcatg	52	29.04	7.8e-05	6.4e-01	4.26	1.79
acctgat	acctgat|atcaggt	49	26.98	8.9e-05	7.3e-01	4.24	1.82
8-mers:							
atcaggtg	atcaggtg|cacctgat	18	5.13	7.6e-06	2.5e-01	5.68	3.51
gtatagta	gtatagta|tactatac	37	16.72	1.3e-05	4.2e-01	4.96	2.21
cagtgaca	cagtgaca|tgtcactg	24	8.93	2.2e-05	7.2e-01	5.04	2.69

Table 3.5

Published promoter elements identified from the analysis of 2-fold **down**-regulated genes by H_2O_2.

Promoter	O/E Ratio	Upstream (bp)	Description	References
5-mers:				
ATATT	1.19 1.13	500 1000	Motif present in the promoter of *rolD* (an Agrobacterium rhizogene)	Elmayan and Tepfer (1995)
ACGTG	1.33	500	**ABRE-like sequence** ABA-responsive elements are enriched in genes involved in response to abiotic stresses e.g. dehydration and cold. E.g. required for etiolation-induced expression of Arabidopsis. *ERD1 (EARLY RESPONSE TO DEHYDRATION)*.	Shinozaki and Yamaguchi-Shinozaki (2000), Simpson *et al.* (2003)
GATAA	1.17 1.16	500 1000	**I-box** Conserved sequence upstream of light-regulated genes of both monocots and dicots	Terzaghi and Cashmore (1995)
TGTCA	1.16	1000	Binding site of a rice BELL homeodomain transcription factor (OsBIHD1) involved in the disease resistance response.	Luo *et al.* (2005)
GGATA	1.16	1000	Binding site of MybSt1 (a potato MYB homolog).	Baranowskij *et al.* (1994)
6-mers:				
AATAAT	1.21	500	**Plant polyA signal**	Joshi (1987)
GATAAG	1.39 1.30	500 1000	**I-box related** Associated with light-responsive promoter regions	Martinez-Hernandez *et al.* (2002)
CATGCA	1.35	1000	**RY repeat** Present in RY/G box of *napA* gene promoter of *Brassica napus*.	Ezcurra *et al.* (1999), Ezcurra *et al.* (2000)
7-mers:				
TATATAA	1.37	500	**TATA box** Required for transcription initiation by RNA polymerases. Present in the 5' upstream region of sweet potato *sporamin A* gene and in the beta-phaseolin promoter	Grace *et al.* (2004)
TATAAAT	1.33	500	**TATA box** Required for transcription initiation by RNA polymerases. Present in the 5' upstream region of a pea legumin gene *(legA)*, a sweet potato *sporamin A* gene and in the beta-phaseolin promoter	Shirsat *et al.* (1989), Grace *et al.* (2004)
TATCCAT	1.49	1000	**Amylase box** Present in 5' upstream region of alpha-amylase genes of rice, wheat and barley.	Huang *et al.* (1990), Hwang *et al.* (1998)

3.2.4 Genes encoding potential ROS protein signalling components

3.2.4.1 Selection of candidate signalling genes for further study

At the time this study was carried out, 41 of the H_2O_2 up-regulated genes were classified either as kinases (10), phosphatases (6) or transcription factors (25), and are shown overleaf in Table 3.6. However, a further 13 kinases, 2 phosphatases and 26 transcription factors were later annotated by the TAIR 6 genome release and (although not known at the time) they are included in Appendix D1 for reference.

Candidate genes for further study were chosen based on the selection criteria outlined in Figure 3.5 (page 84). By step 4 of the selection criteria, there were fourteen candidate genes. The expression patterns of these genes were gauged from publicly available microarray expression data for biological processes known to involve ROS (e.g. environmental stresses). At the time of this candidate gene selection, microarray expression data was available in response to heat stress (Evans and Knight, 2003), UV-B irradiation (Brueggemann and Holub, 2003) and to *Pseudomonas syringae* challenge (De Torres-Zabala and Grant, 2003). All 14 candidate genes exhibited either a 2-fold increase or decrease in transcript abundance in response to at least one of these treatments (Figure 3.6). This supported the involvement of these genes in ROS-related biological processes.

Table 3.6

Putative kinases, phosphatases and transcription factors up-regulated 2-fold by H_2O_2. Only annotations available at the time this study was performed are shown. Asterisks denote genes selected for northern blot analysis.

AGI code	Gene description	H_2O_2 fold induction
Kinases:		
At4g23190	serine/threonine kinase – like protein *	4.79
At5g25930	receptor-like protein kinase – like *	4.37
At1g70530	putative protein kinase	2.73
At4g18950	protein kinase – like protein *	2.68
At3g22060	putative receptor kinase common family *	2.58
At1g09970	putative leucine-rich repeat transmembrane protein kinase *	2.53
At1g73500	putative MAP kinase	2.41
At2g39660	putative protein kinase *	2.40
At2g40500	putative protein kinase	2.26
At5g58350	MAP kinase	2.26
Phosphatases:		
At4g31860	protein phosphatase 2C (PP2C) *	3.25
At4g32950	putative protein phosphoprotein phosphatase	2.98
At4g23570	phosphatase like protein	2.95
At2g33700	putative protein phosphatase 2C *	2.87
At1g08420	putative protein serine/threonine phosphatase alpha *	2.80
At2g30020	putative protein phosphatase 2C *	2.43
Transcription factors:		
At1g27730	salt-tolerance zinc finger protein	8.20
At3g56400	WRKY family transcription factor	7.05
At5g04340	putative C2H2 zinc finger transcription factor	6.88
At4g17490	ethylene-responsive element binding factor (AtERF6) *	6.32
At1g01720	NAC domain protein, putative	5.80
At2g38470	putative WRKY-type DNA binding protein	5.59
At1g62300	WRKY family transcription factor	5.53
At5g59820	zinc finger protein (ZAT12)	5.48
At1g80840	putative WRKY transcription factor	4.85

(Table continues on the following page)

Table 3.6 *(Continues from the previous page)*

At2g24500	putative C2H2-type zinc finger protein	4.64
At2g40140	putative CCCH-type zinc finger protein	4.46
At3g55980	putative protein zinc finger transcription factor	4.01
At1g32240	putative MYB family transcription factor *	3.78
At2g46830	MYB-related transcription factor (CCA1)	2.97
At4g17500	ethylene responsive element binding factor 1 (AtERF1)	2.96
At4g18880	heat shock transcription factor - like protein	2.72
At3g18290	putative zinc finger protein *	2.66
At2g27580	putative zinc finger protein	2.64
At4g31800	WRKY family transcription factor	2.61
At2g40350	DREB subfamily transcription factor	2.54
At4g31550	WRKY family transcription factor	2.45
At2g23320	putative WRKY-type DNA-binding protein	2.36
At5g47230	ethylene-responsive element-binding factor 5 (AtERF5) *	2.27
At4g18170	WRKY family transcription factor	2.27
At4g17230	scarecrow-like 13 (SCL13)	2.19

Figure 3.5

Criteria for selection of candidate genes for further study.

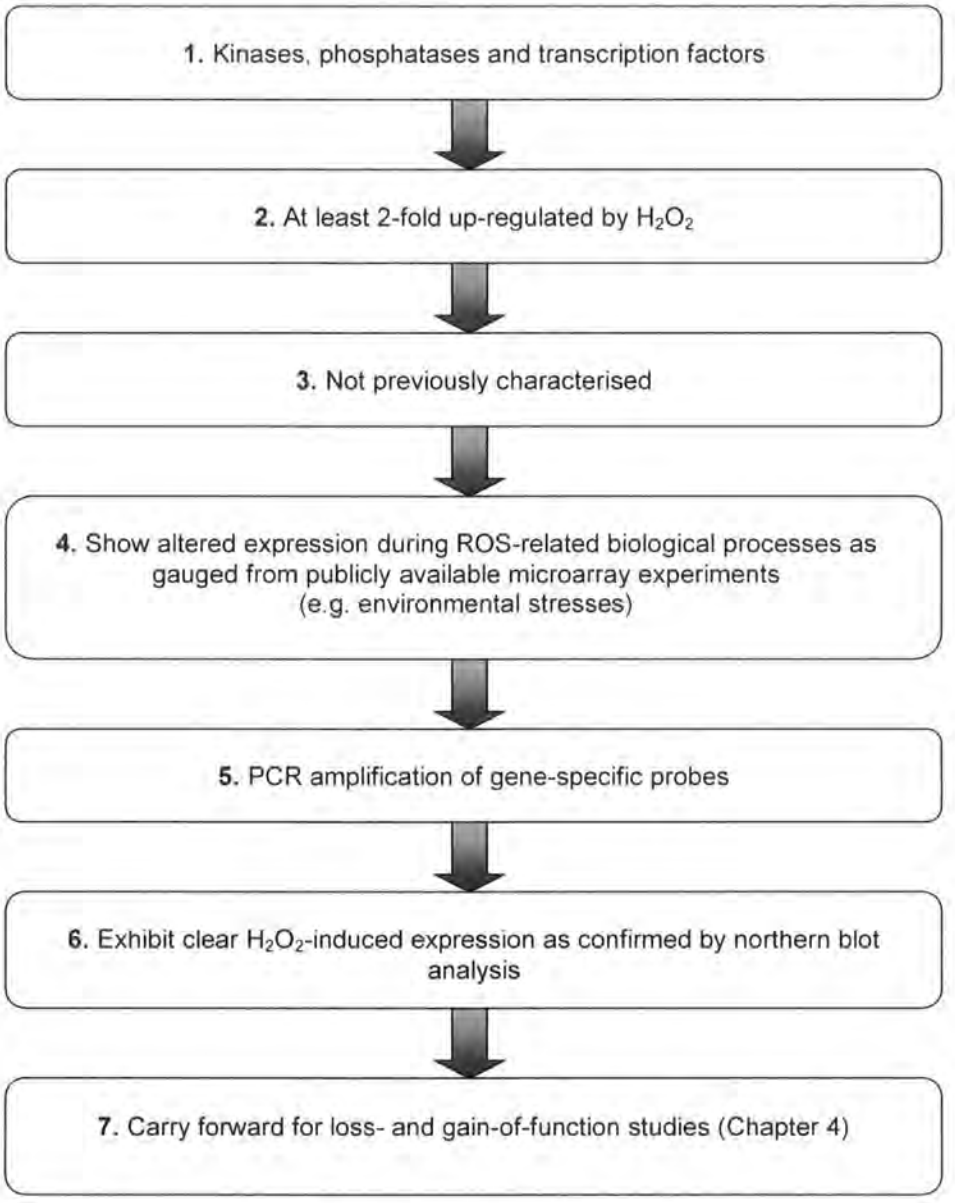
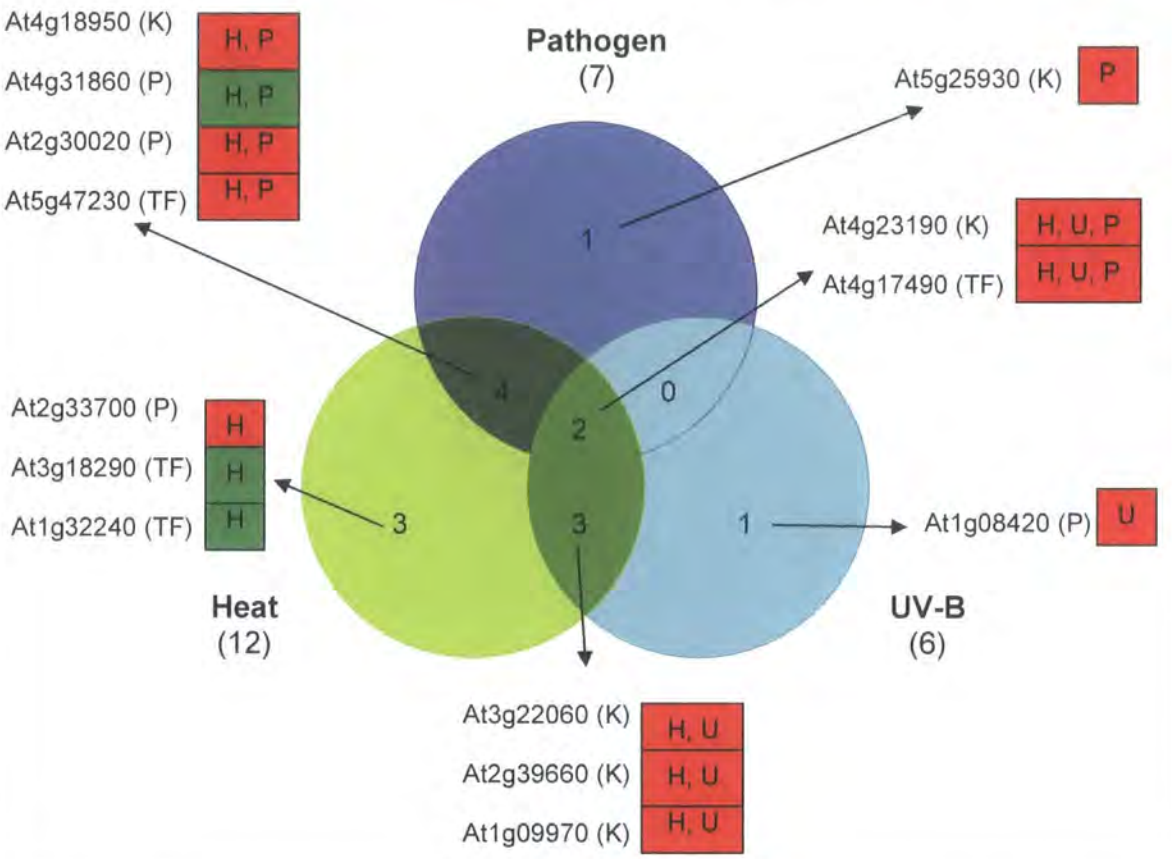


Figure 3.6

Altered expression (at least 2-fold) of candidate genes in response to 3 environmental stresses.



Expression data was obtained from stress-related microarrays (ecotype Columbia) available at the time. Red and green shading denote 2-fold up- or down-regulation respectively in the heat (H), UV (U) or pathogen (P) microarrays. K, P and TF denote kinase, phosphatase and transcription factor respectively.

The heat microarray was performed by Evans and Knight (2003; University of Oxford, Oxford, UK). RNA was extracted from 10-day old seedlings treated for 1 h at 30 °C or 40 °C (for full details please refer to NASCArray experiment 79).

The UV-B experiment was carried out by Brueggemann and Holub (2003; University of Warwick, Warwick, UK). RNA was extracted from 4.5-week old rosettes treated for 1.5 photoperiods (where one photoperiod = 12 h) with supplementary UV-B (280 – 320 nm) in addition to photosynthetically active radiation (400 – 700 nm) (for full details please refer to NASCArray experiment 56).

The pathogen microarray was performed by De Torres-Zabala and Grant (2003; Imperial College, Wye, UK). RNA was extracted from 18-day old leaves 12 h after infiltration with *Pseudomonas syringae* pv. *tomato* DC3000 (for full details please refer to NASCArray experiment 59).

3.2.4.2 Confirmation of H_2O_2-induced expression

Although the microarray experiment lacked replicates, its purpose was as a starting point to identify H_2O_2-regulated genes, rather than to provide a comprehensive analysis of regulation of the transcriptome by H_2O_2. Of the 14 candidate genes chosen, 12 also were up-regulated by H_2O_2 by at least 2-fold on the *oxi1* mutant slides. Northern blot analysis was then used to verify the expression of all selected candidate genes in response to H_2O_2.

Primers were designed by hand to be gene-specific (see Appendix B.2) and used to amplify gene-specific double-stranded DNA probes from cDNA via PCR (Materials and Methods 2.12). Seedlings were treated by the same method of H_2O_2 treatment as that used for the initial microarray experiment (i.e. 7-day old seedlings treated with 10 mM H_2O_2 for 3 h). However, it is important to note that the ecotype used for the northern blots and all subsequent work was Columbia (Col-0). WS-2 was used for the initial H_2O_2 microarray as it was the control and background for the *oxi1* mutant, which was the original purpose of the microarray experiment. However, Col-0 is a more preferable ecotype to use, as it has been sequenced by the Arabidopsis Genome Initiative (AGI), is widely studied and most mutants are available in this background.

Genes with very clear up-regulation in response to H_2O_2 were preferentially chosen over those whose induction was less obvious. Of the 14 candidate ROS-signalling genes tested, 4 of the 6 kinases showed a clear H_2O_2-triggered expression (as shown overleaf in Figure 3.7). However, one of these, At4g23190, was subsequently published as a putative pathogen-inducible cysteine-rich receptor-like kinase (*CRK11*; Chen *et al.*, 2003). Previous work had shown that expression of *CRK11* could be induced during the incompatible interaction with a soil-borne vascular bacterium (*Ralstonia solanacearum*) and by SA treatment (Czernic *et al.*, 1999). This example demonstrates the efficacy of the criteria for candidate gene selection (Figure 3.5).

The 4 phosphatase genes tested all exhibited up-regulation in their transcript levels following H_2O_2 treatment, however At4g31860 and At2g30020 gave the clearest induction (Figure 3.8) and these were selected. Three of the 4 transcription factors showed very clear up-regulation in transcript levels following H_2O_2 treatment (Figure 3.9). In total 8 genes were carried forward for further study (Chapter 4).

Figure 3.7

Induction of putative kinase genes in response to H_2O_2.

AGI code	Northern blot result	Gene description	Microarray fold induction	Further study
	Control H_2O_2			
At5g25930		Receptor-like protein kinase-like protein *(TAIR 6: Protein-kinase family protein)*	4.39	✓
EtBr				
At4g18950		Protein kinase-like protein *(TAIR 6: Putative ankyrin protein kinase)*	2.68	✓
EtBr				
At3g22060		Putative receptor kinase common family protein *(TAIR 6: Lacks kinase domain)*	2.58	✓
EtBr				
At4g23190		Serine/threonine kinase-like protein *(TAIR 6: Putative receptor-like protein kinase CRK11)*	4.79	× Published (Chen *et al.*, 2003)
EtBr				
At2g39660		Putative protein kinase *(TAIR 6: Botrytis-induced kinase 1)*	2.40	× Weak induction on northern blot
EtBr				
At1g09970		Putative leucine-rich repeat transmembrane protein	2.53	× Unconfirmed by northern blot
EtBr				

Northern blot analysis was performed on 7-day old Col-0 seedlings that had been treated for 3 h with 10 mM H_2O_2 or a water control. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg of total RNA per lane). Samples are in triplicate.

Figure 3.8

Induction of putative phosphatase genes in response to H_2O_2. (Detail as Figure 3.7).

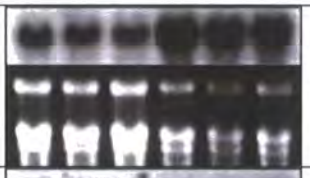
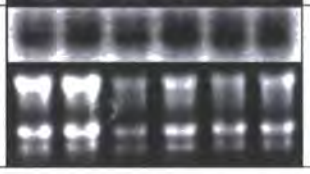
AGI code	Northern blot result	Gene description	Microarray fold induction	Further study
	Control H_2O_2			
At4g31860 EtBr		Putative protein phosphatase 2C	3.25	✓
At2g30020 EtBr		Putative protein phosphatase 2C	2.43	✓
At2g33700 EtBr		Putative protein phosphatase 2C	2.87	× Weak induction on northern blot
At1g08420 EtBr		Putative protein ser/thr phosphatase alpha *(TAIR 6: ser/thre phosphor-esterase family protein)*	3.25	× Weak induction on northern blot

Figure 3.9

Induction of putative transcription factor genes in response to H_2O_2. (Detail as Figure 3.7)

AGI code	Northern blot result	Gene description	Microarray fold induction	Further study
	Control H_2O_2			
At4g17490		Ethylene responsive factor 6	6.32	✓
EtBr				
At5g47230		Ethylene responsive factor 5	2.27	✓
EtBr				
At3g18290		Putative zinc-finger protein	2.66	✓
EtBr				
At1g32240		Putative MYB family transcription factor *(TAIR 6: KANADI family transcription factor)*	3.78	× Unconfirmed by northern blot
EtBr				

3.3 Discussion

Exogenous H_2O_2 application was able to both up-regulate and down-regulate gene expression. Some of the most significant H_2O_2-regulated genes and their biological context are discussed below, with a focus on signalling components. (For the full list of up- and down-regulated genes please refer to Appendices D1 and D2 respectively).

3.3.1 Antioxidants

Transcripts involved in scavenging ROS were clearly up-regulated as gauged from the microarray. For example, genes encoding two APXs (cytosolic *APX1* and stromal APX) and *GPX6* were up-regulated. A dehydroascorbate reductase (*DHAR3*) and a monohydroascorbate reductase (*MDAR2*) were also up-regulated, the products of which help to maintain the reduced cellular pool of ascorbate. In addition, the mitochondrial AOX gene (*AOX1A*) involved in limiting ROS production, and 8 glutathione *S*-transferase (GSTs) genes involved in the detoxification of products such as peroxide- and epoxide-containing metabolites, also exhibited up-regulated transcript levels. The down-regulation of 12 peroxidase transcripts may reflect the requirement of the cell to quench the exogenously added H_2O_2 and repress further H_2O_2 generation. Taken together, these observations demonstrate the capability of the Arabidopsis seedlings to perceive and respond to the exogenous H_2O_2 treatment, as well as providing a protective effect. The proteins encoded by these genes may also function to modulate the levels of H_2O_2 for intracellular signalling

3.3.2 Response to environmental stress

Genes which are involved in protection against protein damage were also significantly up-regulated on the H_2O_2 microarray. For example, genes encoding heat shock or heat shock-related proteins (HSPs) represent an abundant class up-regulated (19 in total). Of these, 9 had fold ratios of more than 10, two of which were more than 100-fold up-regulated. HSPs are able to enhance cellular survival by sequestering oxidatively damaged and denatured proteins (which accumulate under stress conditions) and thus facilitate protein refolding or proteolytic degradation (Banzet *et al.*, 1998). The induction of HSPs by H_2O_2 may therefore lead to increased tolerance of further oxidative stress, as well as contribute towards to tolerance of other environmental stresses. HSPs have previously shown to be up-regulated in

microarray studies of Arabidopsis plants perturbed in the H_2O_2 scavenging enzymes *CAT2* and *APX1* (Pnueli *et al.*, 2003; Vandenabeele *et al.*, 2004). Additionally, the up-regulation of ubiquitin-related gene expression (two poly-ubiquitins, a ubiquitin-specific protease and a U-box domain-containing protein) is suggestive of ubiquitylation-dependent proteolysis of damaged proteins. Such a proteolytic mechanism is also a major event during the induction and execution of cell death (Estelle, 2001).

The up-regulation of cytochrome-*c* transcripts and chloroplast-encoded photosynthesis genes (*psaA*, *psbD*, *ndhF* and *ndhH*), may be a reflection of compensation for oxidative damage to electron-transport components. Cytochrome-*c* (an electron-transport chain component) is a well known mediator in the initiation of apoptosis of mammals: its release from mitochondria into the cytosol triggers the proteolytic activation of a caspase protease cascade (Jiang and Wang, 2004). Studies involving the use of PCD-inducing toxins and abiotic stresses have also demonstrated a translocation of cytochrome-*c* in plants although it does not appear to be universal in plant PCD (Balk and Leaver, 2001).

Other stress-related transcripts up-regulated include disease resistant proteins of the Toll-Interleukin-Resistance (TIR) class, which are involved in pathogen recognition and defence, a mildew resistance protein, *RESPONSIVE TO DESSICATION 2* (*RD2*) and a 23.5-fold induced BCL-2-associated athanogene (BAG) protein (*BAG6*). Plant BAG proteins are homologues of mammalian regulators of apoptosis, and regulate various apoptotic-like processes ranging from response to pathogen attack, to abiotic stress, to plant development.

3.3.3 Second messengers and hormones

A close interaction between H_2O_2 and other signalling agents/molecules has been demonstrated in the literature (e.g. during pathogen defence and guard cell closure). It is well known that a significant amount of cross-talk occurs between ROS and calcium, and the H_2O_2 microarray expression data clearly agrees with this, since the expression of calcium-binding and calmodulin-related proteins was both up- and down-regulated (15 and 7 respectively). Furthermore, an increase in the transcript levels of *NIA1* a nitrate reductase (NR) gene by 2-fold was observed on the H_2O_2 microarray. NR has been demonstrated to mediate the synthesis of the signalling agent nitric oxide (NO) in Arabidopsis guard cells (Desikan *et al.*, 2002). NO mediates ABA-induced stomatal closure and can induce cell death, both of which involve H_2O_2 (Clarke *et al.*, 2000; Neill *et al.*, 2002).

H_2O_2 has previously been demonstrated to trigger transcriptional changes of genes involved in the biosynthesis of hormones (e.g. in CAT deficient tobacco plants exposed to high light intensities; Vandenabeele *et* al., 2003). Work in this chapter supports this observation. For example, the *ALLENE OXIDE CYCLASE 1* (*AOC1*) transcript was 4-fold up-regulated, and it encodes an enzyme crucial for the formation of the correct stereoisomeric JA precursor, *cis*(+)12-oxophytodienoic acid (OPDA; Delker *et al.*, 2006). JA is well known to play a role in regulating plant growth, for example during growth inhibition, leaf abscission and senescence, and has been shown to modulate PCD (Overmyer *et al.*, 2000). There was also a 7-fold up-regulation of the expression of *ACC SYNTHASE 6*, a gene involved in the synthesis of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). A link between ethylene and ROS is well established in the propagation of PCD lesions (Overmyer *et al.*, 2000). In addition, environmental stresses can also induce ethylene formation, and ethylene is able to stimulate a variety of processes including fruit ripening, abscission of leaves and senescence. The expression of 8 auxin-related genes were also up-regulated on the H_2O_2 microarray, including auxin-responsive proteins such as *AUXIN-RESPONSIVE FACTOR 2* (*ARF2*) and auxin efflux carriers. Auxins have been demonstrated to be a basic coordinative signal of plant development. The up-regulation of auxin-related genes suggests a role for ROS in auxin-mediated processes and provides support for a previous study which suggested ROS are able to enhance auxin-driven root growth (Pasternak *et al.*, 2005).

3.3.4 Kinases

Kinase-mediated protein phosphorylation is a rapid response and does not require kinase gene transcription. However, a correlation is often seen between the up-regulation of kinase gene transcription and the corresponding protein levels (Hirt, 1999). This observation was used here to identify potential kinase genes involved in H_2O_2 signalling. The expression of 23 kinases was up-regulated by the exogenous H_2O_2 treatment and 18 were down-regulated. Those up-regulated include transcripts for pathogen responsive kinases. For example, the receptor-like protein kinase (CRK11) which is induced by *Ralstonia solanacearum* (Chen *et al.*, 2003) was initially selected as a candidate gene or further study. Additionally, another gene selected as a candidate (which was later rejected for showing less strong H_2O_2 induction by northern blot analysis than the other candidate genes) was *BOTRYTIS-INDUCED KINASE 1* (*BIK1*). A recent study has characterised this gene (Veronese *et al.*,

2006). The authors demonstrated that mutant *bik1* plants were severely susceptible to necrotrophic fungal pathogens and exhibit altered root growth (producing longer and more root hairs). This work supports a role for BIK1 in modulating the signalling factors required for defence responses and root hair growth, and emphasises the efficacy of the candidate gene selection criteria.

3.3.5 Phosphatases

Transcripts of two tyrosine (Tyr) specific phosphatases were up-regulated on the H_2O_2 microarray. Protein Tyr phosphatases regulate eukaryotic protein phosphorylation events predominantly the inactivation of MAPK cascades (Luan, 1998), and have been identified as the primary target for H_2O_2 in animals (Wu *et al.*, 1998). The phosphatase-related protein SGT1A (suppressor of G2) expression was also up-regulated on the H_2O_2 microarray. This protein along with SGT1B is induced in Arabidopsis leaves upon pathogen infection (Azevedo *et al.*, 2006). SGT1 positively regulates disease resistance conferred by multiple resistance (R) proteins and also developmental responses to auxin (Azevedo *et al.*, 2006).

3.3.6 Transcription factors and promoter elements

Various transcription factor transcripts showed altered expression on the H_2O_2 microarray, suggesting that the subsequent expression of further genes is likely at later time points. Transcription factors with up-regulated expression include those well known to play a key role in stress responses. For example, *ZAT12*, which participates in the response to high light stress (Iida *et al.*, 2000) and plays a role in cold acclimation (Vogel *et al.*, 2005), *DREB2A*, known to be a key regulator in the drought response (Liu *et al.*, 1998) and a salt tolerant zinc finger protein (*STZ*).

The expression of 9 WRKY transcription factor genes were up-regulated and 2 down-regulated in the H_2O_2 microarray experiment. WRKYs are involved in defence, wounding, senescence and plant development (Eulgem *et al.*, 2000; Robatzek and Somissich, 2001). For example, WRKY70 (7-fold up-regulated) acts as a node of convergence for integrating (SA)- and (JA)-mediated signalling events during plant response to pathogens. It is able to activate SA-dependent defence genes and repress JA-regulated genes (Li *et al.*, 2006). WRKY transcription factors modulate gene expression by binding to W-boxes and W-box-like

motifs. As might therefore be expected, this element was significantly over-represented in the promoters of the H_2O_2 up-regulated genes (Table 3.3).

Three ethylene responsive binding factors (ERFs), *ERF1*, *ERF5* and *ERF6* were up-regulated in their transcript levels on the H_2O_2 microarray. ERFs are able to bind to the GCC box sequence found in many stress responsive genes (Fujimoto *et al.*, 2000).

Two heat shock transcription factors (HSFs) including *HSF21* were up-regulated on the H_2O_2 microarray. HSF-binding motifs are present in the promoters of *APX1* (Mittler and Zilinskas, 1992; Storozhenko *et al.*, 1998), *AOX1* as well as in the promoters of many defence genes and transcription factors induced by H_2O_2 such as *ZAT12* (Rizhsky *et al.*, 2004). Moreover, in Drosophila and mammalian cells, the DNA binding of HSFs was shown to be induced *in vitro* and *in vivo* by H_2O_2, suggesting that HSFs can act as direct sensors of H_2O_2 (Zhong *et al.*, 1998).

Transcripts of 4 NAC (NAM [no apical meristem] ATAF1/2, CUC2 [cup-shaped cotyledons]) domain transcription factors were up-regulated on the H_2O_2 microarray including *ATAF1*. Members of this family have been implicated in the regulation of development and differentiation (e.g. *CUC2*; Aida *et al.*, 1997 and *NAP*; Sablowski and Meyerowitz, 1998) including auxin-dependent formation of the lateral root system (*NAC1*; Xie *et al.*, 2000). Others (e.g. *ATAF1* and *ATAF2*) are induced by pathogen attack and wounding (Aida *et al.*, 1997). Environmental stresses can also induce *NAC* genes (e.g. *RD26*; Fujita *et al.*, 2004) all processes involved with ROS.

Down regulation of suppressors might be a mechanism that regulates H_2O_2 driven gene expression. For example, expression of *MYB4* was down-regulated on the H_2O_2 microarray and has previously been shown to regulate the accumulation of a UV protectant. Loss-of-function mutants were more tolerant to UV-B irradiation and had enhanced levels of sinapate esters in their leaves (Jin *et al.*, 2000). *MYB4* therefore functions as a transcriptional repressor of the target gene cinnamate 4-hydroxylase involved in the synthesis of sinapate ester sunscreens.

Abscisic acid response elements (ABREs) are important during the plant's response to abiotic stresses such as dehydration, salinity and cold, all of which are ABA-mediated (Abe *et al.*,

1997). There is evidence that ROS are involved in ABA signalling (Pei *et al.*, 2000). The enrichment of the ABRE motif in the promoter sequences of the H_2O_2 differentially regulated genes may reflect cross-talk among ROS and ABA signalling pathways.

3.3.7 Conclusion

Analysis of microarray data demonstrates that H_2O_2 can modulate the expression of a subset of genes within the Arabidopsis genome at the transcript level. The utility of the microarray system for identifying H_2O_2-responsive genes is authenticated by the detection of altered expression of genes previously found to be H_2O_2 responsive. For example, a previous microarray study in which Arabidopsis cell cultures were exposed to 20 mM H_2O_2 (pooled from 1.5 and 3 h treatments) also showed an up-regulation in transcripts involved in cell rescue and defence (Desikan *et al.*, 2001). Among the expressed sequence tags (ESTs) induced in this previous study were those for heat shock proteins, zinc finger proteins, ERFs, *GST6*, *ZAT12* and *DREB2A*. The exogenous 10 mM H_2O_2 was able to induce genes used to protect against oxidative damage (e.g. *APX1*, *GPX6*) and genes involved in signalling events (e.g. *ZAT12*, *DREB2A*) thus confirming the "dual face" of ROS. Such a transcriptional profile verifies that this method is reliable despite the lack of replicate slides.

Previous work indicates that increased expression of some genes in response to H_2O_2 is transient (Desikan *et al.*, 1998; 2000). Early time-points are likely to reveal candidate genes involved in ROS signal transduction, whilst later time points are likely to uncover potential target genes involved in downstream end responses. The 3 h time point used in this study was originally designed to "capture" both rapidly responding genes and longer term changes in gene expression. However, the microarray presented only a "snapshot" of the transcriptome at a specific moment, and the classification of early and late expressed genes was not possible. A time course study would enable visualisation of the dynamics of the transcriptional response.

It is important to bear in mind that the observed H_2O_2 transcript regulation may be either directly through transcription or via altered RNA stability (or both). Additionally, the observed changes in gene expression do not necessarily correlate with similar changes in protein levels or assume that translation has even occurred. If translation does occur the protein may

still require post-translational modifications or further subunits in order to function. Furthermore, it is clear from other studies that H_2O_2 can alter the activity of cellular proteins (e.g. MAPKs), possibly by interacting directly with target proteins (oxidising residues and therefore altering protein conformation).

The main purpose of this microarray analysis was to identify signal transducers and transcription factors putatively responsive to the initial H_2O_2 treatment. A more detailed functional analysis is necessary to clarify their role within the management of the downstream response.

Chapter 4
Construction and identification of loss- and gain-of-function lines

4.1 Introduction

Having selected 8 candidate ROS-signalling genes for further study (Chapter 3), the next step was to address questions concerning the biological role of these genes. One approach for investigating the gene function is to reduce, or completely abolish gene activity (a loss-of-function mutation). This method is widely used to identify genes that are **necessary** to confer a particular phenotype. Phenotypes associated with such loss-of-function mutations are most often recessive: the mutant can function normally if it retains at least one normal copy of the affected gene (heterozygous). It is therefore imperative to identify those individuals mutated in both alleles (homozygous) in order to reveal the *in vivo* function of the encoded protein.

An essential complement to the loss-of-function approach are studies that examine the effects of atypical gene expression, for example in the "wrong" tissue, at the "wrong" time and/or at abnormally high levels (over-expression). Such gain-of-function analyses are used to identify genes which are **sufficient** to confer a specific phenotype. Over-expression lines are particularly important when studying genes with redundant alleles in the genome and those in which loss-of-function alleles produce either a very subtle mutant phenotype or lead to lethality.

The aim of this chapter was to:

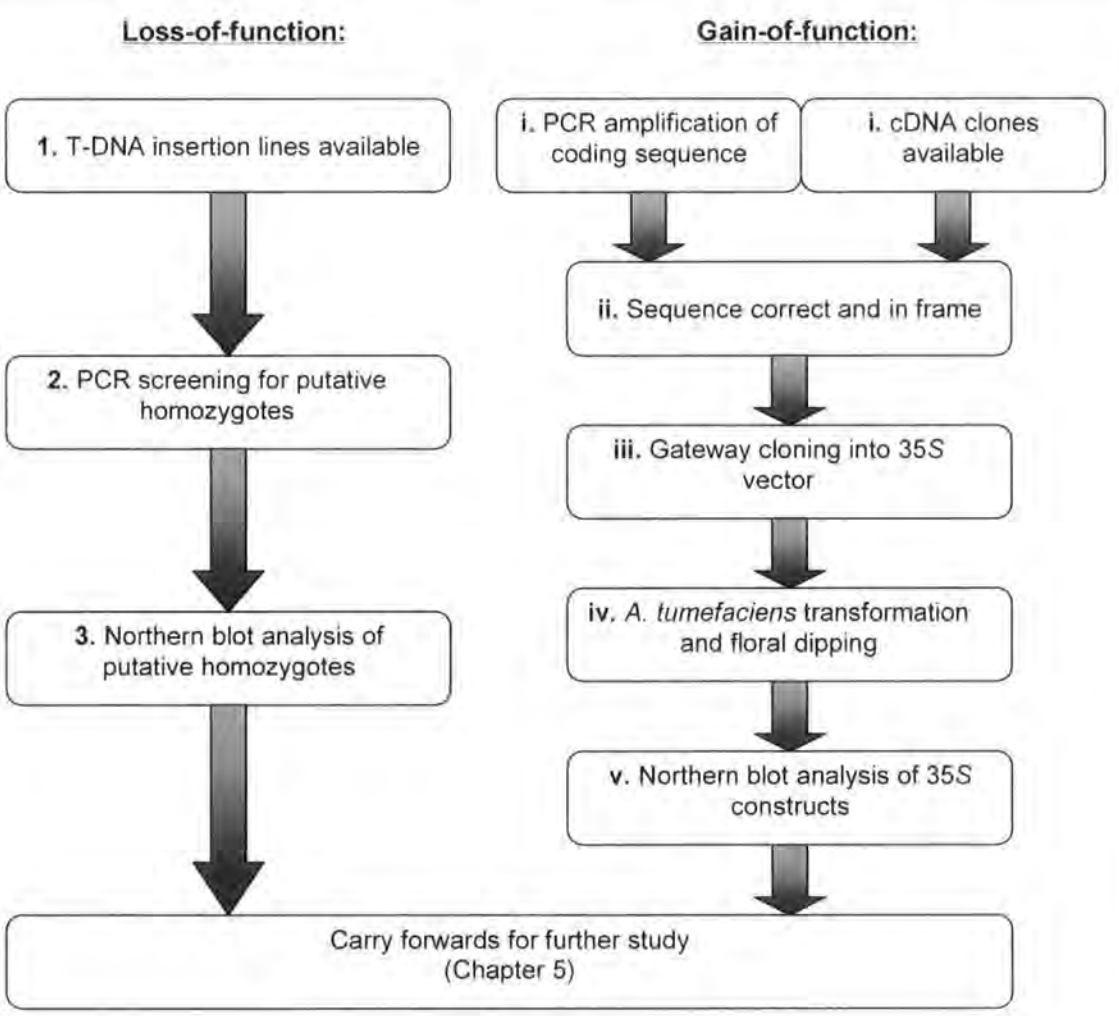
- *Identify homozygous loss-of-function mutants*
- *Construct over-expression lines*
- *Verify the expression levels of the loss-of-function and over-expression lines by northern blot analyses*

4.2 Results

As part of the selection criteria for further study, it was decided that at least one homozygous loss-of-function mutant as well as an over-expression line must be successfully identified in order for a candidate ROS-signalling gene to be carried forward (see Figure 4.1 below for details on the selection criteria).

Figure 4.1

Criteria for selection of candidate genes for further study.



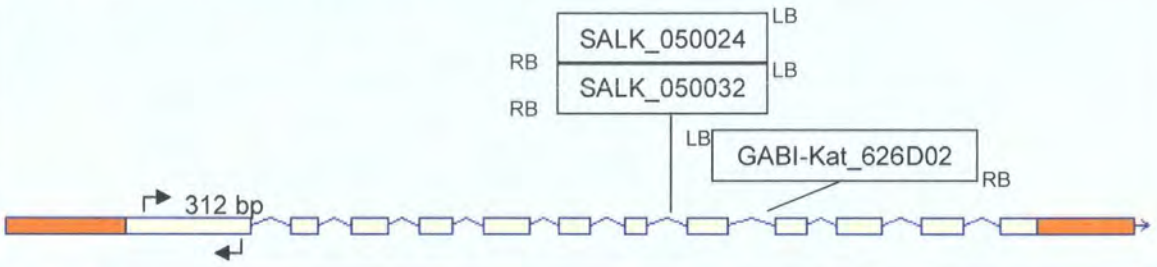
4.2.1 Loss-of-function lines

The publicly available Arabidopsis T-DNA insertion collections of SALK (Alonso *et al.*, 2003), SAIL (Sessions *et al.*, 2002), FLAGdb (Samson *et al.*, 2002) and GABI-Kat (Rosso *et al.*, 2003), were searched for loss-of-function mutants via the SALK Institute Genomic Analysis Laboratory (SIGnAL) T-DNA Express Arabidopsis Mapping Tool (*http://signal.salk.edu/cgi-bin/tdnaexpress*). T-DNA lines were preferentially chosen, where possible, with insertions closest to the transcription and translation start sites at the N-terminal region of the coding sequence. All but one of the 8 candidate genes (At4g31860; protein phosphatase 2C) had available T-DNA insertions. The corresponding seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC; Loughborough, UK), the Arabidopsis Biological Resource Centre (ABRC; Ohio, USA) or the Max Planck Institute (Koeln, Germany). The following two pages show schematic diagrams depicting the locations of the T-DNA insertions for the kinase (Figures 4.2), phosphatase (Figure 4.3) and transcription factor (Figure 4.4) candidate genes. Approximately 20 individual plants per T-DNA insertion line were grown up and screened for homozygous mutations via PCR and northern blot analysis. (Plants grown from the original distribution seed were named T_1 and subsequent generations named T_2, T_3 and so forth).

Figure 4.2

Location of T-DNA insertions in the kinase candidate genes.

At4g18950 (ankyrin protein kinase) 1380 bp



At5g25930 (protein kinase family protein) 3018 bp

At3g22060 (putative receptor kinase common family protein) 759 bp

T-DNA insertion sites and border locations are based on annotations from the SIGnAL T-DNA Express Mapping Tool. Transcript maps are reproduced from the *Arabidopsis thaliana* Integrated Database (ATIDB; *http://atidb.org/*). White regions represent exons, and orange areas denote 5'- or 3'-untranslated regions. Introns are depicted by a single line. Primers used for PCR amplification of probes for northern blot analyses are marked with arrows. LB and RB denote left and right borders respectively. Not drawn to scale.

Figure 4.3

Location of T-DNA insertions in the phosphatase candidate gene. (Detail as Figure 4.2).

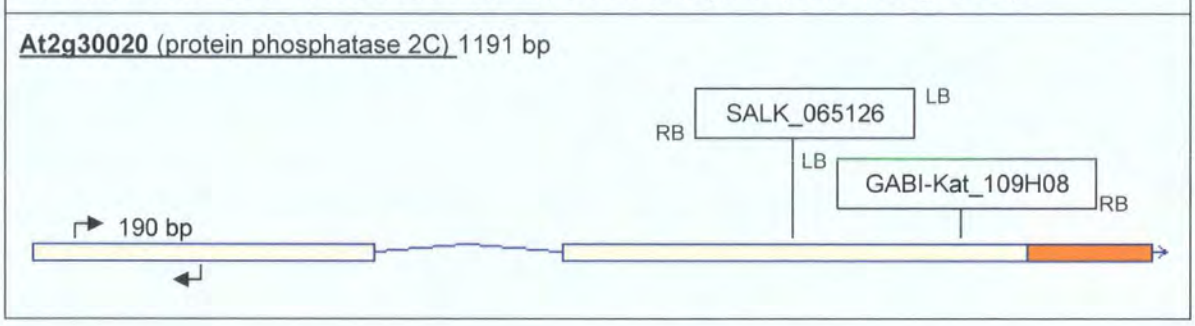
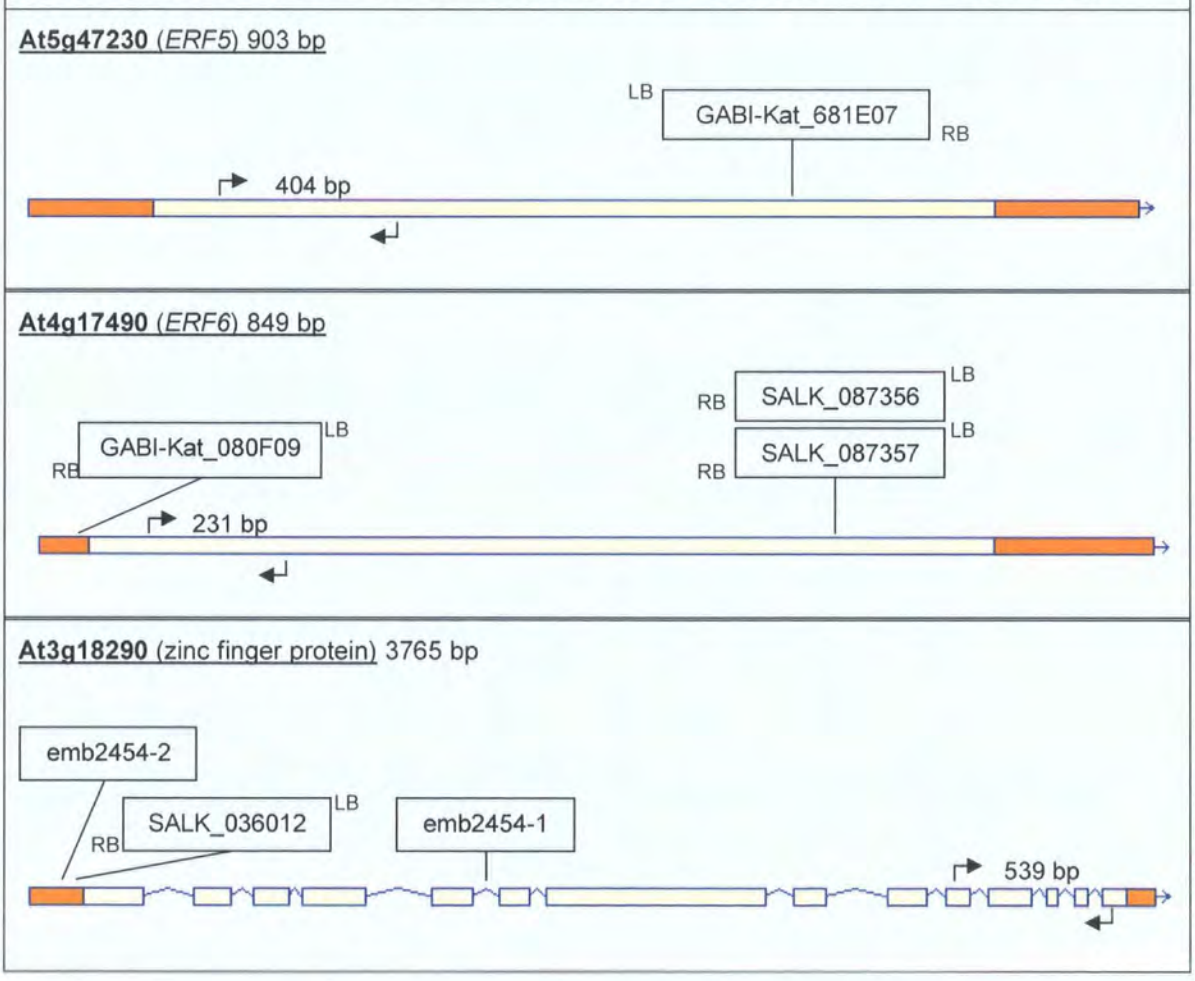


Figure 4.4

Location of T-DNA insertions in the transcription factor candidate genes. (Detail as Figure 4.2).



4.2.1.1 PCR screening method

In order to confirm if plants contained the T-DNA insertion, and if so, whether they were heterozygous or homozygous for the mutation, genomic DNA was extracted from the unopened flower buds of individual T_1 plants from each line (as previously described in Materials and Methods 2.8.1). Suitable primers upstream (5') and downstream (3') of the predicted T-DNA insertion were designed and used in conjunction with the T-DNA border primers for screening (for full primer details please refer to Appendix B.4). The extracted genomic DNA was then used to "seed" PCR reactions (Materials and Methods 2.12) in order to screen for individual T_1 plants containing T-DNA insertions in the gene of interest.

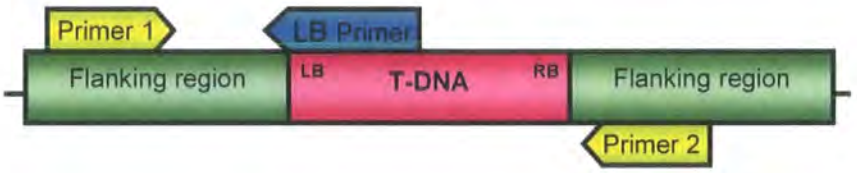
The strategy of the PCR screening method was to perform two reactions per individual plant. Firstly, using a pair of gene-specific primers which flanked the putative site of the T-DNA insertion, and secondly using a gene-specific primer and a T-DNA left border primer (as depicted in Figure 4.5a overleaf). A diagnosis could then be made from the presence or absence of an amplification product as to whether the T-DNA insertion was there. For example, a plant homozygous for the T-DNA insertion will only yield the left border-amplified PCR product, as the excessive size of the T-DNA will prevent amplification of a flanking PCR product. A heterozygote will result in the presence of both the left border-amplified and flanking PCR products. If the plant lacks the insertion in the first instance, or if it had segregated out, then only the gene-specific primers will result in PCR product amplification.

An example of a PCR screen of 5 individual T_1 plants is shown overleaf in Figure 4.5, using two gene-specific flanking primers (Figure 4.5b) and a T-DNA left border primer with a flanking primer (Figure 4.5c). From this, it is possible to deduce that plants 1 and 5 are homozygotes, 4 is a heterozygote, and both 2 and 3 are wild-type segregants.

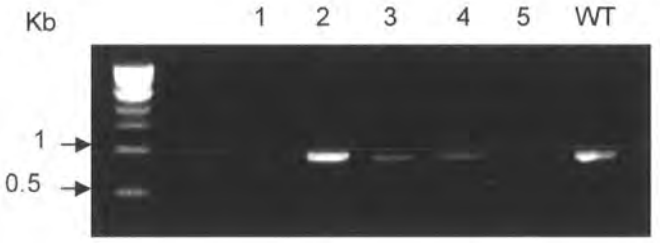
Figure 4.5

PCR screening strategy for identification of T-DNA insertion lines

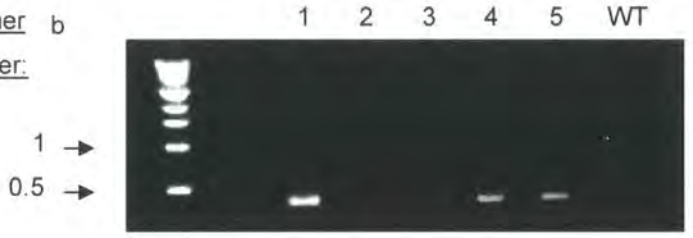
a)



b) PCR with primers 1 and 2:



c) PCR with primer 1 and LB primer:



a) Schematic diagram of primers used for PCR screening. The gene-specific primers (primers 1 and 2) flank the site of the T-DNA insertion and will amplify a product only if there is no T-DNA insertion. The T-DNA-specific left border primer (LB primer) and primer 1 will amplify a product only if the T-DNA insertion is present.

b) Example of an agarose gel electrophoresis of PCR products amplified with 2 gene-specific flanking primers. Lanes 1-5 contain 5 individual plants to be screened plus wild-type (WT)

c) Agarose gel electrophoresis of PCR products amplified with a T-DNA left border primer and a gene-specific flanking primer.

This example is taken from a screen for Gabi-KAT_751D04 in the kinase gene At2g25930.

4.2.1.2 PCR screening results

The PCR screening results are summarised overleaf in Table 4.1. Of the 13 T-DNA insertion lines screened by this method, 5 yielded putative homozygotes. These plants were allowed to self, and genomic DNA was extracted from pooled 7-day old seedlings (approximately 20 plants) of this next generation (T_2). This extracted DNA was used to seed more PCR reactions and verified the initial screening results. Besides the identification of 5 putative homozygotes, 3 lines appeared to lack the T-DNA insertion all together. The remaining 5 lines gave inconclusive results due PCR technicalities (i.e. failure of one or both primer pairs to amplify or amplification of a PCR product of a different size to that predicted). The inconclusive lines and were analysed further by northern blot analysis along with the putative homozygotes (see Section 4.2.1.4 later).

4.2.1.3 Precise location of T-DNA insertions

The exact location of the T-DNA insertion sites (and accuracy of the PCR screens) were determined by DNA sequencing (Materials and Methods 2.14) of the PCR products amplified using the T-DNA left border primers. Insertion sites of the homozygous lines that were ultimately carried forward for further study are shown in Appendix E.

Table 4.1

A summary of the PCR screening results of the available T-DNA insertions for the 8 candidate ROS-signalling genes.

AGI code	Putative ID	T-DNA line	Insert location	PCR screening result		
				Homoz	Absent	Incon
Kinases:						
At4g18950	Ankyrin protein kinase	SALK_050024 SALK_050032 GABI-Kat_626D02	Intron Intron Intron		✓	✓ ✓
At5g25930	Protein kinase family protein	SALK_091274 GABI-Kat_751D04	5' UTR Exon	 ✓		✓
At3g22060	Receptor kinase common family protein	SALK_151902	Intron	✓		
Phosphatases:						
At2g30020	Protein phosphatase 2C	SALK_065126 GABI-Kat_109H08	Exon Exon	 ✓		✓
At4g31860	Protein phosphatase 2C	None available	-	-	-	-
Transcription factors:						
At5g47230	ERF5	GABI-Kat_683E07	Exon	✓		
At4g17490	ERF6	SALK_087356 SALK_087357 GABI-Kat_080F09	Exon Exon 5' UTR	 ✓	 ✓	✓
At3g18290	Zinc finger protein	SALK_036012 emb2454-1 emb2454-2	5' UTR Intron 5' UTR	 - -	✓ - -	 - -

A tick under the column headed "Homoz" denotes successful identification of at least 1 homozygous plant, whilst that under the "Absent" column refers to absence of a T-DNA insertion. "Incon" denotes an inconclusive result from the PCR screening (i.e. due to failure of primer pairs to amplify or the amplification of a product of a size other than that predicted).

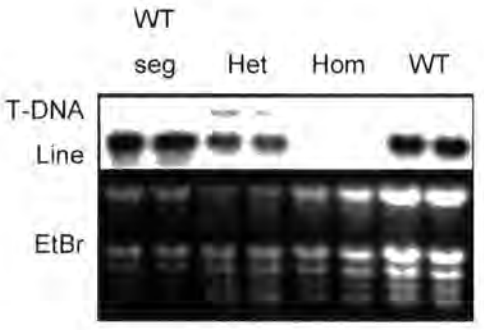
4.2.1.4 Northern blot screening

To extend the PCR screening results, northern blot analyses (Materials and Methods 2.16) were performed on the putative homozygous lines identified and also on those lines where it had not been possible to draw conclusions due to PCR technical problems. Northern blot analysis also allowed visualisation of the effect that the T-DNA insertions had on both the size and abundance of the RNA transcript.

Pooled 7-day old seedlings (approximately 20 per line) from the wild-type and the next generation (T_2) of segregating putative homozygous T-DNA mutants were treated for 3 h with 10 mM H_2O_2 in order to up-regulate candidate gene expression levels (to facilitate transcript visualisation). Total RNA was then extracted and used to perform northern blot analyses from two independent RNA isolations. The same double-stranded gene-specific DNA probes originally used for northern blot analyses of H_2O_2-inducibility (Results Chapter 3.2.3.2) were radio-labelled and hybridised to the membrane-bound RNA. The positions of the probes in relation to the T-DNA insertions are depicted in the previous Figures 4.2, 4.3 and 4.4. An example of a northern blot genotyping screen is shown below in Figure 4.6.

Figure 4.6

Northern blot screening for identification of homozygous T-DNA insertions lines.



"WT seg", "Het" and "Hom" denote wild-type (WT) segregant, heterozygote and homozygote respectively. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg of total RNA per lane). Samples are in duplicate. This example is taken from a screen for SALK_087356 in the *ERF6* gene (At4g17490)

4.2.1.5 Northern blot screening results

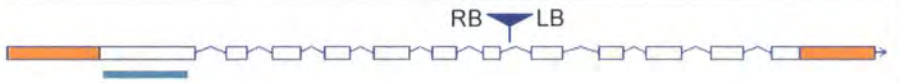
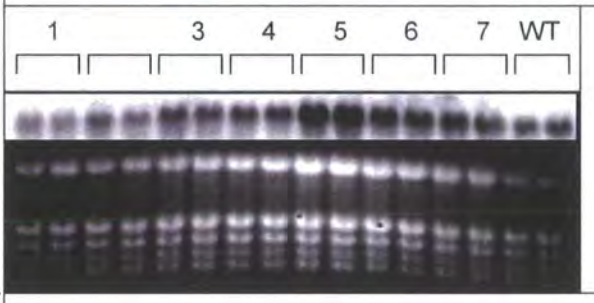
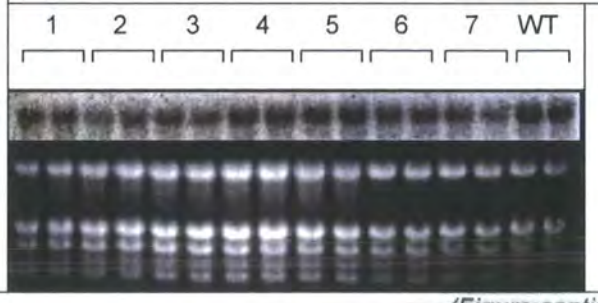
The results of the northern blot analyses for the 10 lines (5 putative homozygous and 5 inconclusive) are shown on the following four pages for the kinase (Figure 4.7), phosphatase (Figure 4.8) and transcription factor (Figure 4.9) T-DNA lines.

The northern blots showed altered transcript levels in the 5 putative homozygous lines identified by PCR. However, only two of these were carried forwards: SALK_151902 which exhibited reduced transcript levels compared to the wild-type (Figure 4.7), and GABI-Kat_681E07 which resulted in a transcript of a smaller size to that of the wild type (Figure 4.9). The remaining three putative homozygous GABI-Kat lines were rejected because of the observed over-expression of the sense transcript (discussed in the following Section 4.2.1.6).

Of the 5 lines that had had given inconclusive results from PCR screening, three were carried forwards: GABI-Kat_626D02, SALK_065126 and SALK_087356 (Figures 4.7, 4.8 and 4.9 respectively). The remaining two inconclusive lines were rejected at this stage as they resulted in transcripts similar in size and abundance to that of the wild-type.

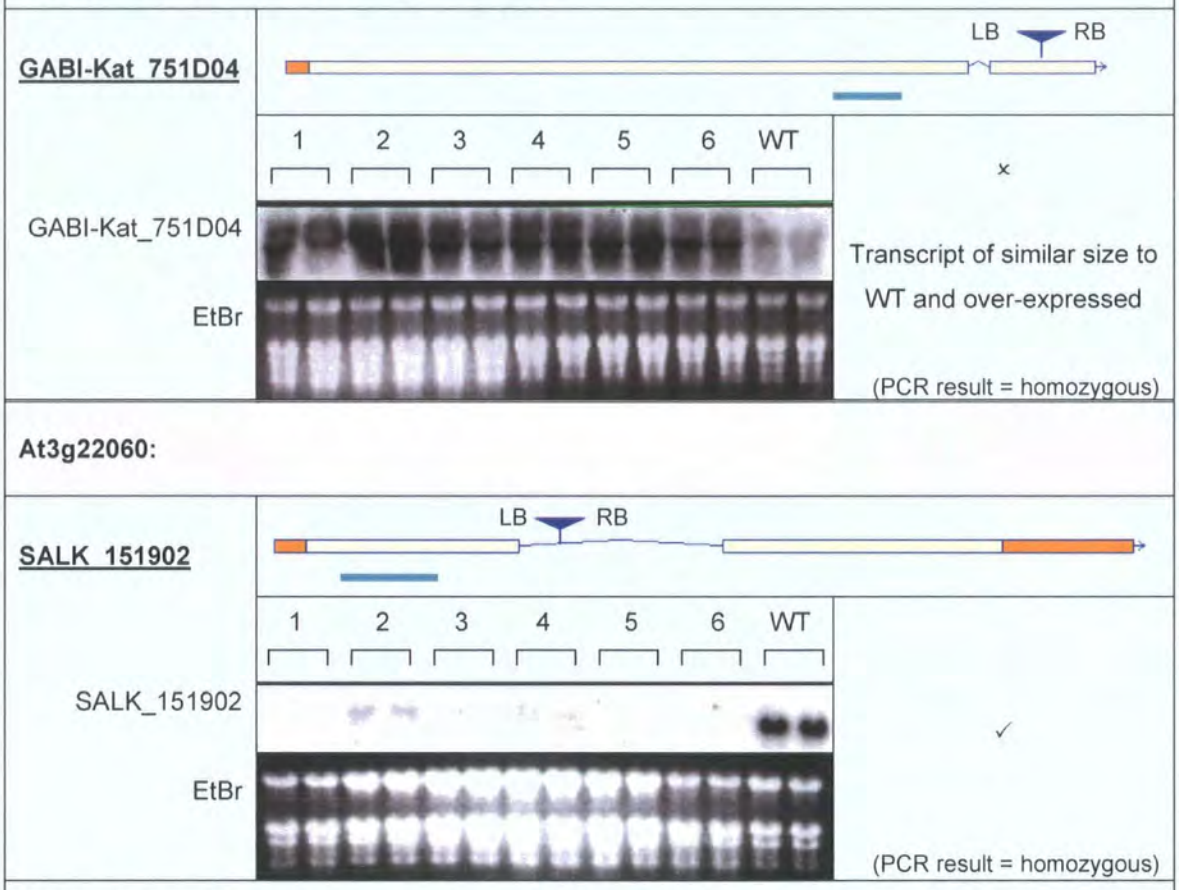
Figure 4.7

Northern blot analysis of putative homozygous T-DNA insertion lines of the kinase genes.

T-DNA line	Northern blot result	Further study
At4g18950:		
SALK_050024		
SALK_050024 EtBr		× Transcript of similar size and abundance as WT (PCR result = inconclusive)
GABI-Kat_626D02		
GABI-Kat_626D02 EtBr		✓ (PCR result = inconclusive)
At5g25930:		
SALK_091274		
SALK_091274 EtBr		× Transcript of similar size and abundance as WT (PCR result = inconclusive)

(Figure continues on the following page)

Figure 4.7 *(Continued from the previous page)*



Pooled 7-day old seedlings (approximately 20 per line) from the wild-type (WT) and the next generation (T_2) of segregating putative homozygous T-DNA mutants were treated with 10 mM H_2O_2 for 3 h. Extracted total RNA from two independent RNA isolations per line (except GABI-Kat 626D02) was probed with the radio-labelled gene-specific double-stranded DNA probes used in Chapter 3. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg of total RNA per lane). Samples are in duplicate. Probes are depicted underneath the gene schematic by a thick pale blue line.

Figure 4.8

Northern blot analysis of putative homozygous T-DNA insertion lines of the phosphatase gene. (Detail as Figure 4.7).

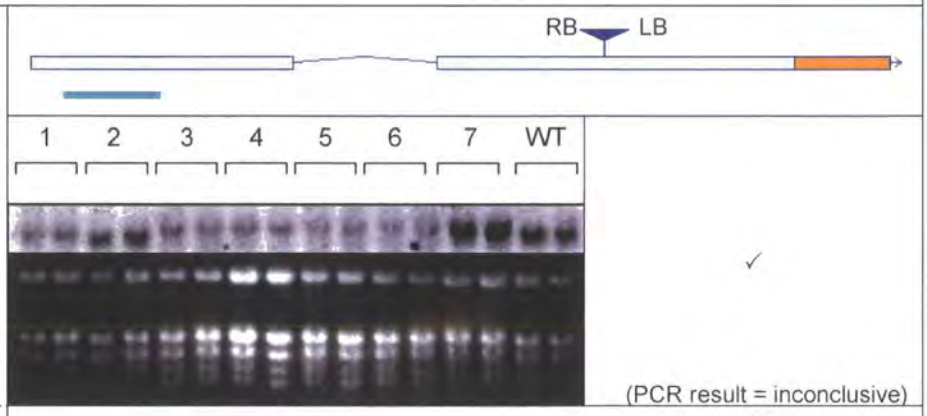
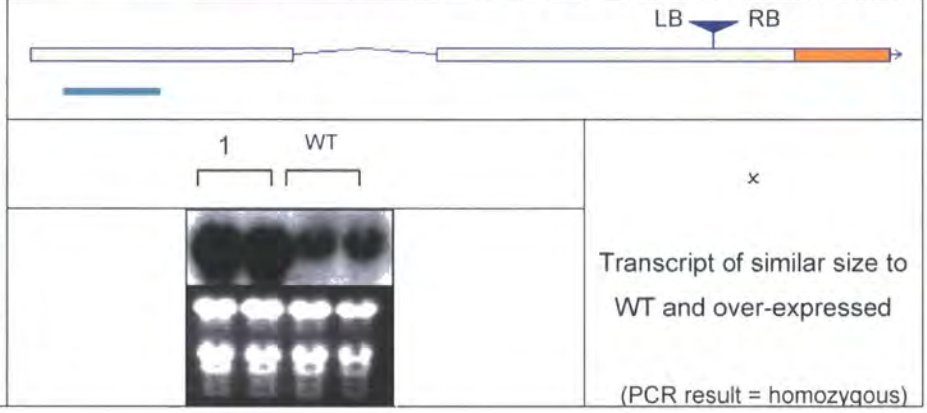
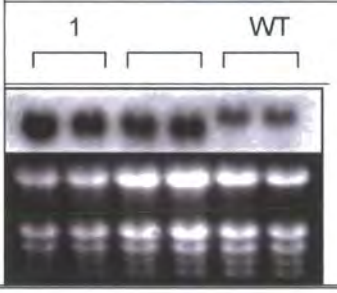
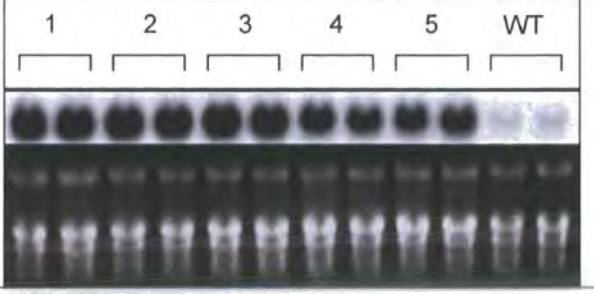
T-DNA line	Northern blot result	Further study
At2g30020:		
SALK_065126		✓ (PCR result = inconclusive)
GABI-Kat_109H08		x Transcript of similar size to WT and over-expressed (PCR result = homozygous)

Figure 4.9

Northern blot analysis of putative homozygous T-DNA insertion lines of the transcription factor genes. (Detail as Figure 4.7)

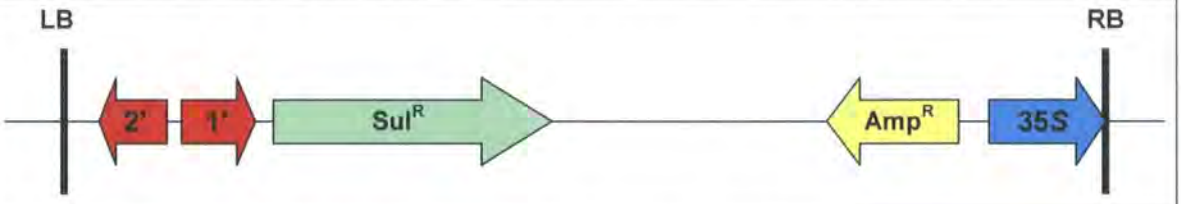
T-DNA line	Northern blot result	Further study
At5g47230:		
GABI-Kat_681E07		
GABI-Kat_681E07 EtBr		✓ (PCR result = homozygous)
At5g47230:		
SALK_087356		
SALK_087356 EtBr		✓ (PCR result = inconclusive)
GABI-Kat_080F09		
GABI-Kat_080F09 EtBr		x Transcript of similar size to WT and over-expressed (PCR result = homozygous)

4.2.1.6 Over-expression of sense transcripts in the GABI-Kat T-DNA insertion lines

A very surprising discovery was that all five of the GABI-Kat T-DNA mutant alleles tested, exhibited over-production of the sense transcript, leading to over-expression instead of loss-of-function of the gene of interest. Upon further investigation, it was realised that the pAC161 vector used to create the GABI-Kat T-DNA insertion lines had been originally designed for activation-tagging (Rosso *et al.*, 2003). Activation-tagging enables a gene of interest to be over-expressed (activated) due to a nearby insertion of a T-DNA carrying a strong promoter at its border. Hence, pAC161 contains the 35*S* promoter at the right border (see Figure 4.10 below). Therefore, if the T-DNA integrated nearby the 5' of a coding sequence in the LB-[T-DNA]-RB direction, then the 35*S* promoter could potentially activate the downstream gene leading to gain-of-function/over-expression. Surprisingly, this feature of the GABI-Kat collection appears not to be realised by the general plant community. Numerous publications exist on using a T-DNA insertion lines from this collection for mutant characterisations, yet there appear to be no articles about using them for activation-tagging purposes.

Figure 4.10

Schematic diagram of the pAC161 vector used to make the GABI-Kat T-DNA lines.



This vector was originally designed for activation tagging, hence the 35*S* promoter (Rosso *et al.*, 2003). The 1'2'-bidirectional promoter is used to drive expression of the sulphadiazine resistance gene (*SUL1*) for selection of transgenic plants, but can be read in either direction. Not drawn to scale.

The T-DNA orientation of 4 of the GABI-Kat lines was in the LB-[T-DNA]-RB direction (thus the 35*S* promoter could drive over-expression of the downstream sense transcript). However, for the fifth GABI-Kat line of *ERF6* (GABI-Kat_080F09) the insertion was in the RB-[T-DNA]-LB direction and was located at the beginning of the only exon, the size of the over-expressed transcript was also similar to that of the wild-type (see Figure 4.9 on the previous page). This result was unexpected, since 35S promoter which could lead to transcript activation was on the other end of the T-DNA in opposite direction to the gene. What then could be causing the observed over-expression of the gene? A closer look at the pAC161 vector showed that the T-DNA also encodes a 1'2'-bidirectional promoter near the left border (Velten *et al.*, 1984). The over-expression may therefore be due to the presence of this promoter potentially leading to activation of the sense transcript. Complete loss-of-transcription was observed in the SALK_087356 line where the T-DNA inserted towards the C-terminal end of the *ERF6* gene (see Figure 4.9 on previous page). This result corroborates the possibility that over-expression was specific to the GABI-Kat pAC161 T-DNA vector.

4.2.1.7 T-DNA insertions may generate antisense RNAs

T-DNAs with strong promoters on their borders may lead to relatively high amounts of antisense transcript production. Northern blot analysis of GABI-Kat_109H08 line resulted in high levels of transcript from the samples containing the T-DNA (Figure 4.8). The size of the transcript was slightly smaller than the wild-type gene transcript. It is possible that the 1' 2' promoter located near the left border of the pAC161 T-DNA vector leads to generation of antisense transcripts. It is unlikely that insertion of T-DNA into this gene is leading to increased sense transcript stability and therefore higher accumulation of RNA, because another T-DNA insertion allele into exon 2 in SALK_065126 line (Figure 4.11) leads to a reduction in the transcript made as shown previously in Figure 4.8. Such high amounts of antisense RNA could lead to silencing of any other gene in the genome if there is sufficient homology between transcripts (discussed later in Section 4.3).

4.2.1.8 The embryo-lethal phenotype

At this point, it was reluctantly decided that there was insufficient time to tackle the challenge of overcoming the embryo-lethal phenotype of the two mutations (emb2454-1 and emb2454-2) in the gene encoding a zinc finger protein (At3g18920). Since embryo lethality will result in no recovery of mutant plants for investigation, one approach to obviate this problem could be to use an embryo-specific rescuing promoter to drive gene expression during very early development. Another approach that may circumvent the lethality problem may be to use an inducible RNA-mediated interference (inducible RNAi) system for conditional gene silencing, (whereby double-stranded RNA inhibits the expression of genes with complementary nucleotide sequences). Therefore the RNAi silencing of the At3g18920 could be initiated at various developmental stages. For example, the Cre/loxP system is one such method used in Arabidopsis, in which 17β-estradiol is used to induce target gene silencing by dsRNA (Guo *et al.*, 2003).

4.2.2 Gain-of-function lines

4.2.2.1 Obtaining the full length coding sequences

This work was carried out at the same time as identification of the T-DNA insertion lines.

In order to obtain full length coding sequences for the 8 candidate genes carried forward from Chapter 3, PCR was used in the first instance to try to amplify the coding regions directly from cDNA. This method was successful for 4 genes: the kinases At4g18950 (1380 bp) and At3g22060 (759 bp), and both ERF transcription factors: At5g47230 (903 bp) and At4g17490 (849 bp). For the remaining 4 candidate genes, which all had relatively large coding sequences (1074 to 3765 bp), the TAIR database was used to identify available full length cDNA clones. These were obtained from ABRC (Ohio, USA), the RIKEN BioResource Centre (Ibaraki, Japan) and the French National Institute for Agricultural Research (INRA) (Paris, France).

The coding regions of the PCR products and cDNA clones were verified via DNA sequencing (see Materials and Methods 2.14). In the case of large DNA regions (>1 Kb), over-lapping lengths were sequenced approximately every 500 bp in order to obtain good quality data. All primers used for coding region amplification and internal sequencing are detailed in Appendices B.3 and B.1 respectively. Two of the 4 cDNA clones gave perfect sequences. However, clone BX819437 of the phosphatase At2g30020 was truncated and clone U21974 of the zinc finger gene resulted in a truncated coding sequence of only 56 bp. A second cDNA clone of the zinc finger protein coding sequence (RAFL09-84-M23) possessed a complete coding sequence. The coding sequence PCRs and cDNA clone sequencing results are summarised overleaf in Table 4.2

By this point, genotyping results for the T-DNA insertion lines had revealed which genes gave no data consistent with possessing a T-DNA insertion (see previous Section 4.2.1.5). These candidates were consequently abandoned for gain-of-function work as they did not meet with the criteria (shown in Figure 4.1 at the beginning of this Chapter). Additionally at this time, the putative receptor kinase common family gene (At3g22060) had been re-annotated on the TAIR 6 database to an unknown protein lacking the protein kinase domain (instead it possesses a domain of unknown function that is usually associated with a kinase domain). It

was decided that this gene was also to be abandoned for further study. Thus only the ankyrin protein kinase (At4g18950) and the two *ERF* genes were taken through all the steps involved in making the gain-of-function constructs (Figure 4.1).

Table 4.2

AGI code	Putative ID	CDS (bp)	PCR	cDNA clone name (and source)	Plasmid vector	Correct sequence in clone
Kinases:						
At4g18950	Ankyrin protein kinase	1380	✓	-		
At5g25930	Protein kinase family protein	3081	×	RAFL15-09-P09 (RIKEN)	Modified pBS-2 (λFLC-1-E)	✓
At3g22060	Receptor kinase common family protein	759	✓	-		
Phosphatases:						
At2g30020	Protein phosphatase 2C	1191	×	BX819437 (INRA)	pCMV-SPORT6	×
At4g31860	Protein phosphatase 2C	1074	×	U14631 (ABRC)	pUNI-51	✓
Transcription factors:						
At5g47230	ERF5	903	✓	-		
At4g17490	ERF6	849	✓	-		
At3g18290	Zinc finger protein	3765	×	U21974 (ABRC)	pUNI-51	×
				RAFL09-84-M23 (RIKEN)	Modified pBS-2 (λFLC-1-B)	✓

4.2.2.2 Gateway method for generation of 35S constructs

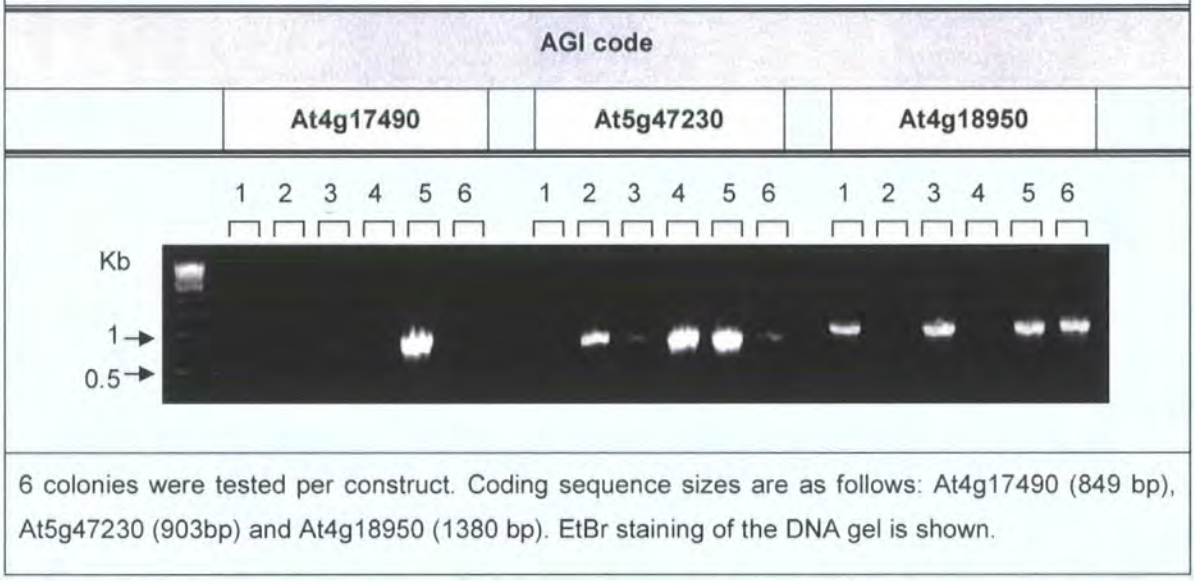
The 35*S* promoter of the Cauliflower Mosaic Virus (CaMV) was used to drive high-level expression of the candidate genes. The constitutive nature of this promoter (gene expressed in all tissues and at all times) enables phenotype screening without *a priori* knowledge as to where the gene is expressed *in planta*. The Gateway Technology cloning procedure (Invitrogen) was used to create 35*S* constructs. This system is based on the site-specific recombination properties of bacteriophage lamda (λ): site-specific attachment (*att*) sites serve as sites for recombination and facilitate transfer of DNA sequences between vectors.

4.2.2.3 Cloning into the entry vector

The full length coding sequences were cloned into the Gateway entry vector (pENTR/D-TOPO; for vector map see Appendix C.2). The coding sequence DNA was inserted between 2 *att*L sites, (*att*L1 and *att*L2) via the TOPO cloning reaction (Materials and Methods 2.18.3) and transformed into competent *E. coli* cells (Materials and Methods 2.19.1.1). Transformed colonies were then selected on kanamycin plates. To verify the presence of the coding sequence, individual colonies (6 per entry clone) were used to "seed" PCR reactions using the coding sequence amplification primers (Appendix B.3). Colony PCR results are shown overleaf in Figure 4.11. The resulting positive single colonies were cultured overnight and their plasmid DNA was extracted (Materials and Methods 2.8.2). The entry vector constructs were then verified by sequencing to ensure they were in frame and mutation free.

Figure 4.11

Colony PCR amplification of full length coding sequences following cloning into the Gateway entry vector.



6 colonies were tested per construct. Coding sequence sizes are as follows: At4g17490 (849 bp), At5g47230 (903bp) and At4g18950 (1380 bp). EtBr staining of the DNA gel is shown.

4.2.2.4 Cloning into the destination vector

The Gateway LR reaction was used to transfer coding sequences from the entry vector into the 35*S* destination vector (pK2GW7; Karimi *et al.*, 2002; for vector map see Appendix C.3). In this reaction the *att*R sites of the destination vector recombine with the *att*L sites of the entry clone to generate the final expression clone. Competent *E. coli* cells were transformed and colonies were selected on spectinomycin plates. Colony PCR was performed, plasmid DNA was extracted from positive colonies and sequenced as previously described (Section 4.2.2.3).

4.2.2.5 Agrobacterium-mediated plant transformation

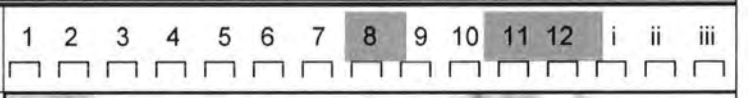
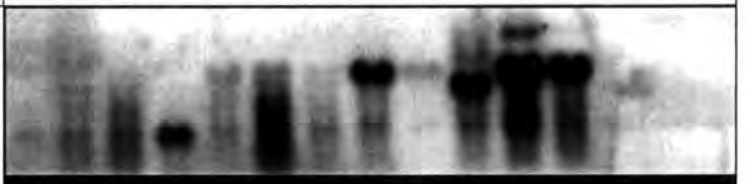
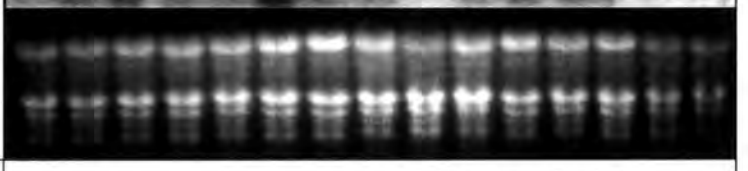
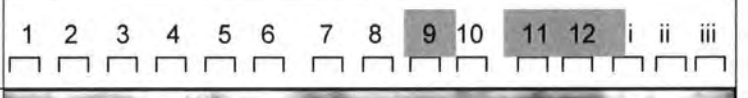
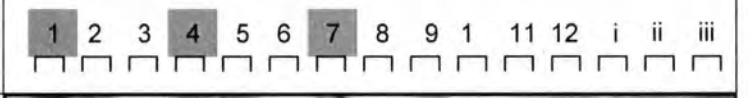
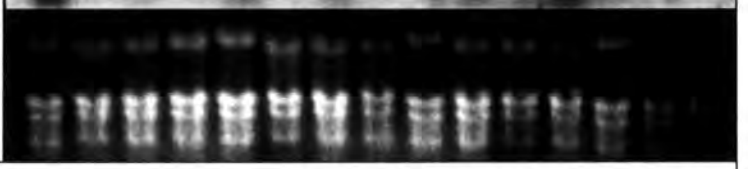
The three 35*S* constructs plus an empty pK2GW7 vector were transformed into *A. tumefaciens* (Materials and Methods 2.19.1.2). *A. tumefaciens* cultures were subsequently used to transform wild-type Col-0 Arabidopsis plants by floral dipping (Materials and Methods 2.19.2). Dipped plants were allowed to self and the resulting seed was collected and selected on kanamycin plates in order to isolate transformants. These plants were then bulked up and the resulting seeds were used for subsequent experimental work.

4.2.2.6 Screening for increased levels of expression

Total RNA was extracted from individual 7-day old seedlings (approximately 12 per 35*S* construct), and northern blot analyses were performed (see Figure 4.12 overleaf). Gain-of-function lines to be used in later experiments were chosen on the basis of clear over-expression of the candidate gene. Not unexpectedly, lines exhibited variation in expression, which is likely to be related to position effects consequent to the random insertion of copies of the transgene in the genome. Therefore three independent transgenic replicate lines were used to control for insertional position effects of the transgenes and are highlighted in blue overleaf in Figure 4.12. The northern blot analyses of At4g18950 revealed transcripts of varying sizes. Only lines with transcript sizes closest to expected were chosen for further study.

Figure 4.12

Northern blot analysis of 35S constructs.

AGI code	Northern blot result
	
At4g18950 Ankyrin protein kinase	
EtBr	
	
At5g47230 ERF5	
EtBr	
	
At4g17490 ERF6	
EtBr	

Total RNA was extracted from pooled 7-day old seedlings (approximately 20 per line) and probed with the radio-labelled gene specific double-stranded DNA probes used in Chapter 3. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg of total RNA per lane). Lines chosen for further study are marked in grey. Empty vector control lines are denoted (i), (ii) or (iii).

4.2.3 Review of candidate genes for further study

Table 4.3 summarises the reasons for not pursuing 11 of the 14 candidate genes originally identified from the H_2O_2 microarray (Chapter 3). The 3 remaining genes, At4g18950, At5g47230 and At4g17490 met all the selection criteria outlined earlier (Figures 3.5 and 4.1) and were studied further. They will be referred to as *APK*, *ERF5* and *ERF6* respectively from this point onwards.

Table 4.3
Summary of the reasons candidate genes were not carried forward for further study.

AGI code	Putative ID	H_2O_2 fold induction	Further study?	Reasons for not carrying forward
Kinases:				
At4g23190	Serine/threonine kinase-like protein [TAIR 6: putative receptor-like protein kinase (CRK11)]	4.79	×	Published (Chen, K 2003)
At5g25930	Receptor-like protein kinase-like [TAIR6: protein kinase family protein]	4.39	×	Unable to identify a loss-of-function DNA line
At4g18950 APK	Protein kinase-like protein TAIR 6: putative ankyrin protein kinase	2.68	✓	-
At3g22060	Putative receptor kinase common family protein [TAIR 6: this protein does not have the protein kinase domain]	2.58	×	Re-annotated as lacking the protein kinase domain
At1g09970	Putative leucine-rich repeat transmembrane protein kinase	2.53	×	Northern blot analysis shows no clear up-

				regulation by H_2O_2
At2g39660	Putative protein kinase [TAIR 6: Botrytis-induced kinase 1 (BIK1)]	2.40	×	Northern blot analysis shows no clear up-regulation by H_2O_2
Phosphatases:				
At4g31860	Putative protein phosphatase 2C	3.25	×	No T-DNA insertions available
At2g33700	Putative protein phosphatase 2C	2.87	×	Northern blot analysis shows only weak induction by H_2O_2
At1g08420	Putative protein ser/thr phosphatase alpha [TAIR6: ser/thr phosphoesterase family protein]	2.80	×	Northern blot analysis shows only weak induction by H_2O_2
At2g30020	Putative protein phosphatase 2C	2.43	×	Unable to obtain the full length coding sequence
Transcription Factors:				
At1g32240	Putative MYB family transcription factor [TAIR 6: KANADI family transcription factor]	3.78	×	Northern blot analysis shows no clear up-regulation by H_2O_2
At3g18290	Putative zinc-finger protein [TAIR 6: embryo defective 2454 (emb2454)]	2.66	✓	Time constraints to technically overcome the embryo lethal phenotype
At5g47230 ERF5	Ethylene responsive element binding factor 5	2.27	✓	-
At4g17490 ERF6	Ethylene responsive element binding factor 6	6.32	✓	-

4.3 Discussion

4.3.1 Loss-of-function lines

The difficulties in obtaining loss-of-function mutants is illustrated by the observation that of the 13 T-DNA lines examined, only 5 were carried through as putative homozygous lines. The remaining 8 appeared to either over-express the transcript (GABI-Kat lines; discussed below in Section 4.3.1.1), or lack the T-DNA insertions altogether (5 SALK lines). The SALK SIGnAL website states that the actual T-DNA insertion site "may be within 0-300 bps" from the point they specify (since the first base provided is the first high quality base in the sequence trace and not necessarily the first base at the insertion site). Therefore, it is possible that for the initial PCR screens, the primers were not designed far enough apart. Another scenario could be that the homozygotes are lethal, however, one would usually expect to see heterozygotes, and this was not the case.

4.3.1.1 Strong promoters in the GABI-Kat T-DNA vector can lead to over-expression

The T-DNA vectors used in generating the GABI-Kat T-DNA insertion mutant collection are not optimally designed for loss-of-function, since all 5 GABI-Kat lines examined in this chapter exhibited very high transcript levels. Additionally, a GABI-Kat T-DNA insertion allele of the Arabidopsis gene *NBS1* was recently shown to induce the partial transcript by over 2000-fold (Waterworth *et al.*, 2007). The presence of strong promoters in the T-DNA vector can therefore lead to over-expression of the sense transcript (or production of antisense RNA, discussed overleaf in Section 4.3.1.2), depending on the position of the promoter in relation to the direction of the native transcript. T-DNA insertions upstream of the translation start site (ATG) would most likely lead to activation of the gene if the promoter direction is towards the translation start site.

Li and colleagues (2006) have demonstrated that T-DNA insertion sites in the Arabidopsis GABI-Kat population are not random, but there is a statistically significant tendency to integrate around the transcription start site: an observation that is also validated in rice (Sallaud *et al.*, 2004). This region is one of the most suitable regions to insert a promoter for activation-tagging/over-expression. Therefore the GABI-Kat T-DNA insertion database may provide an untapped source of gain-of-function lines and help circumvent the problems

associated with amplification and cloning of very large genes. It will also enable the over-expression of endogenous genes in their native locations with all the regulatory sequences at the 3' end.

4.3.1.2 T-DNA insertions may generate antisense RNA

Additionally, depending on their orientation, T-DNAs with strong promoters on their borders may lead to a relatively high amount of antisense transcript production. Generated antisense RNAs could in theory, lead to knocking down of the sense transcript and also cause the degradation of other mRNAs with complementary sequences (e.g. gene family members with high sequence homologies) through the generation of double-stranded RNA (dsRNA). Such dsRNAs are targets for the Dicer enzyme, which cleaves them into short (21-26 nucleotide) interfering RNAs (siRNAs; Novina and Sharp 2004; Brodersen and Voinnet, 2006). The siRNA fragments can then base-pair with complementary sequences and initiate systemic post-transcriptional gene silencing.

4.3.2 Gain of function lines

The varying transcript sizes observed in the 35S over-expression lines are most likely due to tandem insertions of the transgene and consequent recombination. Since over-expression constructs can potentially knockout other endogenous genes in the genome upon insertion (which may further complicate analysis), it was important to analyse several independent lines.

4.3.3 A key role for the phosphatase candidate gene

The PP2C-type phosphatase gene (At2g30020), which was abandoned at this stage (due to cloning problems and time constraints) has very recently been characterised and named *AP2C1* (Schweighofer *et al.*, 2007). In this study, (as was demonstrated in this Chapter), the authors showed that the SALK_065126 was a null line (by semi-quantitative RT-PCR and Southern blotting). Mutant *ap2c1* plants produced significantly higher amounts of JA upon wounding and were more resistant to phytophagous mites (*Tetranychus urticae*). Plants over-expressing *AP2C1* showed lower wound activation of MPK4 and MPK6, reduced ethylene production and compromised innate immunity against the necrotroph *Botyrtis cinerea*. AP2C1

appears to play a key role in regulating stress hormone levels, defence responses and MAPK activities. Again (as with the published *CRK11* and *BIK1* genes) this work adds weight to the efficacy of the criteria for candidate gene selection.

4.3.4 Conclusion

The loss- and gain-of-function lines were analysed at the transcript level only. It is important to bear in mind that the corresponding protein level might not necessarily consent with the altered transcript level.

The next step was to use the identified loss- and gain-of-function lines to ascribe a biological function to the candidate H_2O_2-signalling gene products. This will be addressed in Chapter 5. It is interesting to note that the embryo-lethal nature of the two T-DNA insertion lines in the zinc finger gene (At3g18290), point to a potential role for ROS which so far has not been described in the literature: that of very early development. This gene is therefore an exciting candidate for future work to pursue.

Chapter 5
Functional characterisation of ERF5, ERF6 and APK

5.1 Introduction

Following on from Chapter 4, three genes were carried forward for further study (*ERF5*, *ERF6* and the ankyrin protein kinase gene termed *APK*). Background information on these genes is discussed briefly below.

5.1.1 The ethylene responsive factor (ERF) gene family

ERFs are part of the AP2/ERF transcription factor superfamily, which is defined by the presence of the conserved AP2/ERF DNA binding domain (approximately 60 to 70 amino acids; Riechmann and Meyerowitz, 1998). One hundred and forty-seven genes are postulated to encode proteins containing this domain, of which 122 belong to the ERF family (Nakano *et al.*, 2006). As shown in Figure 5.1 overleaf, the ERF family can be divided into 10 groups (Nakano *et al.*, 2006).

ERF proteins were first isolated from tobacco as factors binding the GCC box (GCCGCC): a short *cis*-acting element found in many ethylene-inducible and pathogenesis-related (*PR*) genes (Ohme-Takagi and Shinshi, 1995; Fujimoto *et al.*, 2000). ERFs can positively or negatively regulate transcription (Fujimoto *et al.*, 2000). For example, a transient expression analysis in tobacco protoplasts revealed that NtERF2 and NtERF4 of tobacco activated GCC box-mediated transcription, whilst NtERF3 repressed it (Ohta *et al.*, 2000).

A wide range of biological functions have been described for *ERF* genes including response to environmental stresses, pathogen attack and hormones. A summary is shown in Table 5.1 on page 128. However, despite the likelihood that these genes play important roles in many plant physiological processes, most of the members of the ERF family have yet to be studied.

Figure 5.1

An un-rooted phylogenetic tree of Arabidopsis ERF proteins. Names of *ERF* genes that have already been reported are shown. Image reproduced from (Nakano *et al.*, 2006).

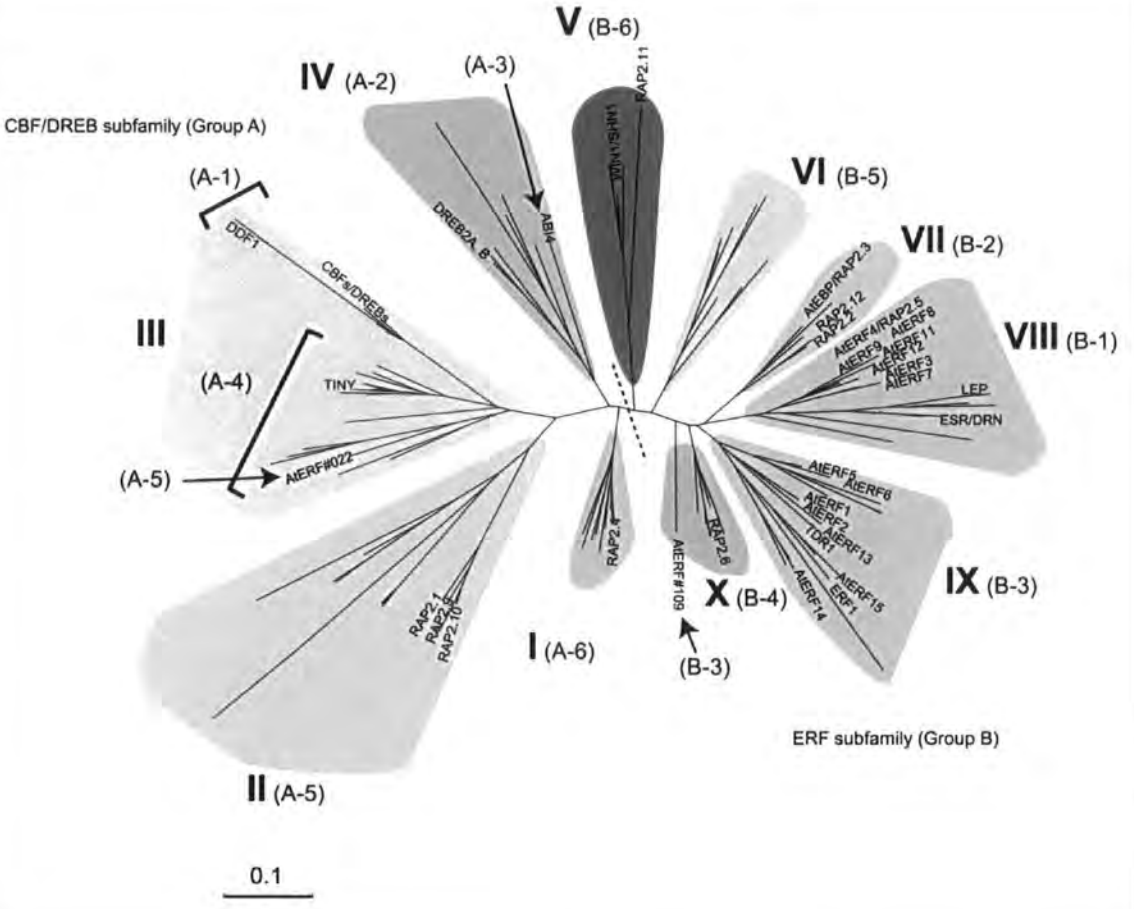


Table 5.1

Examples of *ERF* genes with reported biological functions. Reproduced from Nakano *et al.* (2006).

Group	Gene	Function	Method	Species	Reference
I (a)	*WXP1*	Wax accumulation	Over-expression	Mt	Zhang *et al.* (2005)
III (c)	*CBF1 to 4 DREB1A to D*	Freezing, drought and salt tolerance	Over-expression	At	Liu *et al.* (1998), Gilmour *et al.* (2000), Haake *et al.* (2002)
III (e)	*TINY*	Growth regulation	Activation tagging	At	Wilson *et al.* (1996)
IV (b)	*ABI4*	ABA response, and sugar signalling	Knock-out	At	Finkelstein *et al.* (1998), Arenas-Huertero *et al.* (2000), Huijser *et al.* (2000)
VI	*Pti6*	Disease resistance	Over-expression	Le	Zhou *et al.* (1997), Gu *et al.* (2002)
	Tsi1	Salt tolerance and disease resistance	Over-expression	Nt	Park *et al.* (2001)
VII	*JERF3*	Salt tolerance	Over-expression	Le	Wang *et al.* (2004)
	CaPF1	Freezing tolerance and disease resistance	Over-expression	Cr	Yi *et al.* (2004)
VIII (a)	*AtERF4*	Ethylene, JA and ABA response	Over-expression, Knock-out	At	Yang *et al.* (2005), McGrath *et al.* (2005)
	AtERF7	ABA response	Over-expression, RNA(i)	At	Song *et al.* (2005)
VIII (b)	*ESR1/DRN*	Organ identity	Activation tagging	At	Banno *et al.* (2001), Kirch *et al.* (2003)
	BD1	Floral meristem identity	Knock-out	Zm	Chuck *et al.* (2002)
IX (a)	*ORCA3*	Indole alkaloid biosynthesis	Activation tagging	Cr	Van der Fits and Memelink (2000)
	OPBP1	Salt tolerance and disease resistance	Over-expression	Nt	Guo *et al.* (2004)
	Pti4	Disease resistance	Over-expression	Le	Guo *et al.* (2004)
IX (c)	*ERF1*	Disease resistance	Over-expression	At	Solano *et al.* (1998), Berrocal-Lobo *et al.* (2002)
	Pti5	Disease resistance	Over-expression	Le	Gu *et al.* (2002), He *et al.* (2001)
	NtERF5	Disease resistance	Over-expression	Nt	Fischer and Droge-Laser (2004)
	TERF1	Salt tolerance	Over-expression	Le	Huang *et al.* (2004)
	AtERF14	Disease resistance	Over-expression Knock-out	At	Onate-Sanchez *et al.* (2007)
X (a)	*ABR1*	ABA response	Knock-out	At	Pandey *et al.* (2005)

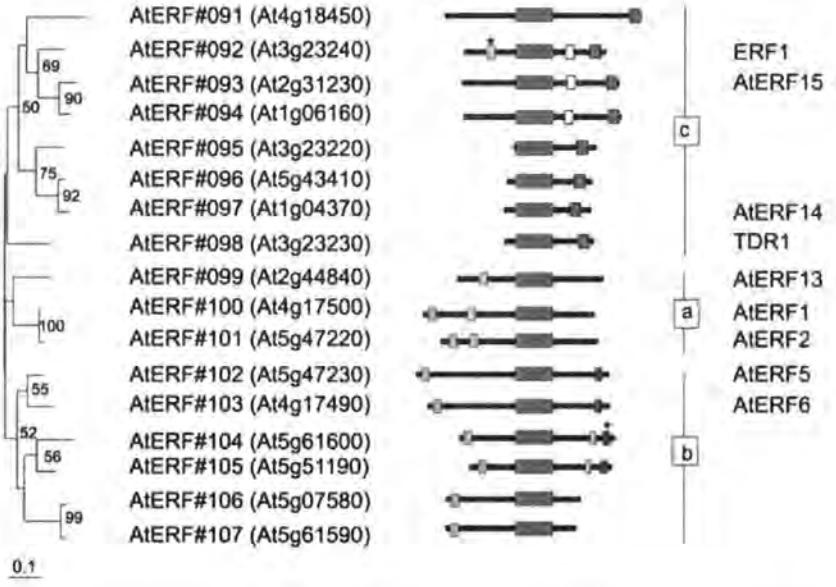
At, Cr, Le, Mt, Nt and Zm denote *A. thaliana*, *Catharanthus roseus*, *Lycopersicon esculentum*, *Medicago truncatula*, *Nicotiana tabacum* and *Zea mays* respectively.

5.1.1.1 ERF5 and ERF6

As shown in the previous phylogram (Figure 5.1) ERF5 and ERF6 are both members of group IX(B-3) based on their alignment of the AP2/ERF domain (Figure 5.2.). There is strong conservation between the two proteins, as shown overleaf by the comparison of their amino acid sequences (Figure 5.3).

Figure 5.2

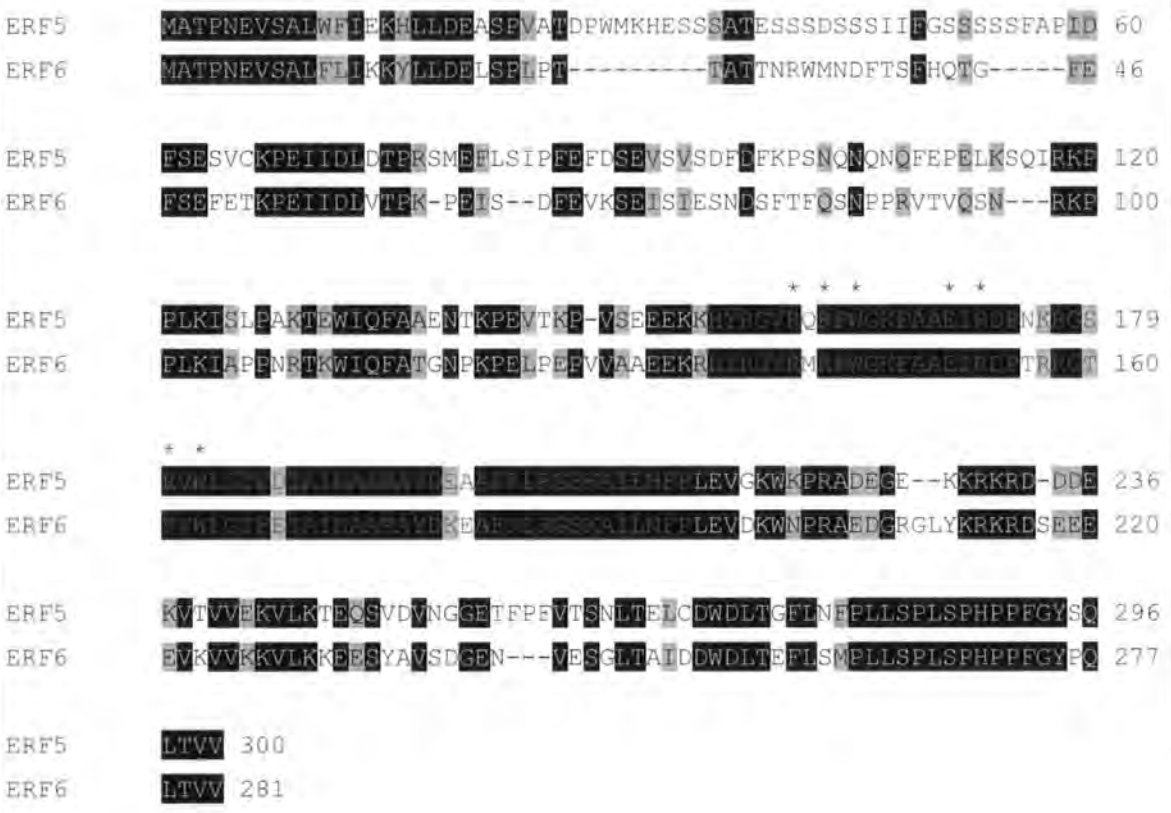
Phylogenetic relationships and protein structure schematics among the Arabidopsis ERFs from group IX (B-3). Image reproduced from Nakano *et al.* (2006).



The AP2/ERF domain is denoted by a grey box. Coloured boxes represent conserved amino acid motifs. Bootstrap values from 100 replicates were used to assess the robustness of the phylogenetic tree (Nakano *et al.*, 2006). Boot strap values > 50 are shown.

Figure 5.3

Alignment of ERF5 and ERF6 amino acid sequences.



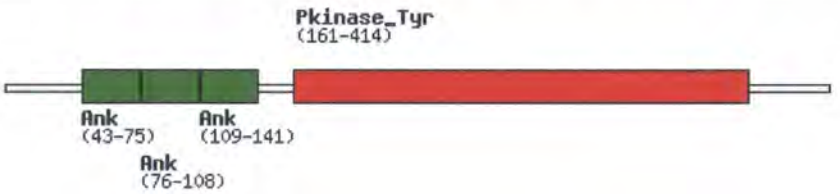
Black and grey shading indicate identical and conserved amino acid residues respectively. The AP2/ERF domain is shown in red. Asterisks represent amino acids residues that directly make contact with DNA (Allen *et al.*, 1998). Sequences were obtained from the TAIR database and aligned using the ClustalW multiple sequence alignment program (*http://www.ebi.ac.uk/clustalw/*).

5.1.2 Ankyrin protein kinase (APK [At4g18950])

As shown below in Figure 5.4, the APK protein consists of a tyrosine protein kinase domain preceded by 3 ankyrin repeats: tandemly repeated modules of approximately 33 amino acids which mediate protein-protein interactions by specific and tight binding to target polypeptides (Sedgwick and Smerdon, 1999).

Figure 5.4

Protein prediction of APK showing the 3 ankyrin repeats and tyrosine protein kinase domain.



Amino acid locations are indicated in parentheses. Image produced using Scansite (*http://scansite.mit.edu/*).

APK was found to share high amino acid identity to five other Arabidopsis ankyrin protein kinases (see Figure 5.5 below). The highest match was with At3g58760 (60 % identical) and a comparison of these two amino acid sequences is shown overleaf in Figure 5.6.

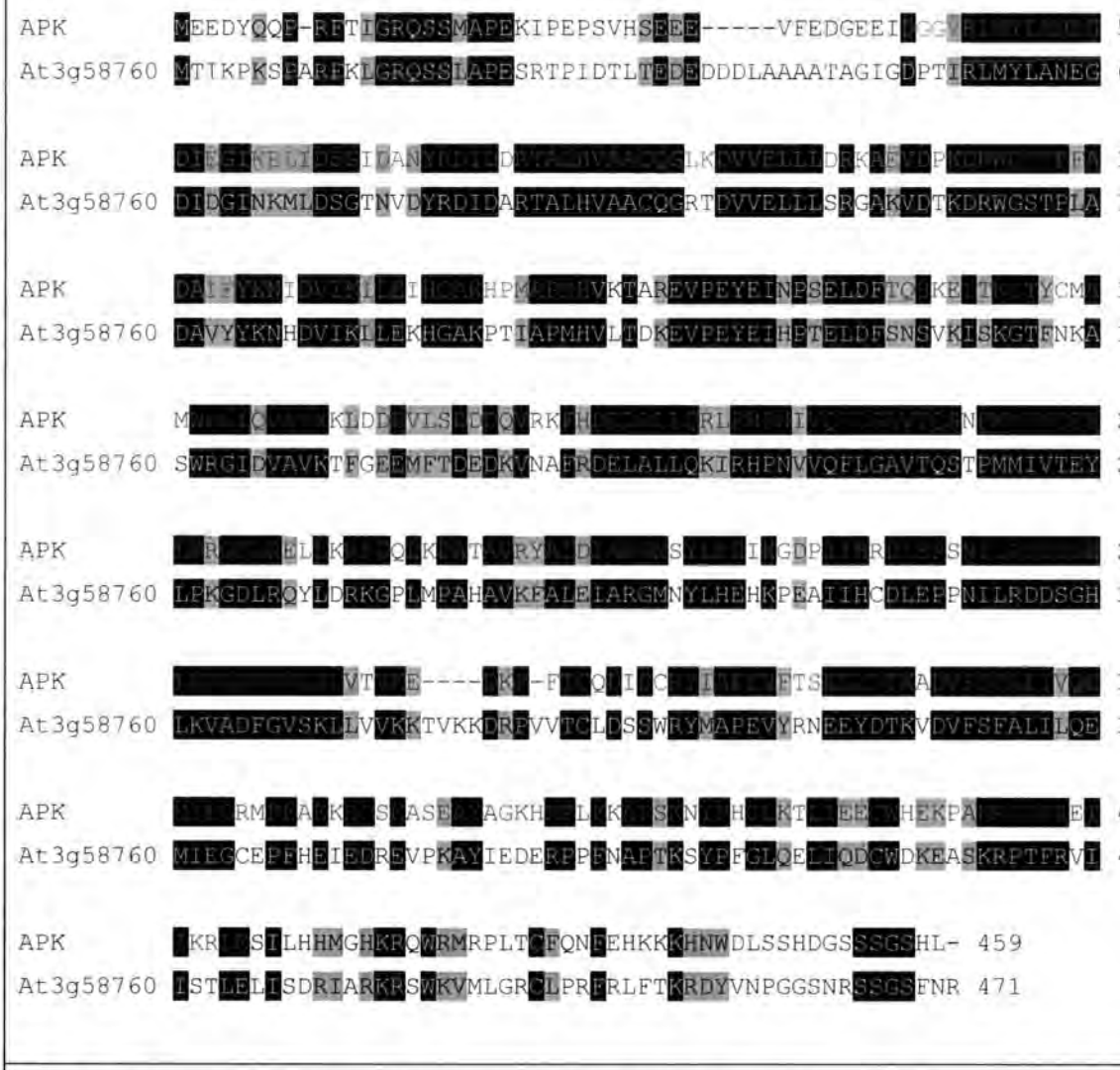
Figure 5.5

Phylogram of APK and its 5 closest relatives in Arabidopsis.

Amino acid similarity was searched using the Basic Local Alignment Search Tool (BLAST) (*http://www.arabidopsis.org/wublast/index2.jsp*). Image produced using ClustalW (*http://www.ebi.ac.uk/clustalw/*).

Figure 5.6

Amino acid sequence alignment of APK with its closest relative in Arabidopsis, a putative ankyrin protein kinase (At3g58760).



The 3 ankyrin repeats are shown in green, whilst the tyrosine protein kinase domain is shown in red. Sequences were obtained from the TAIR database and aligned using the ClustalW multiple sequence alignment program (*http://www.ebi.ac.uk/clustalw/*).

The aim of this chapter was to:

Determine the biological function(s) of ERF5, ERF6 and APK by:

1. *Examining gene expression profiles in response to ROS, environmental stressors and hormones.*

2. *Assessing the T-DNA insertion mutants and over-expressor lines for altered phenotypes during development, and in response to a variety of environmental stress and hormone treatments*

5.2 Results

ERF5, *ERF6* and *APK* were examined in response to various ROS-related stimuli in order to determine their biological function. This study can be divided into 2 parts. Firstly, a detailed analysis of the expression profiles of these three genes was compiled (Section 5.2.1). Secondly, the phenotypic effect of reduced and increased expression of these genes was investigated (Section 5.2.2).

It should be noted that part-way through this work, the laboratory was relocated from the University of Oxford to Durham University. This caused significant disruption due to differences in terms of the experimental growth conditions and equipment/facilities available.

5.2.1 Part 1: Profiling gene expression

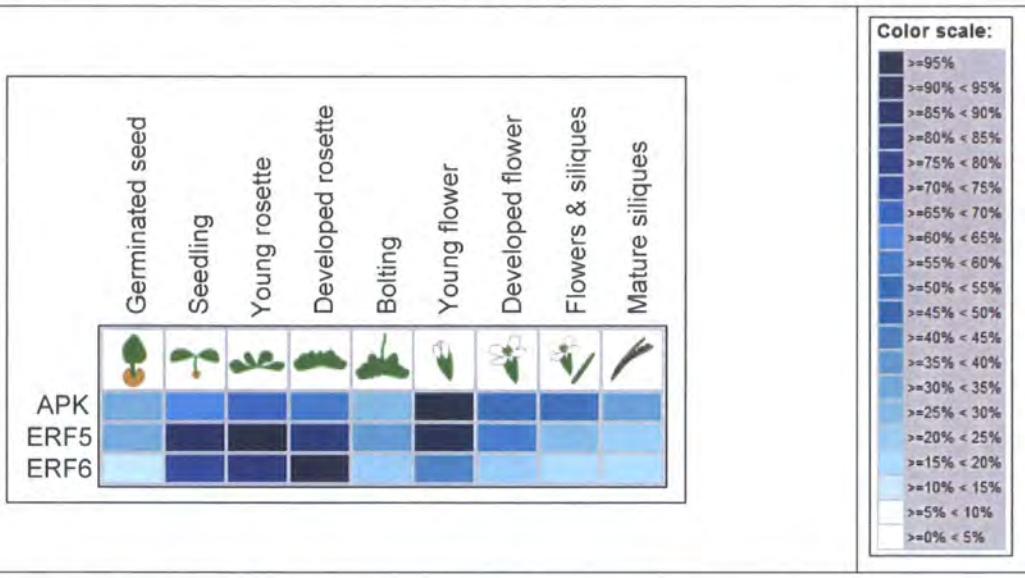
Northern blot analyses of wild-type Col-0 seedlings were undertaken in response to a variety of treatments. This work was supplemented with publicly available microarray data from the 2005 AtGenExpress Project performed by The Arabidopsis Functional Genomics Network (AFGN). Due to experimental difficulties associated with the laboratory relocation, in some instances it was not possible to obtain a complete set of data for all three genes. Where data for one of these genes is absent, the results are presented in Appendix F.

5.2.1.1 Development and tissue specificity

The comprehensive developmental microarrays of AtGenExpress were used to gauge expression of the three candidate genes throughout the plant life cycle. As shown overleaf below in Figure 5.7, all three were expressed most highly during early developmental stages and again at early flowering. Figure 5.8 shows that all three genes exhibited very similar tissue specificity profiles, with expression being highest in the roots.

Figure 5.7

Expression of *ERF5*, *ERF6* and *APK* during Arabidopsis development.



Expression levels are based on Affymetrix microarray data available at the Genevestigator microarray analysis tool (*https://www.genevestigator.ethz.ch*). Image produced using the Genevestigator Gene Chronologer tool. The average categorical values for each gene are normalised such that the category with the highest average signal intensity value (or "expression level") obtains the darkest colour. White corresponds to signal intensity = 0.

Figure 5.8

Expression of *ERF5*, *ERF6* and *APK* across different Arabidopsis tissues.

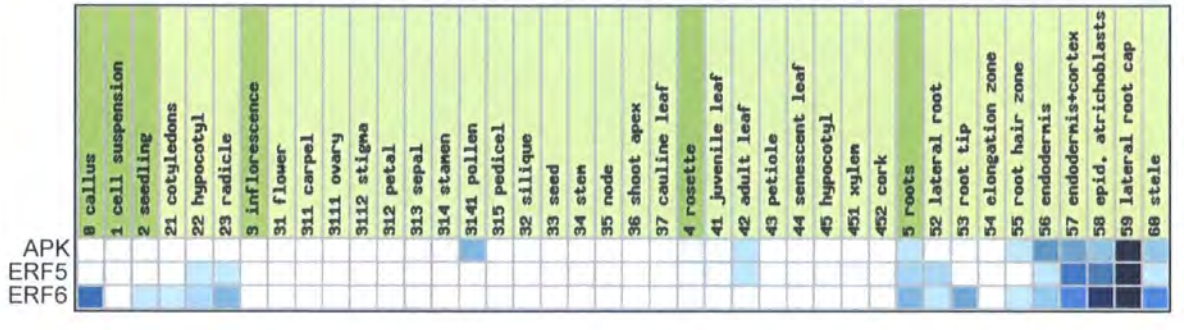


Image produced using the Genevestigator Gene Atlas tool. Detail as in Figure 5.7.

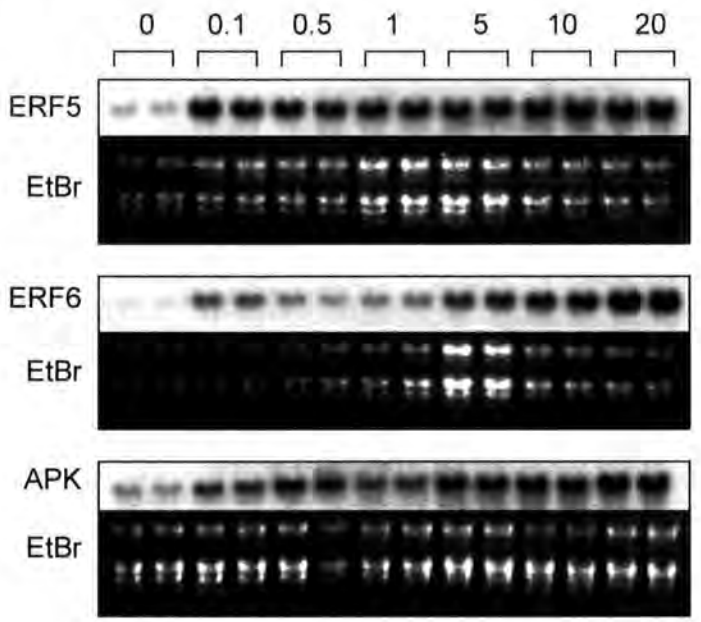
5.2.1.2 Response to ROS

5.2.1.2.1 Hydrogen peroxide

The sensitivity of the genes in response to a range of H_2O_2 concentrations was monitored by northern blot analysis. Wild-type seedlings were treated for 3 h with a final concentration of 0.1 to 20 mM H_2O_2 (Figure 5.9; Materials and Methods 2.5). The transcript levels of *ERF6* and *APK* gradually increased as the H_2O_2 concentration rose, being highest at the maximum concentration tested. *ERF5* showed a more constant expression pattern across the whole concentration series.

Figure 5.9

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to different H_2O_2 concentrations.

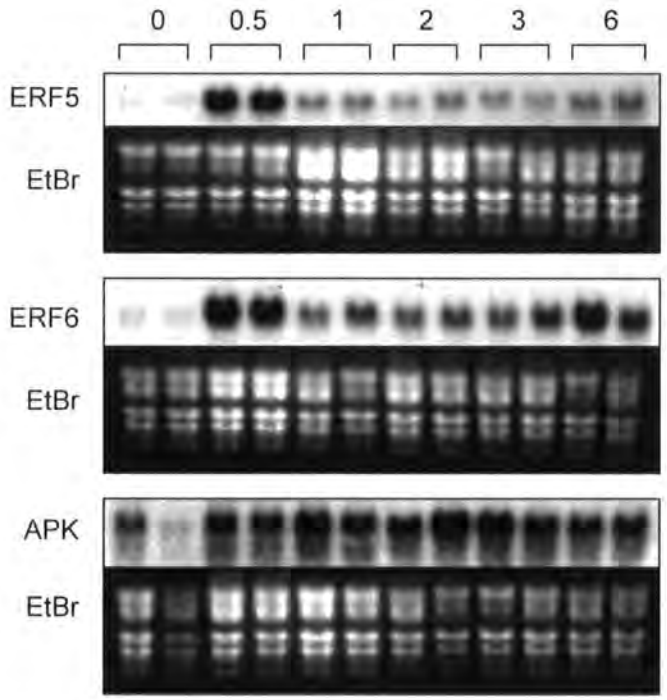


Wild-type seedlings (10 days old) were incubated for 3 h in water prior to a 3 h H_2O_2 treatment with a final concentration of either 0.1, 0.5, 1, 5, 10 or 20 mM (in the growth cabinet). Water was used the control treatment (0). Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

In order to examine how early or late the H_2O_2-induced expression changes occurred and if these alterations were sustained, a time course was performed and analysed by northern blot. Wild-type seedlings were treated with 10 mM H_2O_2 for 0.5 to 6h (Figure 5.10; Materials and Methods 2.5). Strikingly, transcript levels of both *ERF5* and *ERF6* were significantly higher at the earliest time point (0.5 h) than any other tested. *APK* expression appeared to be much more constant across all the time points tested.

Figure 5.10

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to different lengths of H_2O_2 treatment.



Wild-type seedlings (10 days old) were incubated for 3 h with water prior to a 10 mM H_2O_2 treatment for either 0.5, 1, 2, 3 or 6 h (in the growth cabinet). Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

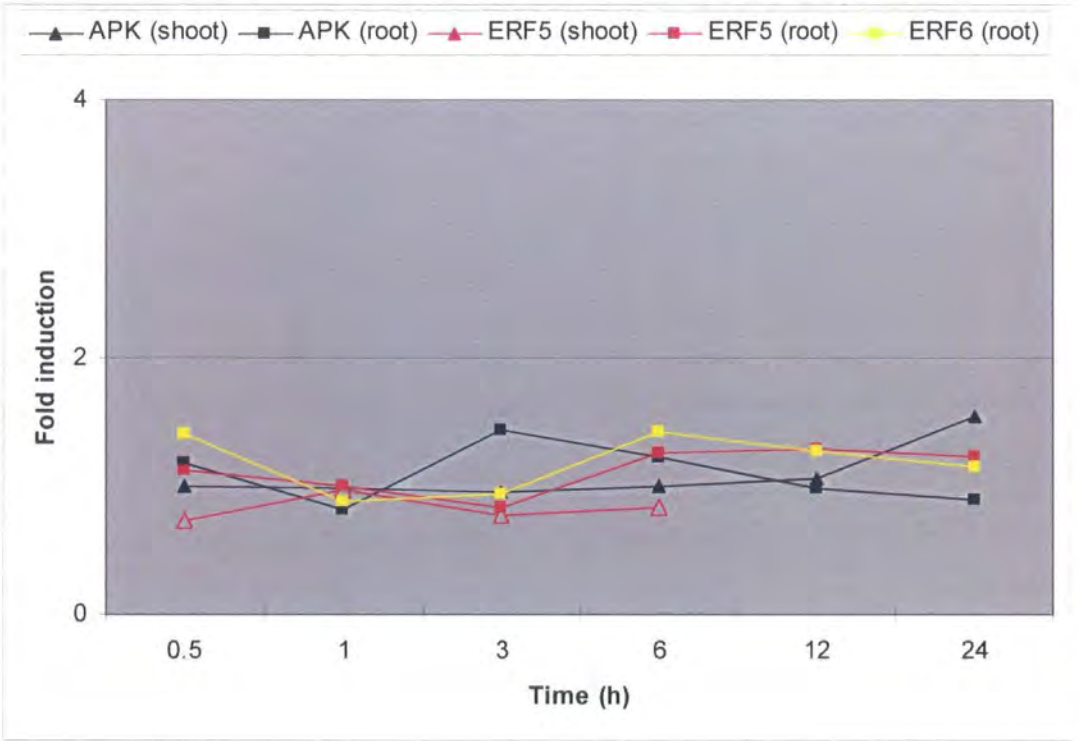
5.2.1.2.2 Superoxide

The effect of superoxide generators on gene expression was also investigated. Northern blot analyses of *ERF5* and *APK* in response to different concentrations of menadione (0.5 to 50 µM) is shown in Appendix F.1 (Materials and Methods 2.5; performed in the light). Both exhibited extremely strong induction but only with the highest concentration tested. The level of induced expression of *APK* was strikingly higher compared to the 10 mM H_2O_2 treatment.

Surprisingly, as shown below in Figure 5.11, the AtGenExpress microarray data revealed no alteration in transcript levels in response to 10 µM of the $O_2^{\bullet -}$-generator methyl viologen.

Figure 5.11

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to methyl viologen treatment.



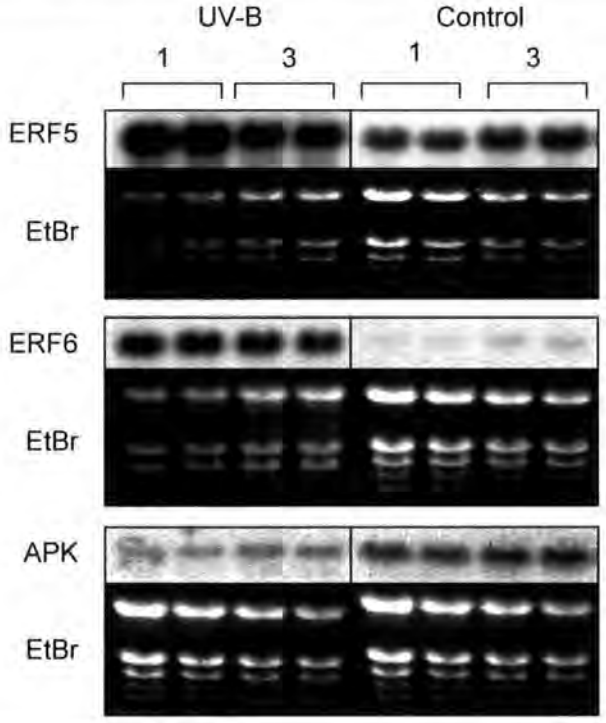
A final concentration of 10 µM methyl viologen was added to liquid media of 16-day old wild-type Col-0 plants. Shoot and root tissue was harvested separately at 0.5, 1, 3, 6, 12 and 24 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 143.

5.2.1.3 Response to UV-B

The effect of UV-B exposure on transcript levels was examined. Northern blot analyis was performed on seedlings exposed to UV-B (1 J/cm^2; approximately 45 s; Materials and Methods 2.5.1). Both *ERF5* and *ERF6* exhibited a pronounced up-regulation in expression at 1 and 3 h post-treatment as shown below in Figure 5.12. *APK* expression appeared to be down-regulated at both time points tested.

Figure 5.12

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to UV-B treatment.

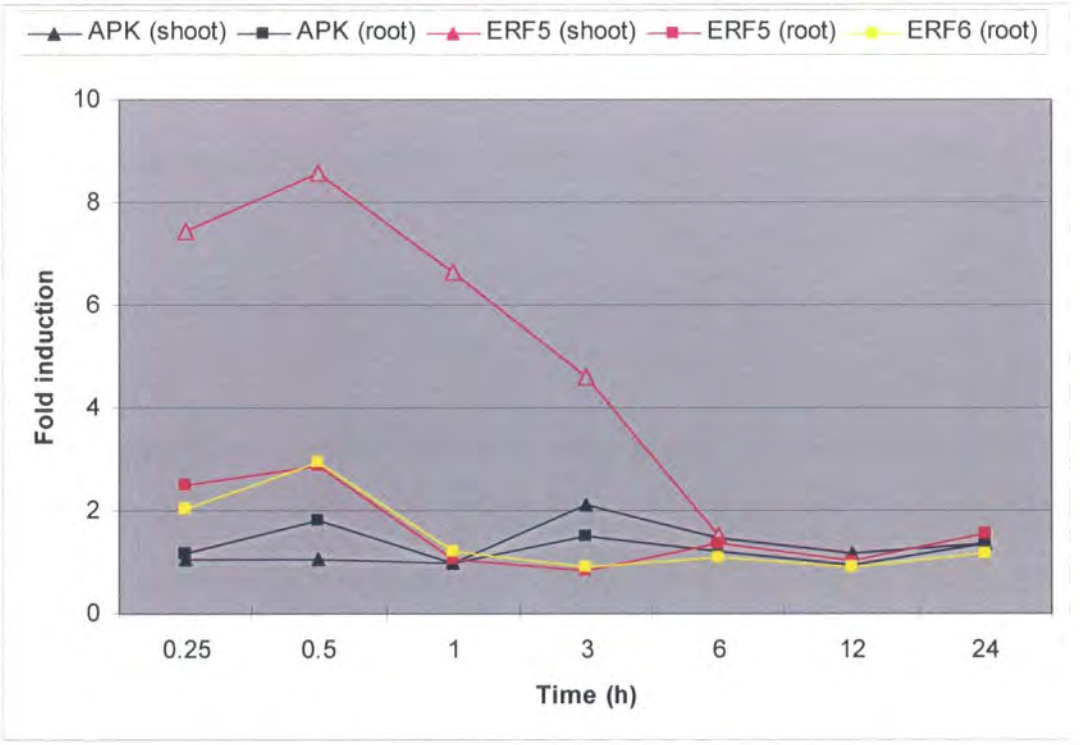


Plates of 10-day old wild-type seedlings were irradiated with 1 J/cm^2 (approximately 45 s) of UV-B in a UV cross-linker by removing the lid and then were immediately placed back into the growth chamber. Control sample plates were taken out of the growth chamber and their lids were removed for the same length of time. Tissue was harvested at 1 or 3 h post-treatment. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

ERF5 expression in shoots was also highly induced during the first 3 h period post-UV-B treatment in the AtGenExpress microarray data (Figure 5.13). Although no data was available for *ERF6* expression within shoot tissue (due to absence calls), it was over 2-fold induced in roots within 0.5 h. However, in contrast to the northern blot data, *APK* transcript levels were weakly up-regulated at the 0.5 and 3 h time points in at AtGenExpress microarray experiment.

Figure 5.13

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to UV-B treatment.



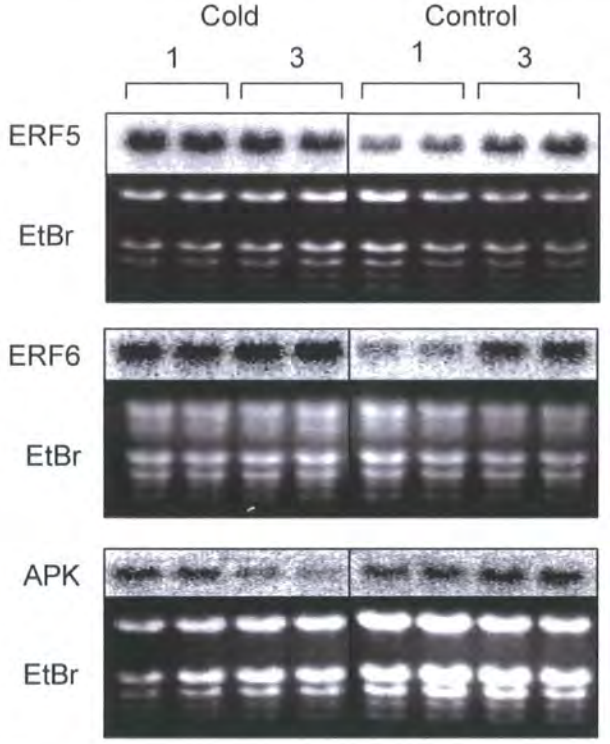
Wild-type Col-0 plants (16 days old) were treated for 15 min with UV-B (1.18 W/m^2 Philips TL40W/12). Shoot and root samples were harvested at 0.25, 0.5, 1, 3, 6, 12 and 24 h post treatment. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 144.

5.2.1.4 Response to cold

Transcript levels were examined by northern blot analysis following a 5 °C cold treatment (Materials and Methods 2.5.1). Both *ERF5* and *ERF6* showed similar expression patterns, with a strong up-regulation at the 1 h time point. *APK* was down-regulated following the 3h cold treatment (Figure 5.14).

Figure 5.14

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to cold treatment.

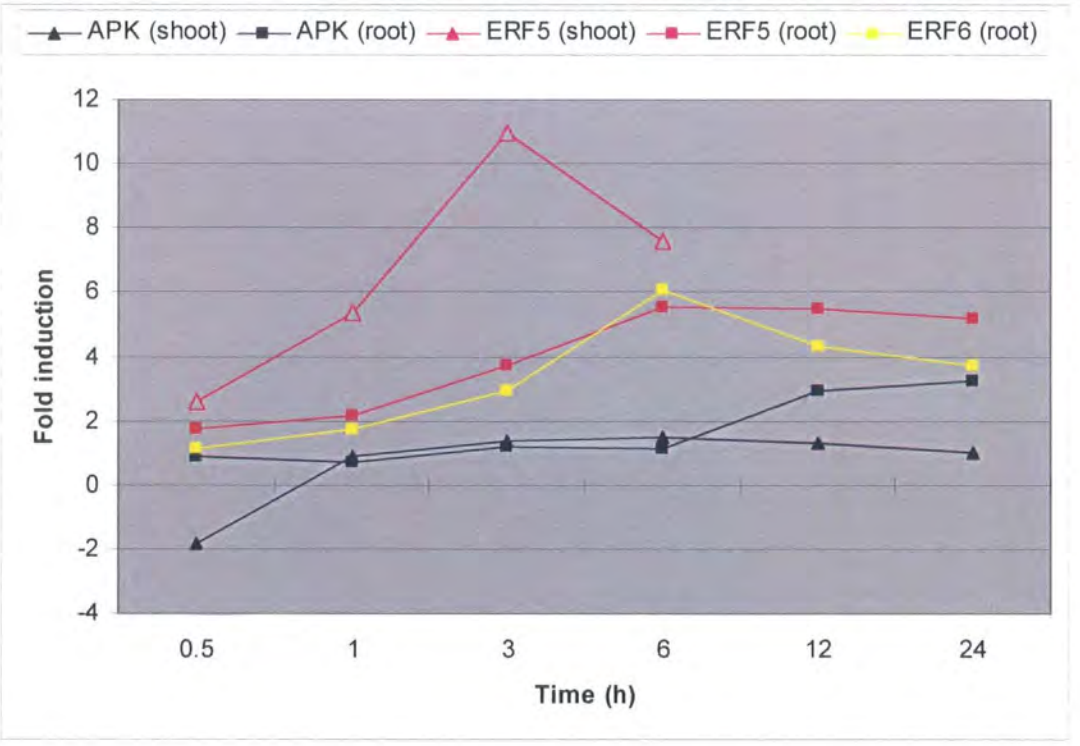


Wild-type seedlings (10 days old) were incubated in 10 ml of water for 3 h prior to treatment at 5 °C or 20 °C (control) for 1 or 3 h (in the dark). Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

The AtGenExpress data confirmed this observed cold-induction induction of *ERF* expression (Figure 5.15). For *APK*, although the AtGenExpress data showed a 2-fold repression in shoots at 0.5 h, there appeared to be no change at the 3 h time point (in contrast to the northern blot). This microarray experiment also detected induction of *APK* expression at later time points: by over 3-fold at 12 and 24 h in root tissue.

Figure 5.15

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to cold treatment.



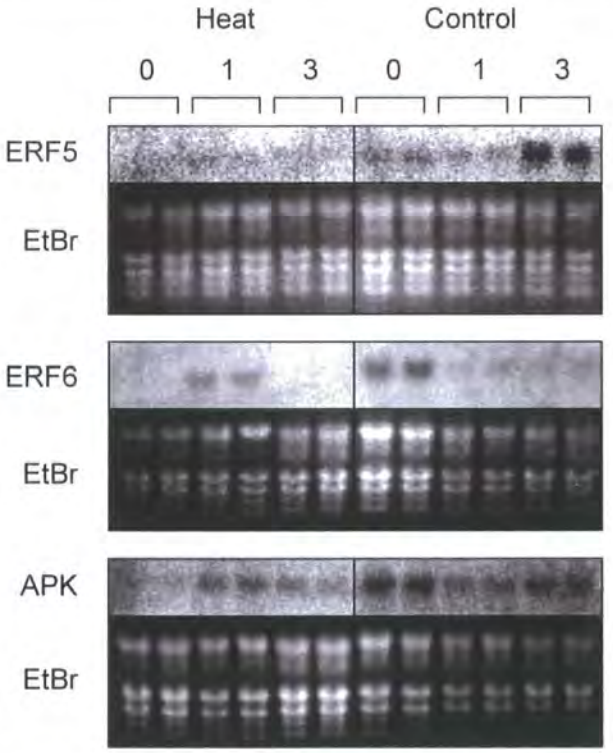
Wild-type Col-0 plants (16 days old) were placed in a 5 °C cold room for 0.5, 1, 3, 6, 12 or 24 h. Shoot and root samples were harvested. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 138.

5.2.1.5 Response to heat

The expression of the three genes was examined following a 1 h 40 °C heat treatment (Materials and Methods 2.5.1). Samples were collected either immediately, 1 or 3 h post-treatment and analysed by northern blot analysis (Figure 5.16). The expression of all three genes appeared to be down-regulated immediately after treatment (0 h), although *ERF6* showed some induction at the 1 h time point.

Figure 5.16

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to heat treatment.



Wild-type seedlings (10 days old) were treated on plates for 1 h at 40 °C or 20 °C (control) and then placed back into the growth cabinet. Tissue was harvested at 0, 1 or 3 h post-treatment. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

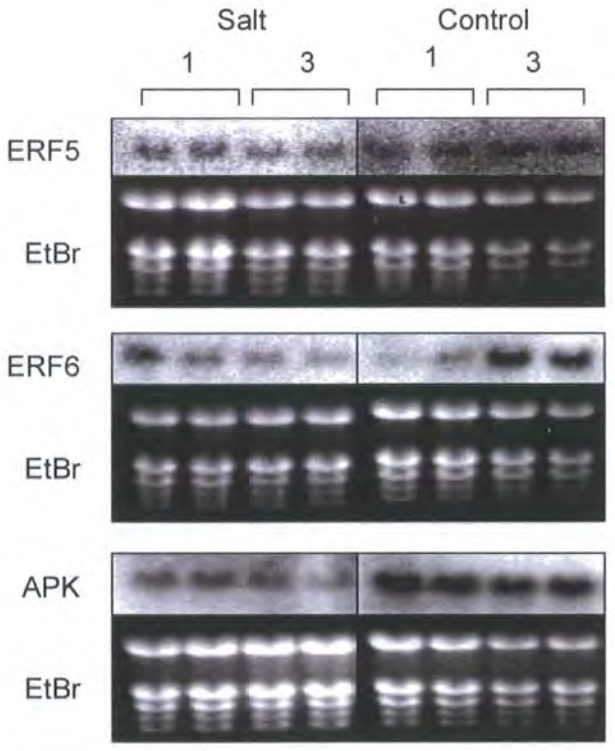
The AtGenExpress data is not included in here as, since the sample data files had been mis-annotated on the TAIR database. The TAIR website states that "the AtGenExpress heat stress time course had been mistakenly labelled. The links to the correct data files from the experiment page are presently being fixed."

5.2.1.6 Response to salt

In order to examine the effect of salt on transcript level, seedlings were treated with 0.44 M NaCl (Materials and Methods 2.5.1). Figure 5.17 shows the resulting northern blot analysis. All three genes exhibited down-regulation of expression at at least 1 time point, with *APK* being the most strongly repressed.

Figure 5.17

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to NaCl.

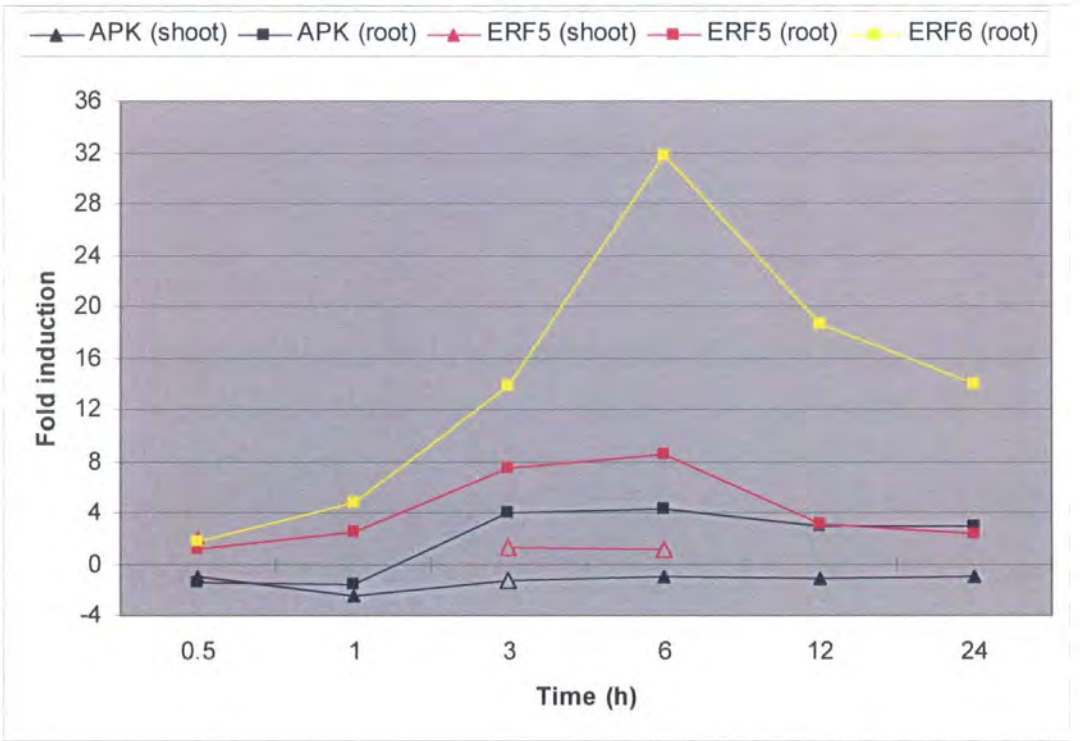


Wild-type seedlings (10 days old) were incubated for 3 h in water prior to treatment with 0.44 M NaCl or water (control) for 1 or 3 h. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

In contrast, the AtGenExpress data showed an induction in expression levels with a lower NaCl concentration (150 mM; Figure 5.18). Transcript levels were up-regulated in the roots of all three genes after a 3 h treatment. However, *APK* was repressed at the 1 h time point in agreement with the northern blot result.

Figure 5.18

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to salt treatment.



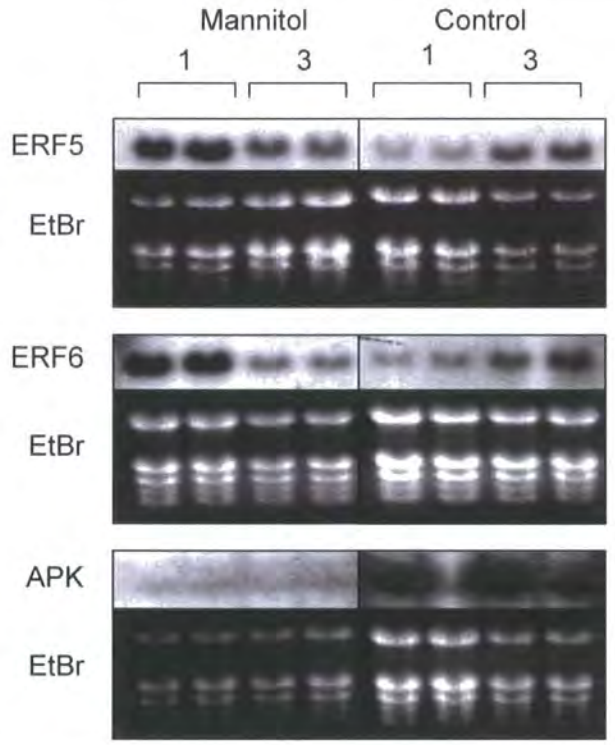
A final concentration of 150 mM NaCl was added to liquid media of 16-day old wild-type Col-0 plants. Shoot and root tissue was harvested separately at 0.5, 1, 3, 6, 12 and 24 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 140.

5.2.1.7 Response to osmotic and drought stress

The response to a 0.22 M mannitol treatment was monitored by northern blot analysis (Materials and Methods 2.5.1; Figure 5.19). *ERF5* and *ERF6* were both strongly up-regulated at the 1 h time point, whilst *APK* expression appeared to be repressed (although it was difficult to be certain as there were high background levels on the northern blot).

Figure 5.19

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to mannitol.

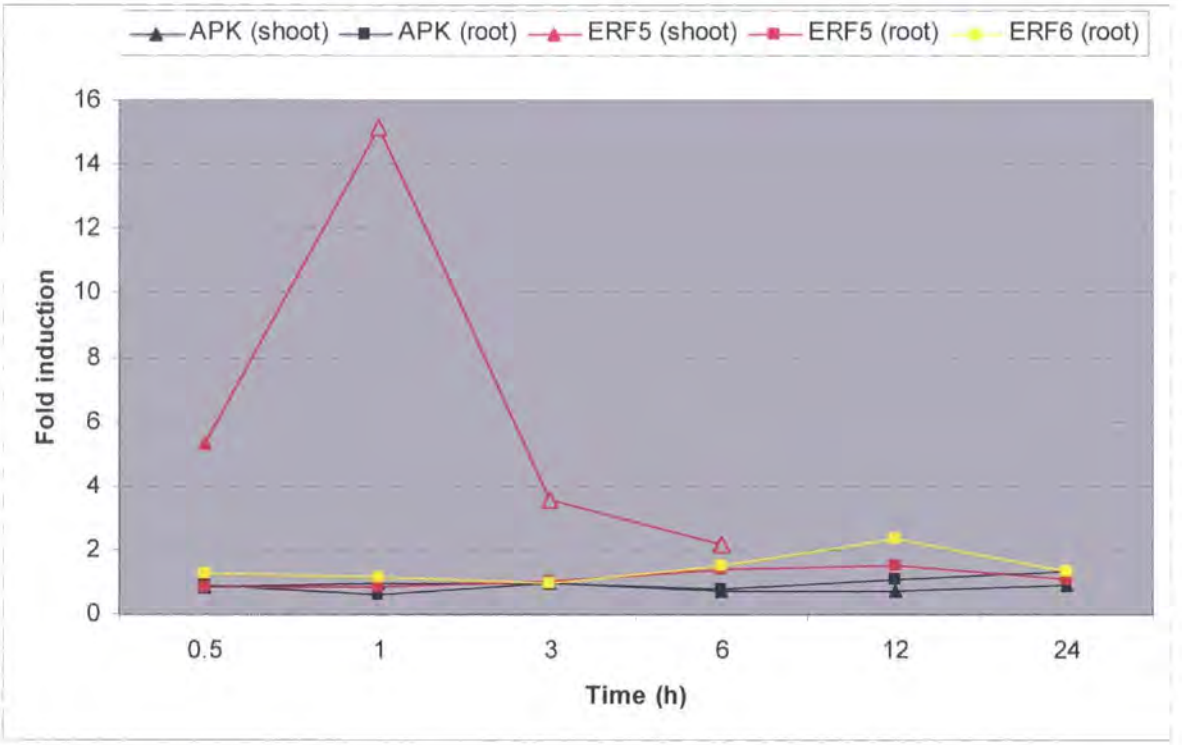


Wild-type seedlings (10 days old) were incubated for 3 h in water prior to treatment with 0.22 M mannitol or water (control) for 1 or 3 h. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

The AtGenExpress microarray experiment used a higher concentration of mannitol (300 mM) and confirmed that *ERF5* (shoots) exhibited strongly increased expression during the initial 3 h treatment (*ERF6* shoot data was missing due to absence calls; Figure 5.20). *APK* showed no change in transcript levels across all time points.

Figure 5.20

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to mannitol treatment.

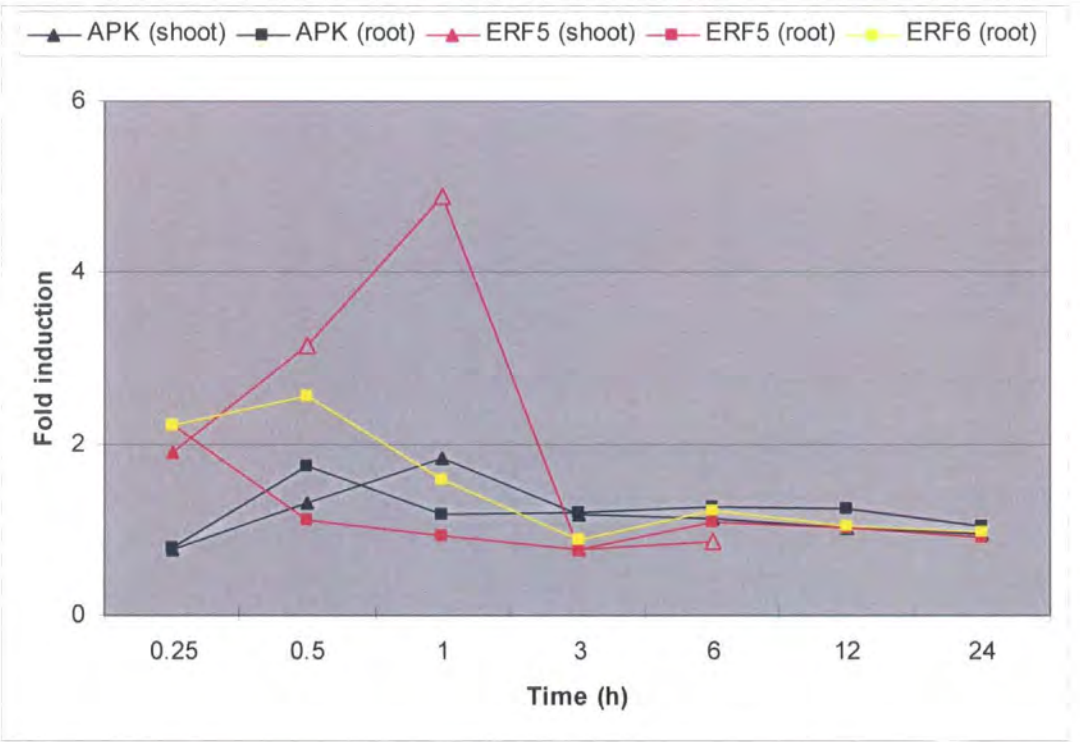


A final concentration of 300 mM NaCl was added to liquid media of 16-day old wild-type Col-0 plants. Shoot and root tissue was harvested separately at 0.5, 1, 3, 6, 12 and 24 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 139.

Expression of both *ERFs* (but not *APK*) was also induced at the early time points following exposure to a dry air stream (Figure 5.21).

Figure 5.21

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to drought treatment.



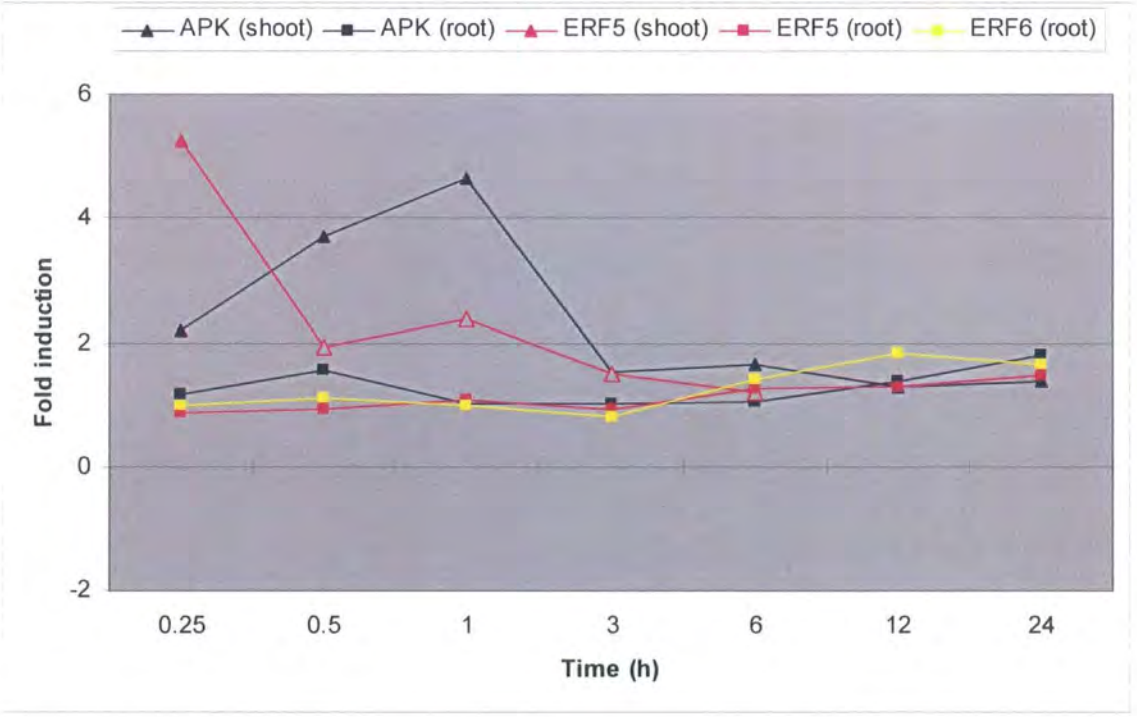
Wild-type Col-0 plants (16 days old) were treated for 15 min with a dry air stream (10 % loss of fresh weight) and then incubated in closed vessels. Shoot and root tissue was harvested separately at 0.25, 0.5, 1, 3, 6, 12 and 24 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 141.

5.2.1.8 Response to wounding

Transcript levels in response to pin puncture-mediated wounding were gauged from the AtGenExpress microarray data (Figure 5.22). Here, *ERF5* expression was rapidly induced in shoot tissue after 15 min. *ERF6* gave little change in transcript levels, although the shoot data was absent. *APK* expression gradually increased to a peak at 1 h following wounding.

Figure 5.22

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to wounding.



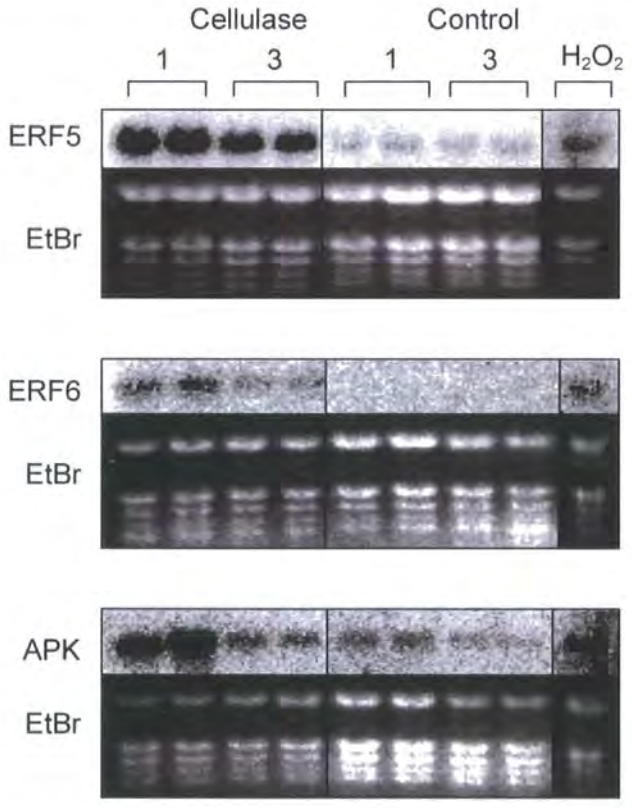
Wild-type Col-0 plants (16 days old) were punctured with pins. Shoot and root tissue was harvested separately at 0.25, 0.5, 1, 3, 6, 12 and 24 h post-treatment. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 145.

5.2.1.9 Response to pathogen elicitors

Transcript levels were investigated following treatment with pathogen-derived compounds (Materials and Methods 2.5.1). Firstly, all three genes were very strongly up-regulated after a 1 h treatment with 0.1 % cellulase as analysed by northern blots (Figure 5.23). *ERF5* maintained high expression levels at the 3 h time point.

Figure 5.23

Northern blot analysis of *ERF5*, *ERF6* and *APK* in response to cellulase.



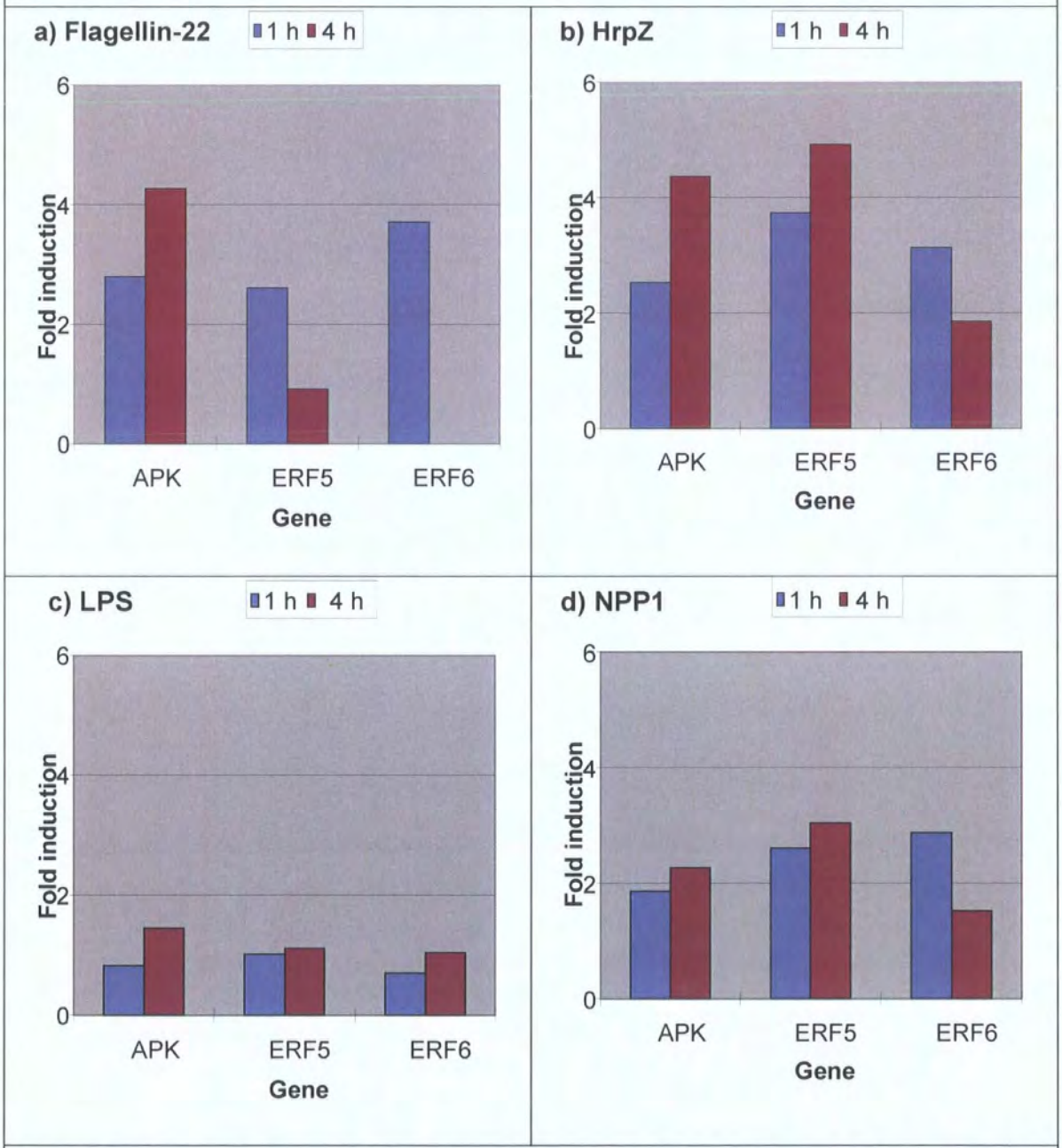
Wild-type seedlings (10 days old) were incubated for 3 h in water prior to treatment with 0.1 % cellulase or water (control) for 1 or 3 h. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate.

Secondly, northern blot analysis of *ERF5* and *ERF6* in response to 1 μM of the bacterial elicitor flagellin-22 was also performed and is shown in Appendix F.2. Both showed strong induction after 1 h, and again *ERF5* also exhibited strong induction at the 3 h time point. In agreement with this, the AtGenExpress microarray data showed up-regulated expression of all three genes by flagellin-22 at 1 h (see Figure 5.24a overleaf). However, in contrast to the northern blot analysis, by 4 h *ERF5* levels had returned to normal whilst *APK* was 4 fold up-regulated.

Expression of all three genes was also induced by another bacterial elicitor, harpin (HrpZ) (Figure 5.24b) and weakly by the oomycete-derived necrosis-inducing Phytophthora protein 1 (NPP1; Figure 5.24d). No fold change was observed following infiltration with the *Pseudomonas syringae* lipopolysaccharide (LPS; Figure 5.24c).

Figure 5.24

AtGenExpress microarray data analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to pathogen elicitors.



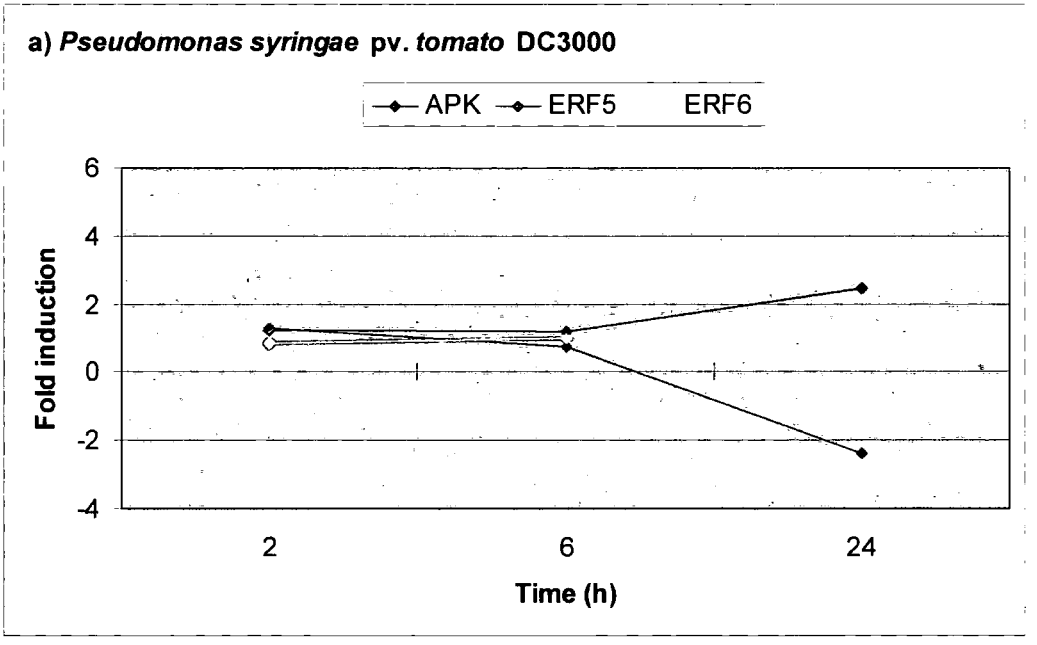
Leaves of 5-week old wild-type Col-0 plants were infiltrated with **a)** 1 µM flagellin-22 **b)** 1 µM HrpZ **c)** 100 1 µg/ml LPS **d)** 2 µM NPP1. Tissue was harvested at 1 and 4 h post treatment. Fold inductions were calculated from present only calls. For full details please refer to NASCArray experiment 122.

5.2.1.10 Response to pathogen infection

AtGenExpress data was also examined for the expression of the three genes following infiltration of 5-week old plant leaves with virulent, avirulent, type III secretion system deficient and non-host strains of the bacteria *Pseudomonas syringae* (shown in Figure 5.25 below and on the following two pages). *APK* expression was 2-fold induced at the 24 h time point with all 4 strains. *ERF5* transcript levels were repressed at 24 h by the virulent strain but 2 fold up-regulated 2 h post inoculation with the avirulent and non-host strains. *ERF6* was 2-fold induced by the avirulent strain but weakly repressed by the type III secretion system deficient strain after 6 and 2 h respectively.

Figure 5.25

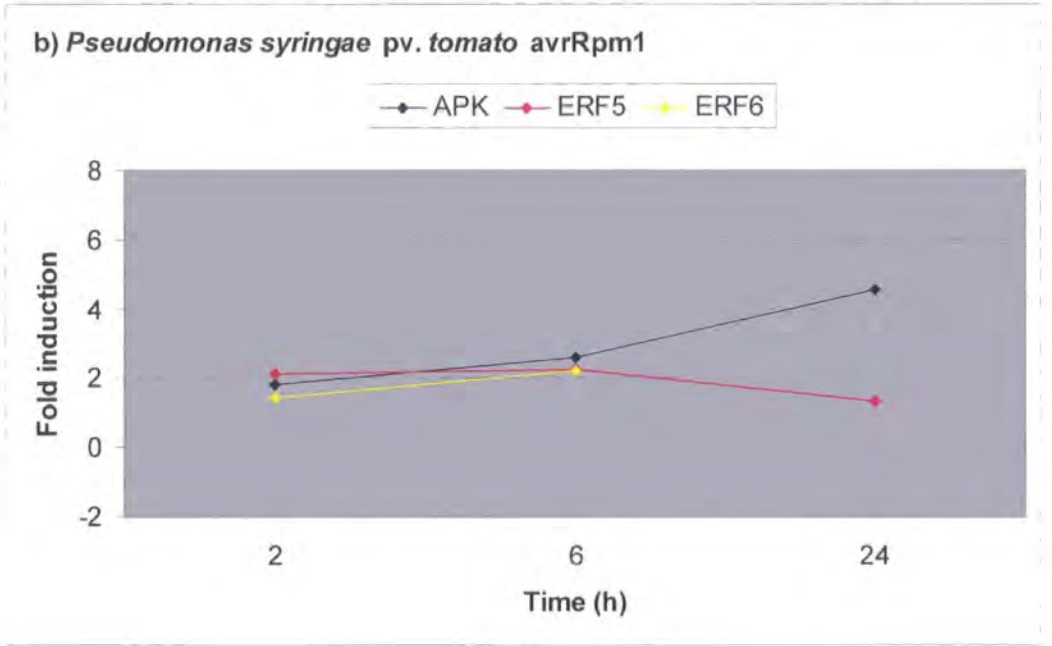
AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to **a)** virulent **b)** avirulent **c)** type III secretion system deficient and **d)** non-host strains of *Pseudomonas syringae*.

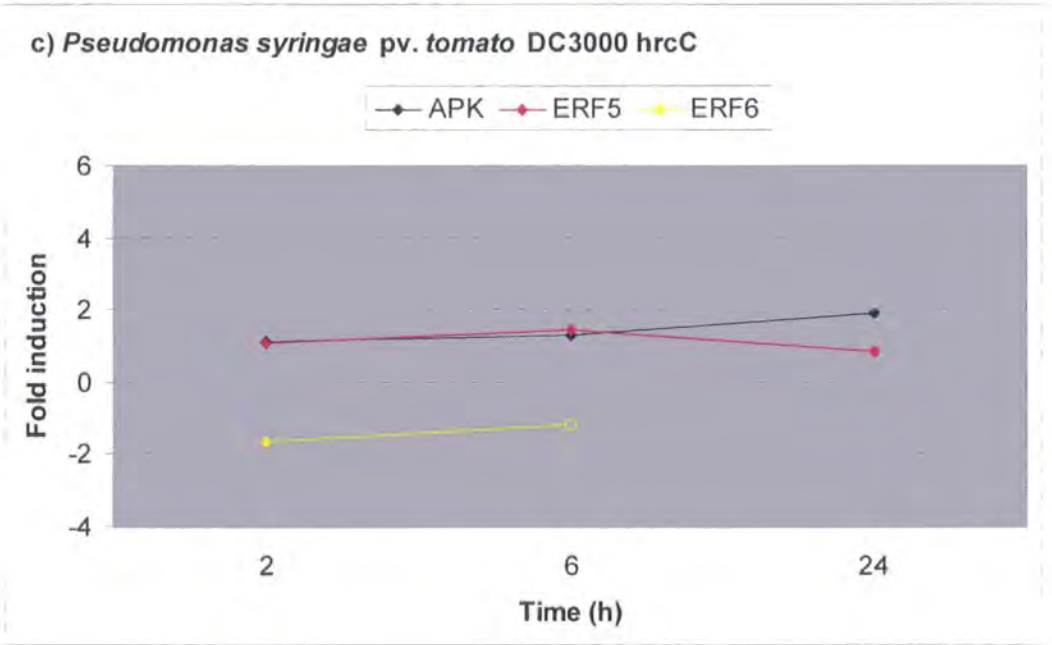


Wild-type plants (5 weeks old) were inoculated with 1 x 10^8 cfu/ml in 10 mM $MgCl_2$. Tissue was harvested 2, 6 and 24 h post inoculation. Control samples were inoculated with 10 mM $MgCl_2$. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 123.

(Figure continues on the following page)

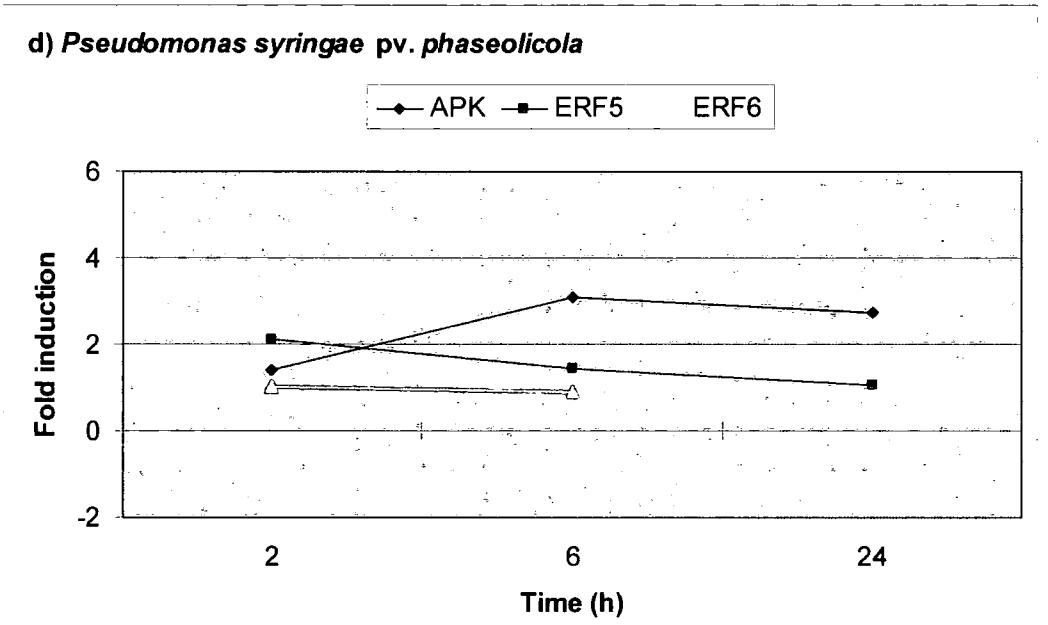
Figure 5.25 *(Continued from the previous page)*





(Figure continues on the following page)

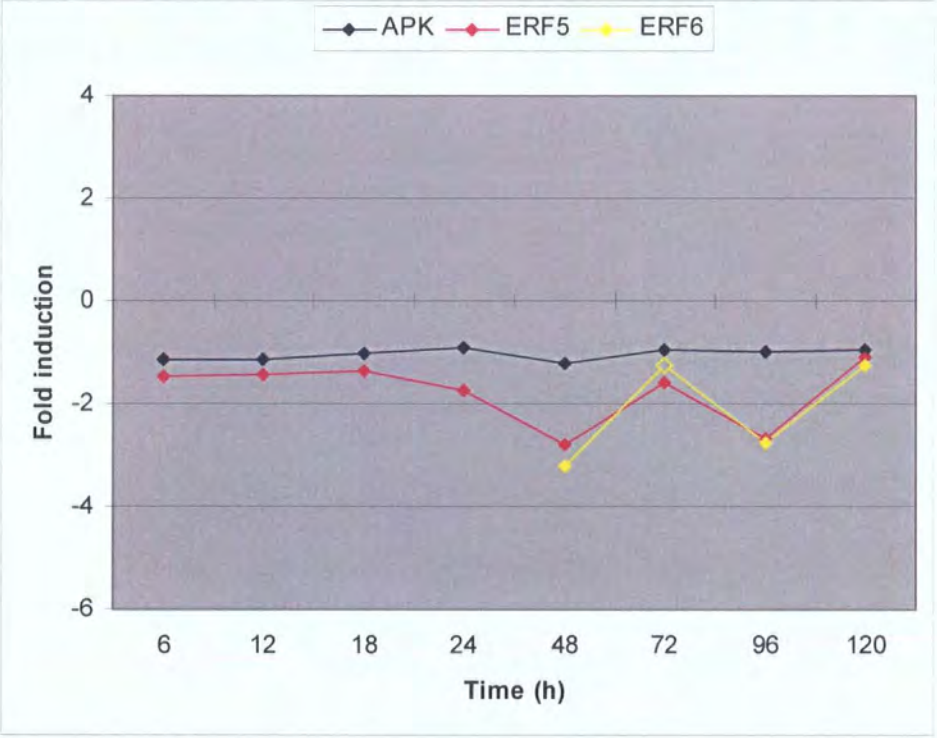
Figure 5.25 *(Continued from the previous page)*



The AtGenExpress project also performed microarrays following inoculation with fungal pathogens. According to this data, expression of both *ERFs* was repressed 24 h after inoculation with the biotroph *Erisiphe orontii* (powdery mildew) whilst *APK* showed no change (Figure 5.26).

Figure 5.26

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to *Erisiphe orontii* infection.

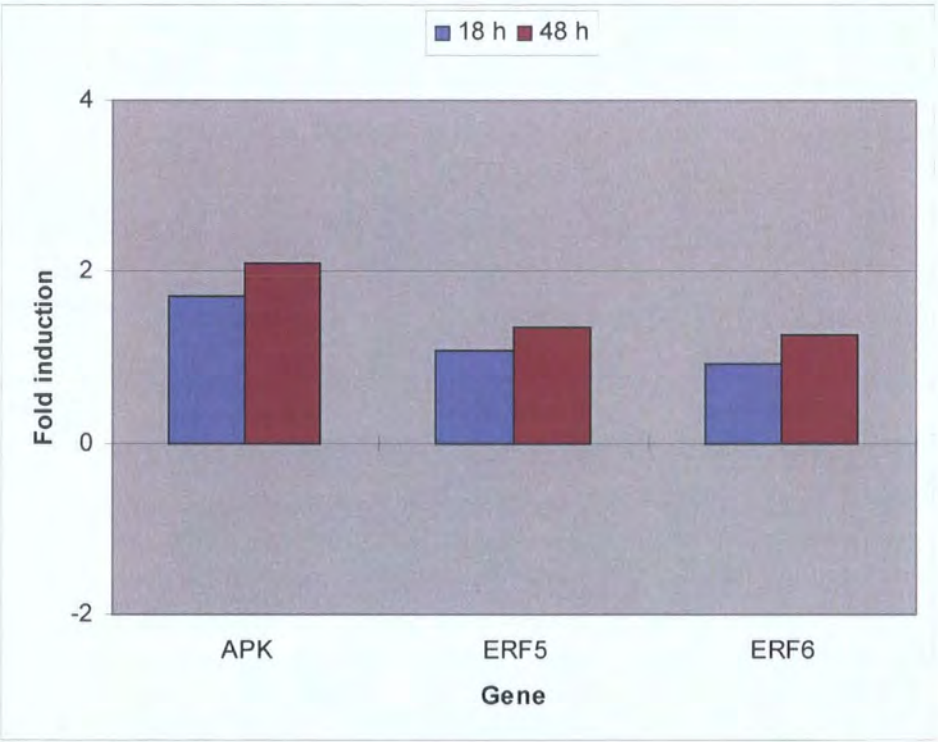


Leaves of 31-day wild-type plants were inoculated via a settling tower. Tissue was harvested 6, 12, 18, 24, 48, 72, 96 and 120 h post inoculation. Control samples were mock inoculated by placing in the control tower for 15 min. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 169.

Infection with the necrotroph *Botrytis cinerea* (grey mould rot) was also examined (Figure 5.27). Only *APK* was induced, by approximately 2-fold.

Figure 5.27

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to *Botrytis cinerea* infection.

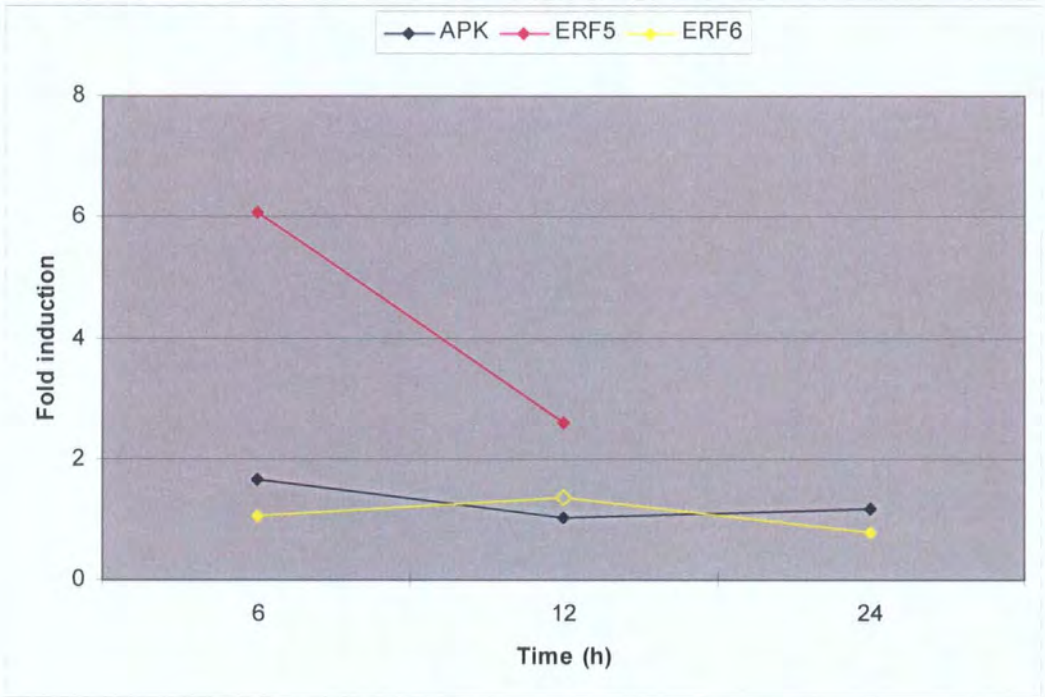


Leaves of 4-week old wild-type plants were inoculated with 4-5 drops of 5 x 10^5 spores. Controls were inoculated with 24 g/L potato dextrose broth medium. Tissue was harvested 18, or 48 h post-inoculation. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 167.

Infection with the oomycete *Phytophthora infestans* (potato late blight) only induced expression of *ERF5* (by 6 fold after 6 h; Figure 5.28).

Figure 5.28

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to *Phytophthora infestans* infection.



Leaves of 5-week old wild-type plants were inoculated with 5 x 10^5 spores in water. Tissue was harvested 6, 12 and 24 h post-inoculation. Control samples were mock inoculated with water. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 123.

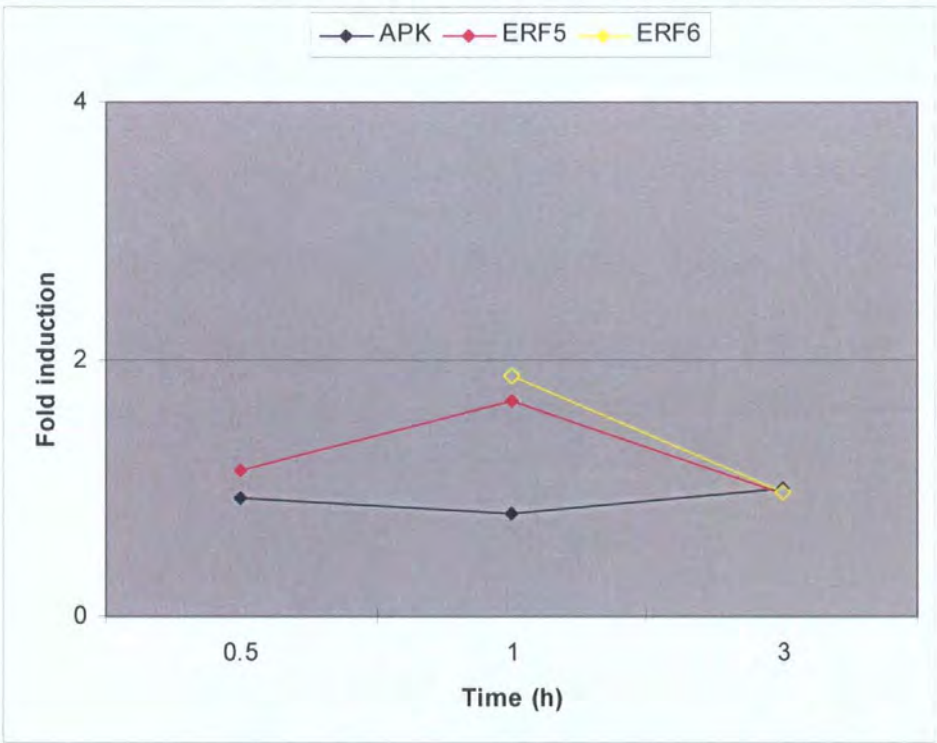
5.2.1.11 Response to hormones

5.2.1.11.1 Ethylene

Response to ethylene was assessed by exogenous ACC application (Materials and Methods 2.5.1). Northern blot analysis of *ERF5* and *APK* transcript levels following a 100 µM ACC was performed and the results are shown in Appendix F.3. Only *ERF5* showed a weak induction (at the 3 h time point). The AtGenExpress microarray data also showed a weak induction in expression of both *ERFs* following a 10 µM ACC treatment (Figure 5.29).

Figure 5.29.

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to ACC treatment.



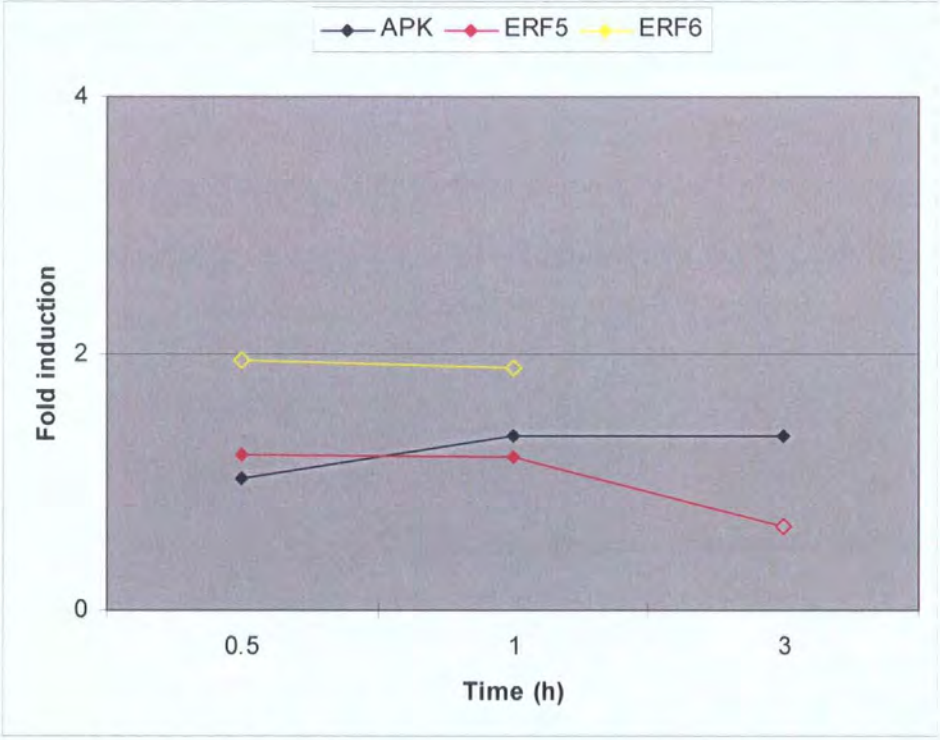
Wild-type seedlings (7 days old) were treated with 10 µM ACC and tissue harvested after 0.5, 1 and 3 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 172.

5.2.1.11.2 Methyl jasmonate

Northern blot analysis following a 100 µM meJA treatment was performed (Materials and Methods 2.5.1). A clear increase in transcript levels of *ERF5* and *APK* RNA was seen at the 1 and 3 h time points (shown in Appendix F.4). However, the AtGenExpress microarray data only revealed *ERF6* to be responsive to a 10 µM treatment (Figure 5.30).

Figure 5.30

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to methyl JA treatment.



Wild-type seedlings (7 days old) were treated with 10 µM methyl JA and tissue harvested after 0.5, 1 and 3 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 174.

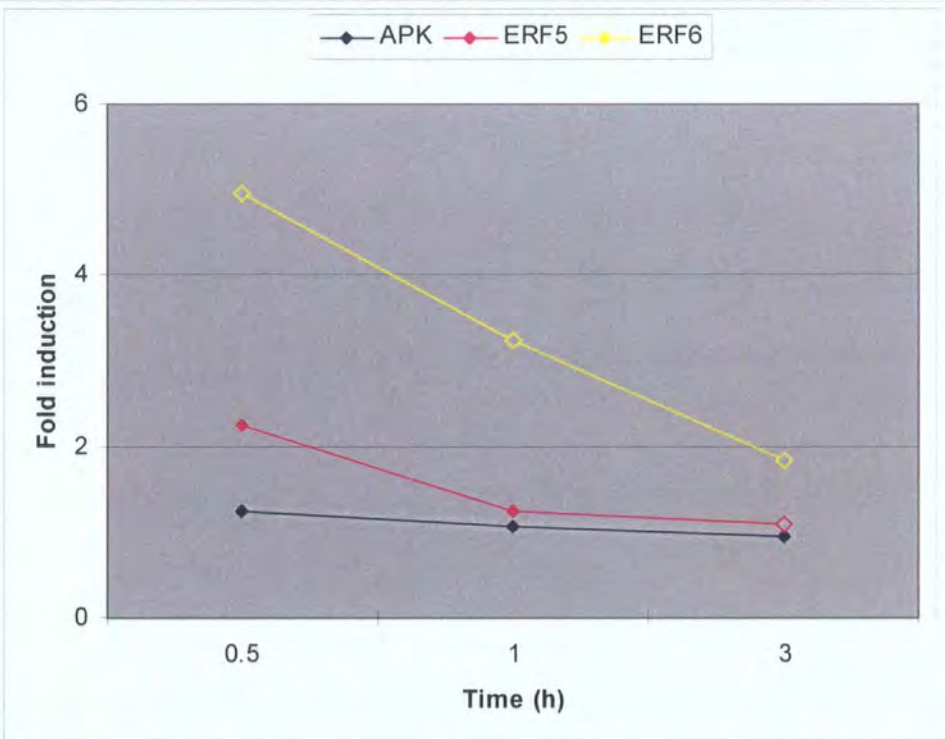
5.2.1.11.3 Auxin

Northern blot analysis of *ERF5* and *APK* was performed following treatment with 1 µg/ml naphthaleneacetic acid (NAA; Materials and Methods 2.5.1). The results are shown in Appendix F.5. *ERF5* RNA levels were up-regulated after both 1 and 3 h, whilst *APK* showed no change.

In agreement with the northern blot analyses the AtGenExpress data also showed *ERF5* to be induced at 0.5 h after application of 1 µM 3-indoleacetic acid (IAA), as well as no change in *APK* expression levels (Figure 5.31). In addition, *ERF6* expression (for which no northern blot analysis was performed) was highly up-regulated in the AtGenExpress data.

Figure 5.31

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to auxin treatment.



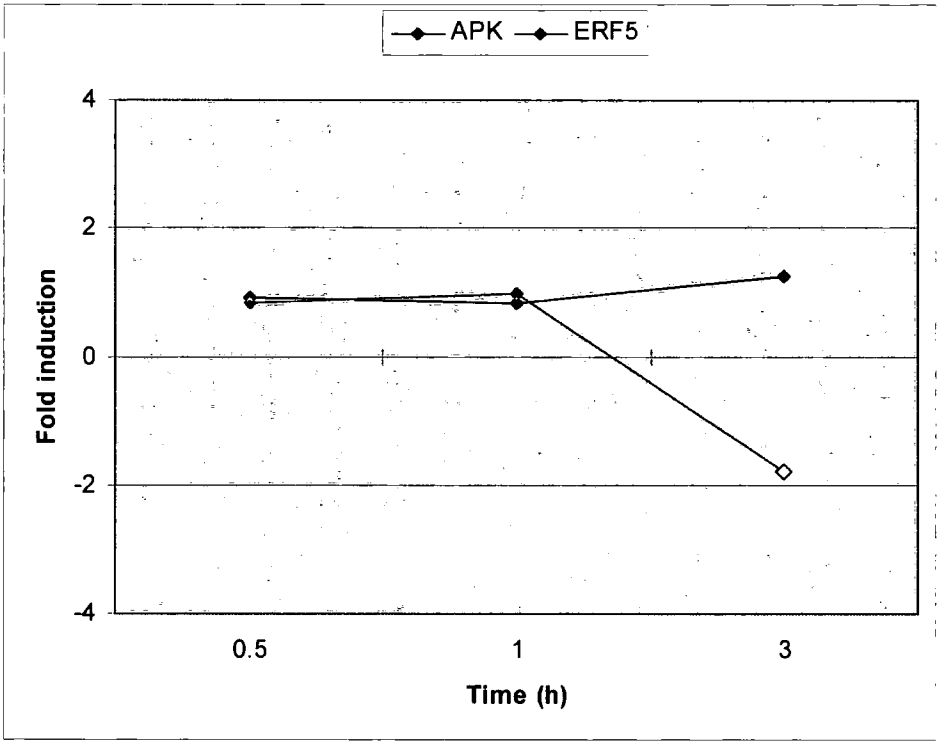
Wild-type seedlings (7 days old) were treated with 1 µM IAA and tissue harvested after 0.5, 1 and 3 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 175.

5.2.1.11.4 Abscisic acid

Northern blot analyses were performed on *ERF6* and *APK* following treatment with 100 µM ABA (Materials and Methods 2.5.1). The results are shown in Appendix F.6 and revealed the transcript levels to be up-regulated: *ERF6* expression was strongly induced after 1 h although by 3 h levels had returned to normal, whilst *APK* expression was up-regulated at both the 1 and 3 h time points, although to a lesser extent. However, the AtGenExpress data showed neither gene to have increased expression in response to a lower concentration (10 µM) of ABA (Figure 5.32). No AtgenExpress data was available for *ERF6* (due to absence calls).

Figure 5.32

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to ABA treatment.



Wild type seedlings (7 days old) were treated with 10 µM ABA and tissue harvested after 0.5, 1 and 3 h. Fold inductions were calculated from present calls only. Open symbols denote absence of a replicate sample. For full details please refer to NASCArray experiment 176.

5.2.1.11.3 Salicylic acid

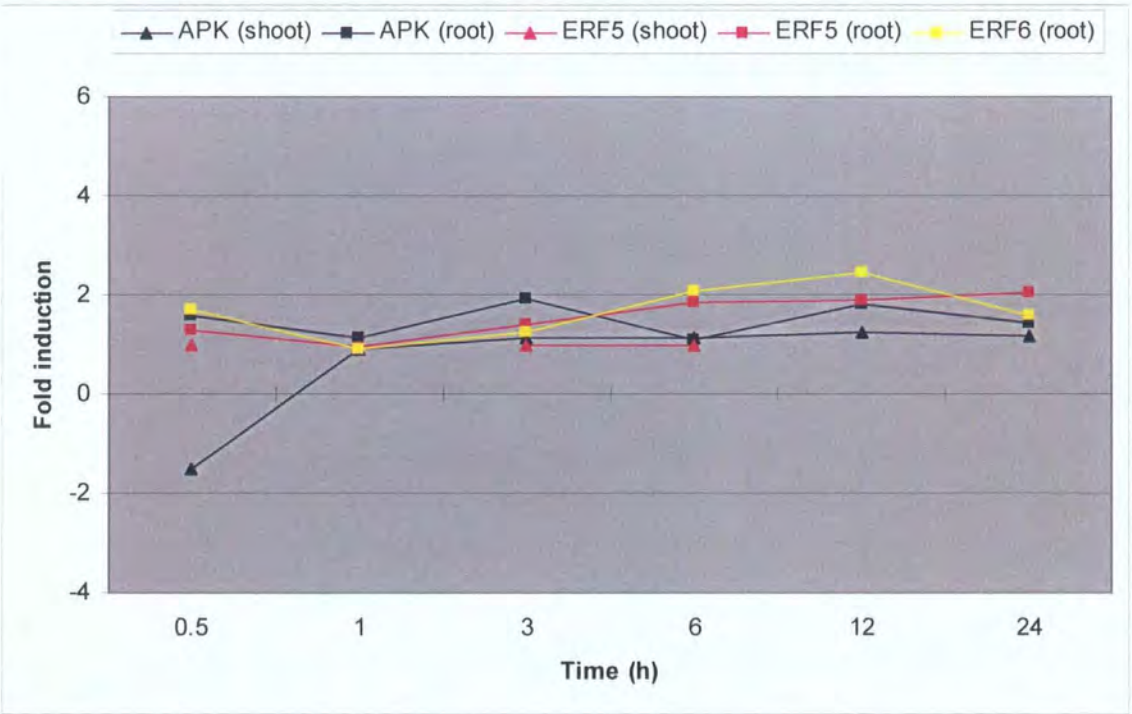
The transcript levels of *ERF6* and *APK* were assessed by northern blot analysis in response to a 100 µM SA (salt) treatment (Materials and Methods 2.5.1). As shown in Appendix F.7, both genes exhibited no change in expression. No AtGenExpress data was available for SA.

5.2.1.12 Response to genotoxic stress

Genotoxic stress was assessed by the AtGenExpress Project, via application of the DNA-damaging compounds bleomycin and mitomycin C (Figure 5.33). Both the *ERFs* showed increased expression between 6 and 24 h in roots. *APK* showed weak induction of expression at the 3 h time point and again at 24 h in roots.

Figure 5.33

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to genotoxic stress.



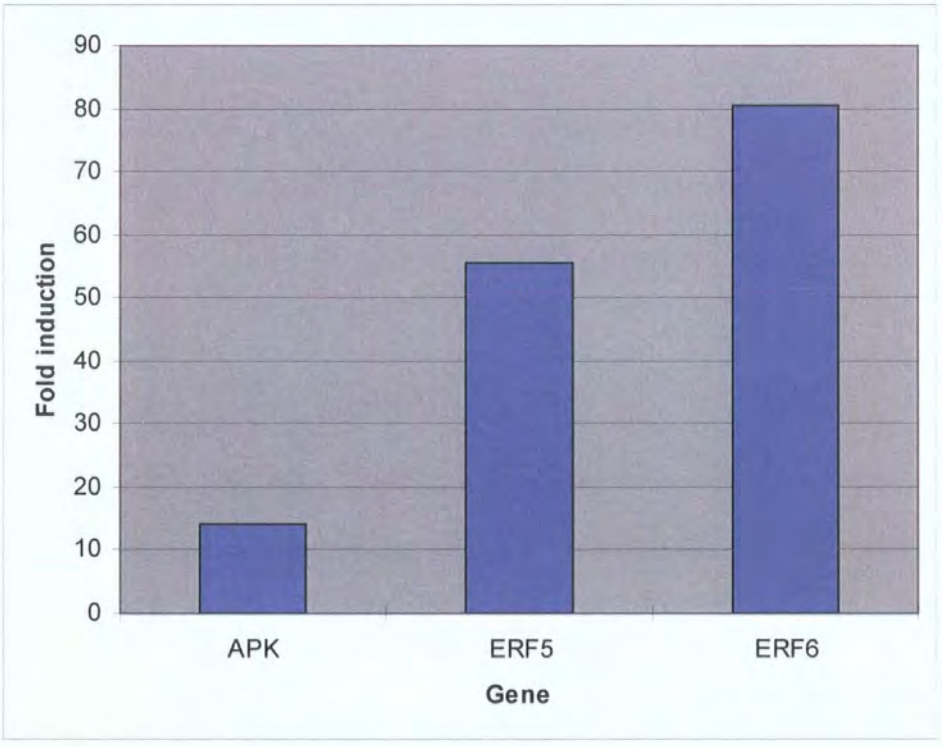
Bleomycin (1.5 µg/ml) and mitomycin (22 µg/ml) was added to the liquid media of 16-day old wild-type plants. Shoot and root tissue was harvested 0.5, 1, 3, 6, 12 and 24 h post treatment. Fold inductions were calculated from present calls only. For details please refer to NASCArray experiment 142.

5.2.1.13 Response to cycloheximide treatment

The AtGenExpress microarray data showed that the protein synthesis inhibitor cycloheximide (CHX) had a profound up-regulation effect on the expression of all three genes (Figure 5.34). Expression of *ERF6* was 80-fold up-regulated, whilst that of *ERF5* and *APK* was 56- and 14-fold induced respectively.

Figure 5.34

AtGenExpress microarray analysis of fold induction of *ERF5*, *ERF6* and *APK* in response to cycloheximide (CHX) treatment.



Wild-type seedlings (7 days old) were treated with 10 µM CHX and tissue harvested after 3 h. Fold inductions were calculated from present calls only. For full details please refer to NASCArray experiment 189.

5.2.2 Part 2: Functional characterisation of loss- and gain-of-function lines

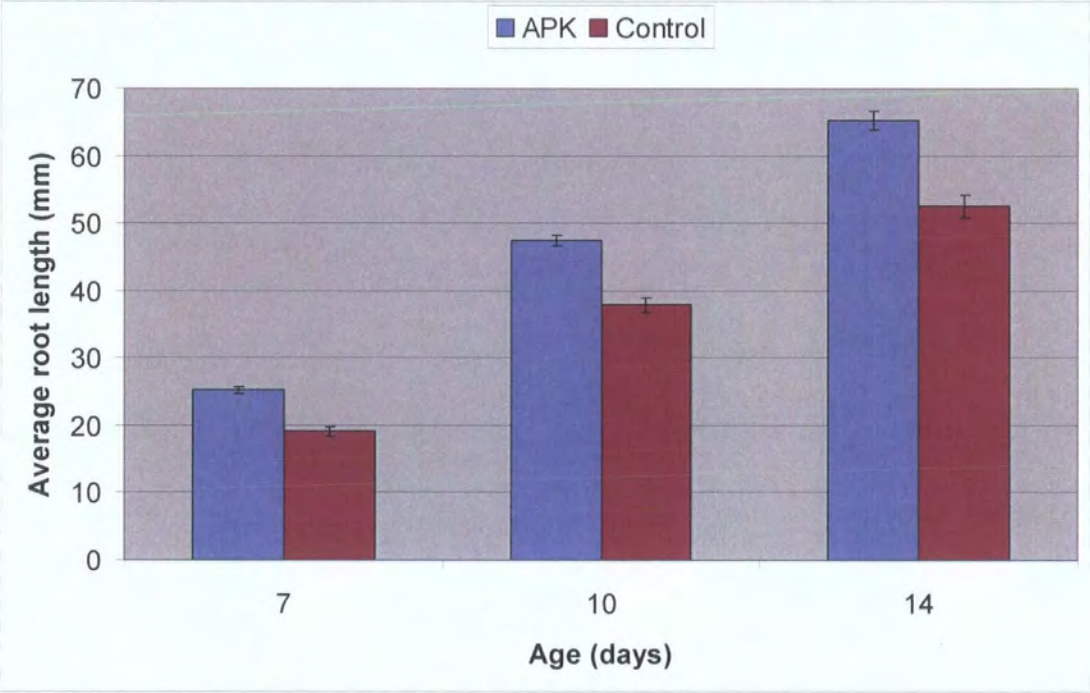
The loss- and gain-of-function lines (Chapter 4) were subjected to a variety of treatments in according to the availability of facilities. The results of these primary screens were assessed, and only investigated further if a noticeable difference was observed from the control plants. Screens were designed to test for both susceptibly and tolerance. Growth and development of the lines was also monitored for abnormal phenotypes. The expression profile data (Part 1) was used as a basis to direct investigations against particular stimuli.

5.2.2.1 Development

Growth of the T-DNA insertion and over-expressor lines was monitored throughout the plant life cycle according to the General Growth Protocol provided by the Arabidopsis Gantlet Project of Western Washington University, USA (*http://thale.biol.wwu.edu/index/html*; Materials and Methods 2.7). Plants were grown on vertical plates for 14 days and then transferred onto soil. Characteristics monitored included size, shape and colour of cotyledons and leaves, root length and branching, time of flowering, flower morphology and senescence. Initially, the three *APK* over-expressor lines tested exhibited longer root lengths compared to the empty vector control lines (see Figures 5.36 and 5.37 overleaf). However, upon repetition, this feature was not observed again. No other differences were observed between any of the lines tested compared to control plants.

Figure 5.36

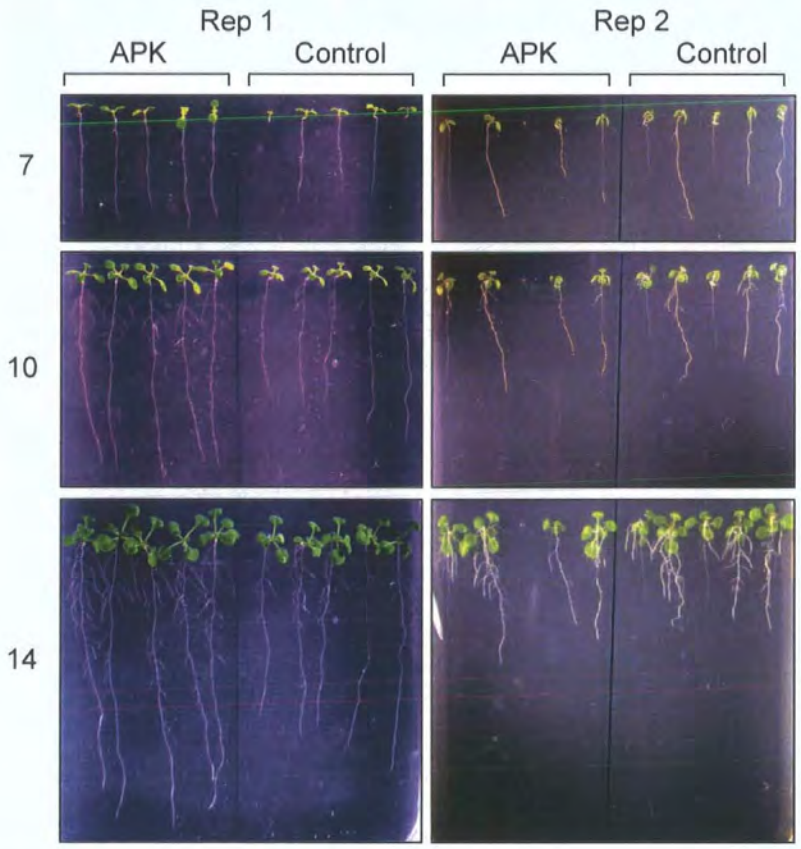
Average root lengths of the three *APK* over-expressing lines compared to the three empty vector control lines in the initial screen. Upon repetition no difference was observed.



Root lengths were measured at day 7, 10 and 14 from photographs using the ImageJ image analysis tool (*http://rsb.info.nih.gov/ij/*).

Figure 5.37

Example of root lengths of an *APK* over-expressing line in two replicate experiments.

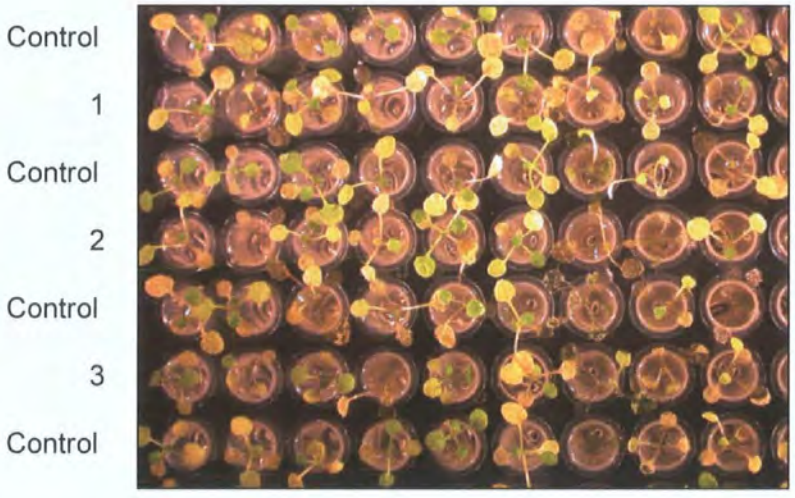


In replicate 1 (rep 1) the root lengths of the *APK* over-expressing line were noticeably longer compared to the empty vector control line. However, this was not seen in a subsequent replicate experiment (rep 2). Images are shown of 7, 10 and 14 day old plants. 4 plates of 3 over-expressing lines were monitored per experiment (40 plants in total).

Additionally, dark-induced senescence was monitored by the method shown below in Figure 5.35 (Materials and Methods 2.7.1). No difference was observed when compared to the control lines.

Figure 5.35

An example of the dark-induced senescence screen.



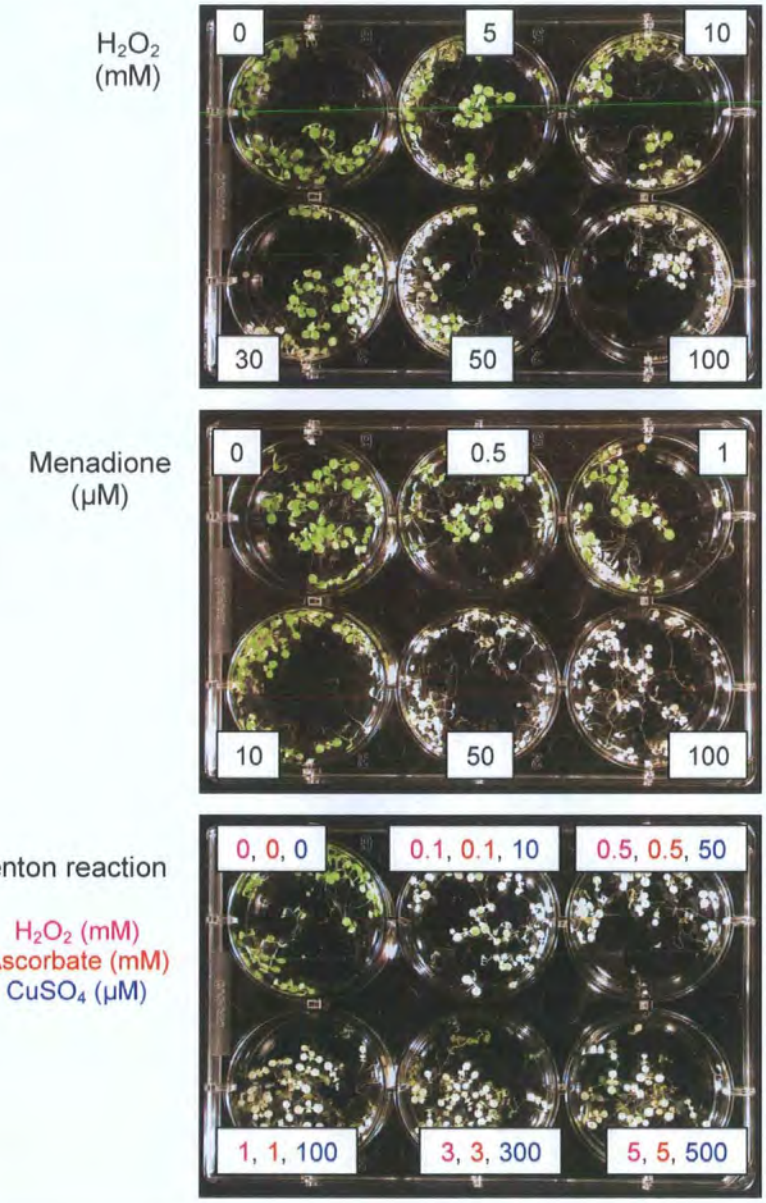
Seedlings (5 days old) were cut at the hypocotyl base and placed in 0.2 ml microfuge tubes filled with water. The container was then wrapped in foil, placed in the growth cabinet and monitored for 10 days. Image taken after 4 days. This example is taken from a screen for three *APK* over-expressing lines (1 to 3) against three empty vector (control) lines.

5.2.2.2 Oxidative stress

T-DNA insertion and over-expressor lines were examined in their ability to withstand oxidative stress, exerted by different concentrations of H_2O_2 (5 to 100 mM), the superoxide generator menadione (0.5 to 100 µM) and the Fenton reaction (to generate hydroxyl radicals; see Materials and Methods 2.6.1.4 for reagent concentrations). The time and extent of bleaching of seedling cotyledons was assessed visually. No noticeable difference was observed over 5 days compared to the control lines. An example of the screen is shown overleaf in Figure 5.38.

Figure 5.38

An example of the oxidative stress screen.



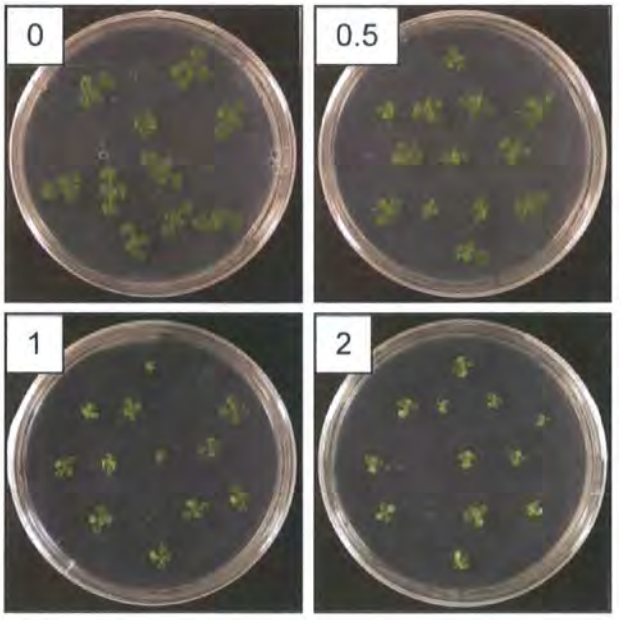
Seedlings (10 days old) were incubated for 3 h in water prior to incubation with different concentrations (as indicated) of H_2O_2, menadione, the Fenton reaction or water (control). The time and extent of cotyledon bleaching was monitored over 5 days. Image taken after 3 days. This example shows wild-type seedlings.

5.2.2.3 UV-B stress

Seedlings of T-DNA insertion and over-expressor lines were exposed to 0, 0.5, 1 or 2 J/cm^2 of UV-B in a cross-linker (Materials and Methods 2.6.1.6). The plants were monitored daily for bleaching and growth retardation up to 10 days post-treatment. An example is shown below in Figure 5.39. No noticeable difference was observed between the loss- and gain-of-function lines compared to the controls.

Figure 5.39

An example of the UV-B screen.

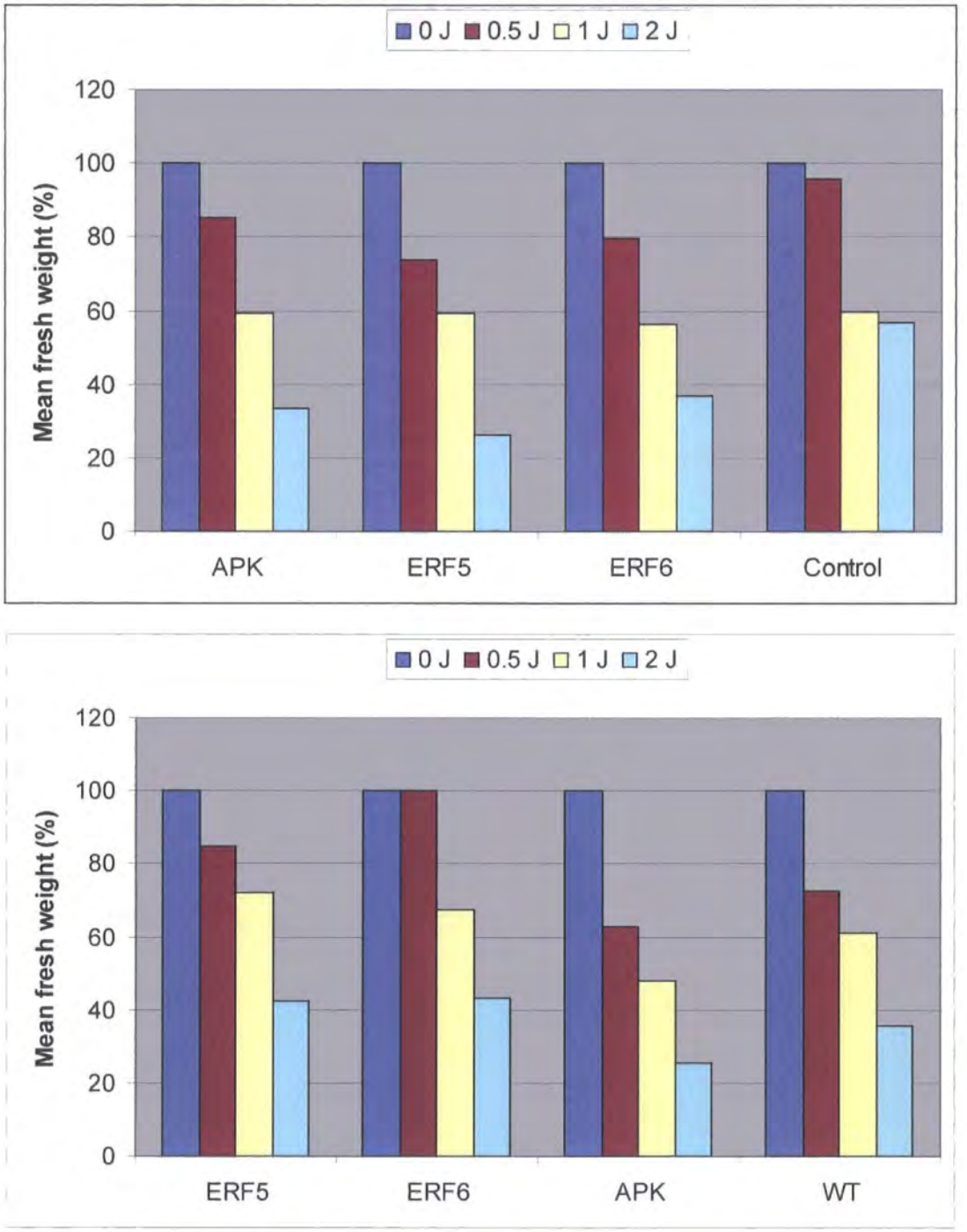


Seedlings (10 days old) were treated with 0.5, 1 or 2 J/cm^2 of UV-B in a cross-linker. The lids of control plates (0) were removed for the same length of time (approximately 45 to 90 s). Plants were monitored daily for bleaching and growth retardation 10 days post-treatment. Image taken 7 days post-treatment. This example shows wild-type seedlings.

At 10 days post-treatment, fresh weights were measured and are shown on the following two pages for the T-DNA insertion mutants (Figures 5.40) and over-expressing lines (Figure 5.41). As a general tend, fresh weight decreased as the strength of UV-B increased.

Figure 5.40

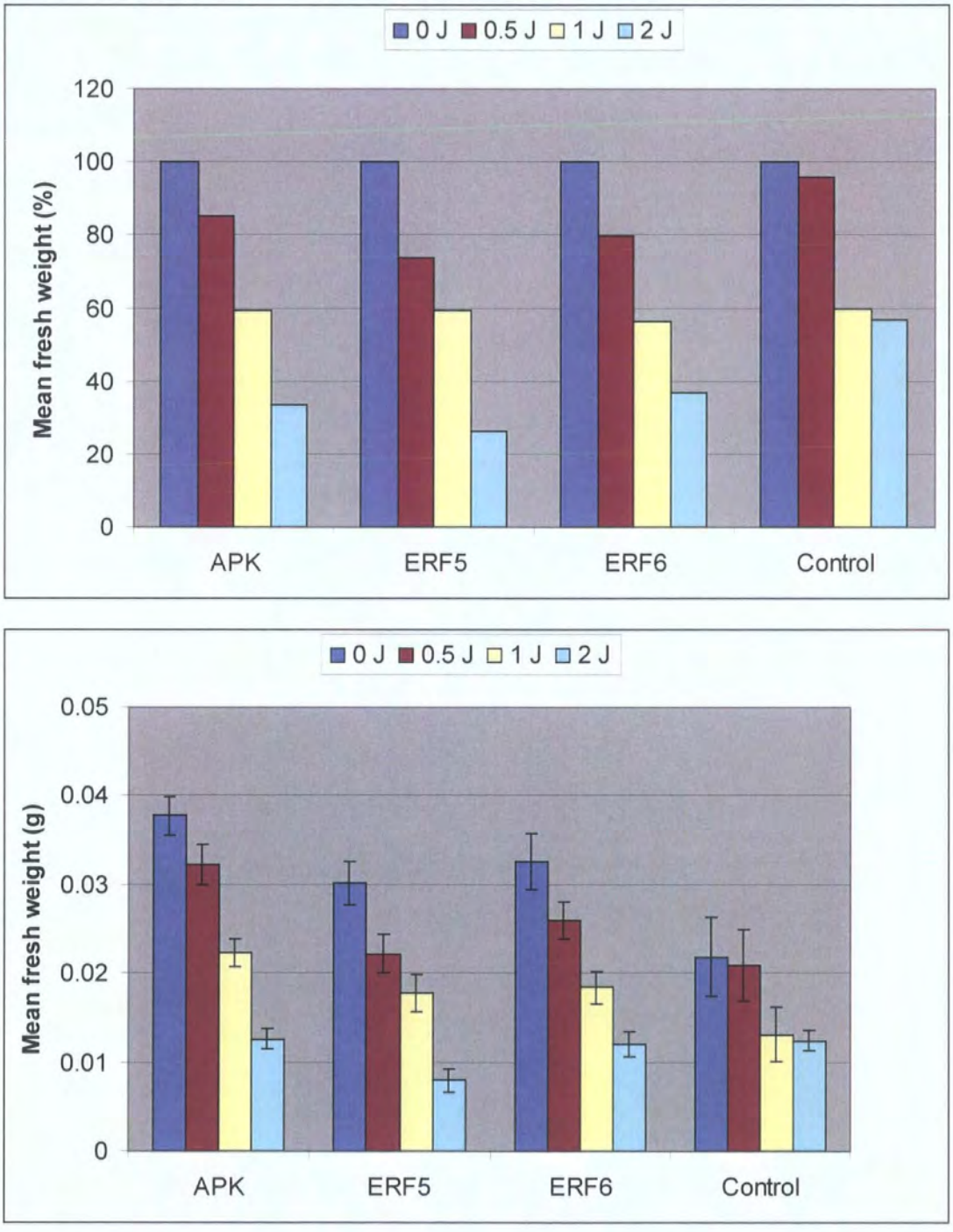
Average fresh weights of **T-DNA insertion lines** following UV-B treatment.



Plants (10 days old) were treated with 0, 0.5, 1 or 2 J/cm^2 of UV-B. Fresh weights were measured 10 days post-treatment. **a)** Fresh weights expressed as a percentage relative to the untreated plants **b)** Raw values of mean fresh weights including standard error bars.

Figure 5.41

Average fresh weights of **over-expressor lines** following UV-B treatment.



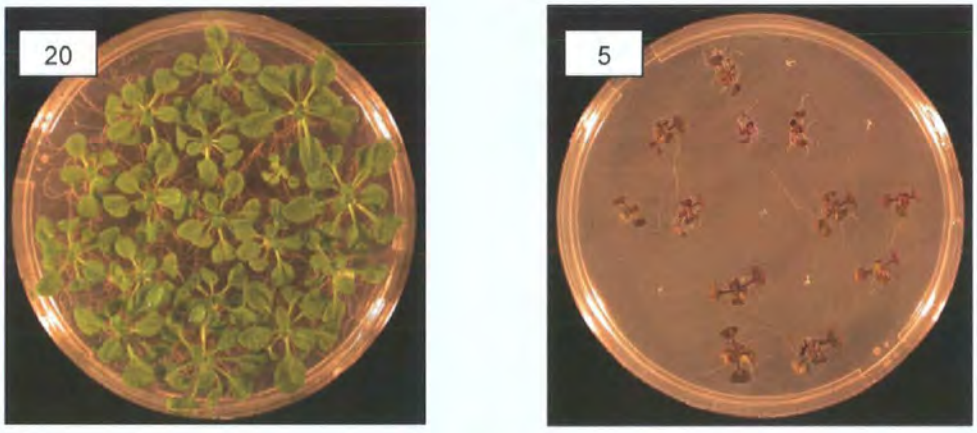
Plants (10 days old) were treated with 0, 0.5, 1 or 2 J/cm^2 of UV-B. Fresh weights were measured 10 days post-treatment. **a)** Fresh weights expressed as a percentage relative to the untreated plants **b)** Raw values of mean fresh weights including standard error bars.

5.2.2.4 Cold stress

Seedlings of T-DNA insertion and over-expressor lines were maintained in controlled growth cabinets at either 5 °C or 20 °C (Materials and Methods 2.6.1.1). Plants were monitored daily for up to 2 weeks. Significant anthocyanin accumulated which could not be overcome by lowering the light levels (Figure 5.42). However, no noticeable difference in growth or general health was observed compared to the control plants.

Figure 5.42

Example of anthocyanin accumulation after 14 days at 5 °C.



Seedlings (10 days old) were maintained in a growth cabinet at either 5 °C or 20 °C (control). Image taken after 14 days. This example shows wild-type plants.

In addition, plants were transferred on to peat and maintained at either 5 °C or 20 °C for 6 weeks. Again, no noticeable difference in growth or overall health was observed when compared to the control lines. An example is shown below in Figure 5.43.

Figure 5.43

Examples of wild-type plants after 6 weeks at 5 °C.

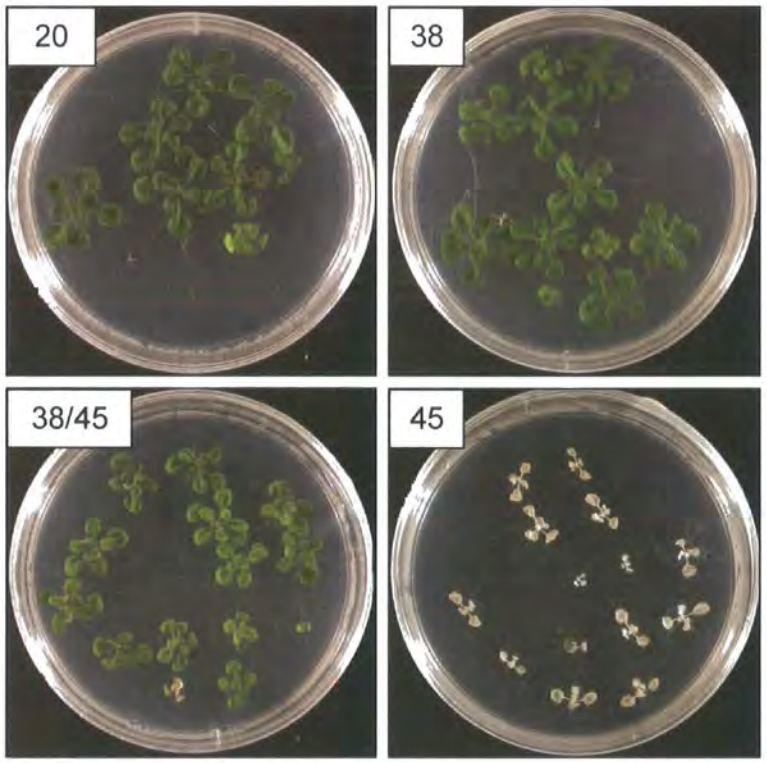
This example shows an *APK* over-expressor line (test line) and an empty vector line (control).

5.2.2.5 Heat stress

The experiments were designed to test for susceptibility or tolerance to heat stress and the ability to acclimate to heat. As shown below in Figure 5.44, 14-day old seedlings were treated with one of four treatments (Materials and Methods 2.6.1.3): either 38 °C (1.5 h), 38 °C (1.5 h) then 45 °C (2 h), 45 °C (2 h) or maintained at 20 °C (control). Plants were monitored daily for survival up to 18 days post-treatment.

Figure 5.44

Example of the heat screen.

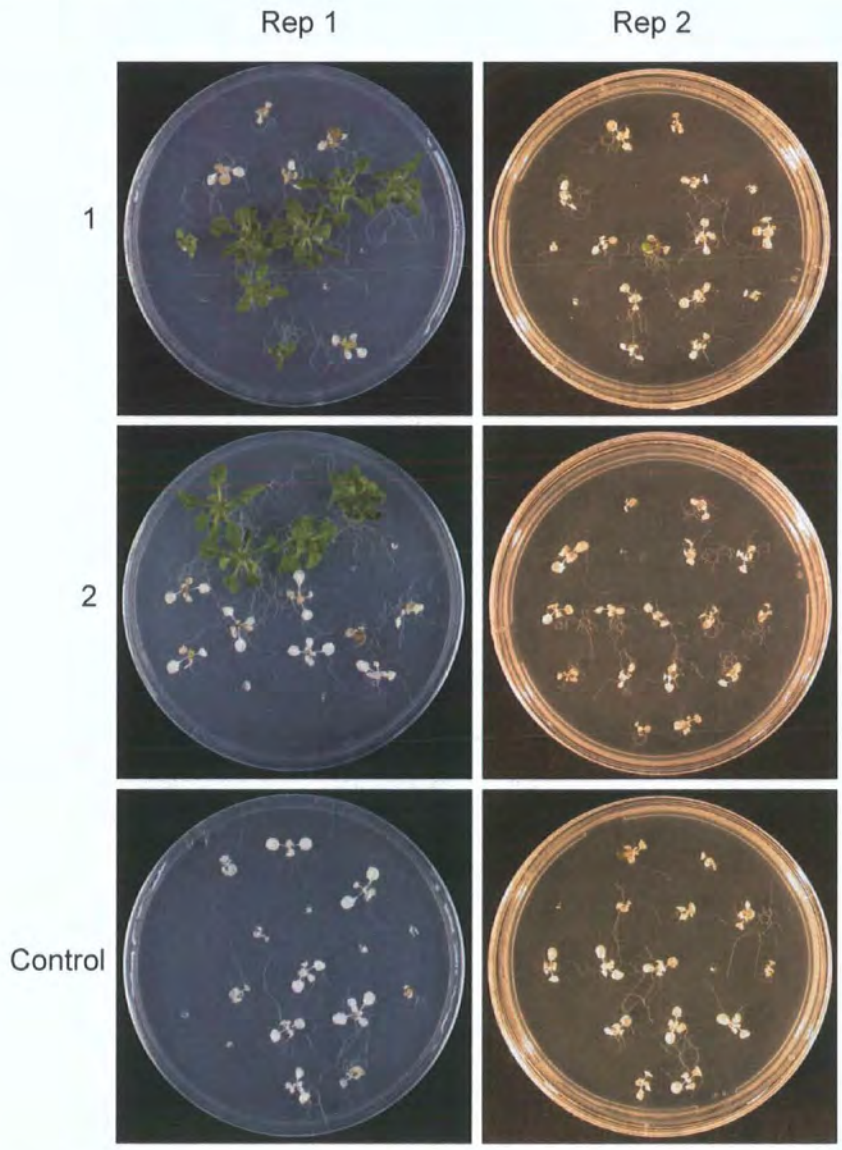


Plants (14 days old) were treated at either i) 38 °C for 1.5 h ii) 38 °C for 1.5 h then 45 °C for 2 h iii) 45 °C for 2 h or iv) maintained at 20 °C (control). Image taken 7 days after treatment. This example shows wild-type plants.

The initial screen revealed that after 2 h at 45 °C, half of the seedlings of two of the 3 *ERF5* over-expressing lines survived (Figure 5.45 below). However, this result was unable to be repeated in subsequent experiments.

Figure 5.45

Tolerance of the two *ERF5* over-expressing lines following 2 h at 45 °C.



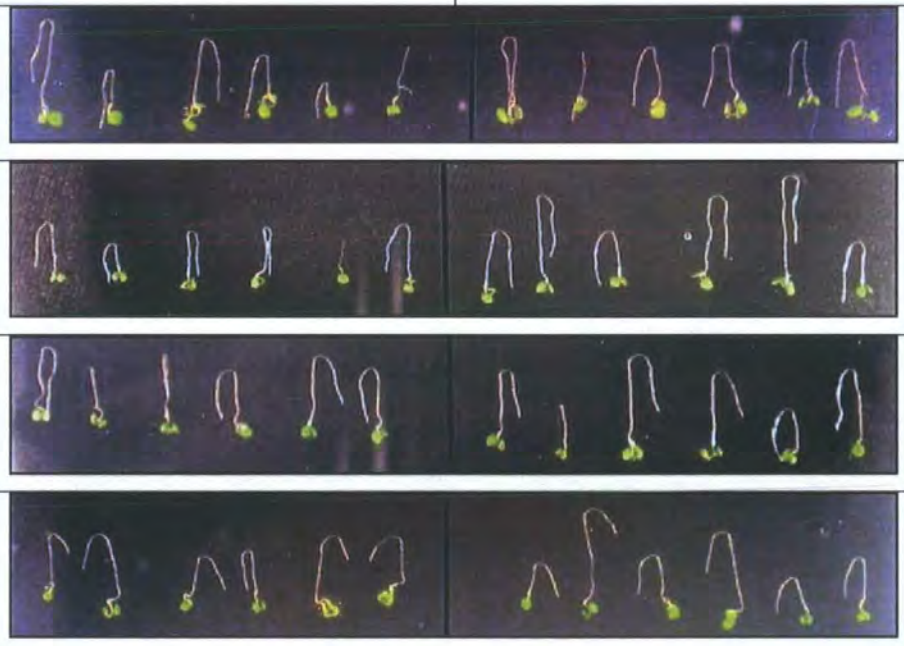
In replicate 1 (rep 1) both lines exhibited survival, However, this was not seen in a subsequent experiments e.g. replicate 2 (rep 2). Images are shown 18 days post-treatment

5.2.2.6 Mannitol stress

Five day old seedlings were transferred to vertical plates containing different concentrations of mannitol (0, 100, 200 and 300 mM) as described in Materials and Methods 2.6.1.2. Plates were turned through 180° and plants grown for 3 further days to examine root growth. An example is shown below in Figure 5.46. No difference was observed between the T-DNA insertion or over-expressing lines compared to the controls.

Figure 5.46

An example of a root reorientation screen against mannitol.



Seedlings (5 days old) were transferred onto plates containing 0, 100, 200 or 300 mM mannitol and turned through 180°. Plates were placed in the growth room. After 3 days root growth was examined. This example shows an *ERF6* over-expressor line compared to an empty vector (control).

5.2.2.7 Salt stress

Loss- and gain-of-function lines were examined for susceptibility or tolerance to salt by root reorientation screen, of the type previously described for mannitol (Section 5.2.2.6). Response to 50, 100, 150 and 200 mM NaCl was visually assessed (Materials and Methods 2.6.1.5), but no difference was observed from the control plants (data not shown).

5.2.2.8 Biotic stress

Tolerance to virulent and avirulent *Pseudomonas syringae* pv. *tomato* strains was investigated by dipping method (Tornero and Dangl, 2001; Materials and Methods 2.6.2.). No difference was observed in the loss- and gain-of-function lines compared to the control plants (data not shown).

5.2.2.9 Hormones

5.2.2.9.1 Ethylene

Lines were germinated on plates of 10 μM ACC which increases root hair density by producing ectopic hairs (Dolan, 2001). The roots of 5-day old seedlings were visually assessed under a dissecting microscope for altered root length and root hair formation (Materials and Methods 2.6.3.2). No difference was observed between the T-DNA insertion or over-expressing lines when compared to the control lines (data not shown).

5.2.5.9.2 Auxin

The effect of NAA was assessed by germinating lines on vertical plates and at 4 days old were transferred to vertical plates containing 0, 0.1, 1 or 10 µM NAA (Materials and Methods 2.6.3.1). The position of the root tip was marked at this time, and 3 days later root growth was assessed (Figure 5.47). No difference was observed in either the loss- or gain-of-function when compared to the control lines.

Figure 5.47

An example of an auxin screen for root length.

Seedlings (4 days old) were transferred to plates containing 0, 0.1, 1 or 10 µM NAA. The root tip positions were marked and the plates returned to the growth room for 3 days. This example shows an *ERF5* over-expressor line compared to an empty vector (control).

5.3 Discussion

5.3.1 Gene expression profiles (Part 1)

Altered transcript levels of the three candidate ROS-signalling genes (*ERF5*, *ERF6* and *APK*) were observed in response to many different factors (e.g. cold, UV-B and pathogen response), suggestive of a role in the response of Arabidopsis to these stimuli. However, some discrepancies between the expression data from northern blot analyses and the AtGenExpress microarray data were detected. This may be due to the different ages of plants treated and variations in treatment methods and conditions.

The broad response to different stresses might result from the activation of gene expression by ROS which accumulate in plant cells in response to many stress conditions. *ERF5* was able to exert a more pronounced induction of expression in response to lower H_2O_2 concentrations than either *ERF6* or *APK*. Assuming that the detected transcript levels are representative of the corresponding protein levels, this may reflect a difference in ROS sensitivity: such that *ERF5* is involved in response to lower level ROS signals (e.g. for ROS signalling), whilst *ERF6* and *APK* may respond to higher level ROS accumulation (e.g. during oxidative stress). Furthermore, *ERF5* expression is highly responsive (within 1 h) to many stress stimuli, pointing to a perhaps to a potential role for it as a very sensitive responder to ROS signals.

The observation that all three genes were highly up-regulated by cycloheximide treatment (which blocks protein translation but not transcription) may indicate that their expression is independent of *de novo* protein synthesis. This is often the case for induction of early genes, and indicates a primary response to the stimulus via modification of pre-existing components (Herschman, 1991). Cycloheximide has also been reported to cause apoptosis in mammalian cells (Ledda-Columbano *et al.*, 1992), so it is possible that the candidate genes may be responding to a PCD stress.

5.3.2 Functional characterisation of the loss- and gain-of-function lines (Part 2)

The second part of this Chapter aimed to couple the observed transcript changes with altered phenotypes, in order to test for the sufficiency and/or necessity of these genes. T-DNA insertion mutants and over-expressor lines were assessed for altered phenotypes following various treatments. Under the specific conditions tested, the three gene products appeared to be neither necessary nor sufficient for the normal plant phenotype (or they played too small a part to be seen). The initial observations of enhanced root length of the *APK* over-expressing lines and heat tolerance of the *ERF5* over-expressors were not seen upon subsequent repeat experiments. However, the same growth cabinets and conditions could not be used in the repeats (due to relocation of the laboratory). The screens for abnormal phenotypes were by no means exhaustive, and more extensive work (particularly with a variety of pathogens) may uncover abnormal phenotype(s).

5.3.3 Conclusion

Apart from the difference in H_2O_2 sensitivity, both *ERF5* and *ERF6* exhibited very similar gene expression patterns in response to a wide range of treatments. *ERFs* form a large subfamily and many members are regulated by the same stimuli and potentially bind the same promoter elements. Therefore a high level of functional redundancy may exist. The generation of an *erf5/erf6* double mutant may overcome this. Another way to aid the functional characterisation of redundant transcription factors is to use chimeric repressors to facilitate targeted repression of the gene of interest. An example in plants is that of CRES-T (chimeric repressor silencing technology) which utilises the EAR (ERF-associated amphiphilic repression) repression domain motif (of 12 amino acids). Arabidopsis transcription factors fused to the EAR motif act as dominant repressors and suppress the expression of specific target genes, even in the presence of the redundant transcription factors (Hiratsu *et al.*, 2003).

Future work with APK might include disruption of essential residues required for kinase ATP binding. For example, site-specific mutagenesis of the lysine residue in the ATP anchor can reveal kinase function (Zhang *et al.*, 1994; Nirmala *et al.*, 2006). This would be advantageous over work with the loss-of-function mutants as the mutated alleles would be dominant negative alleles analogous to CRES-T.

Chapter 6

Microarray analyses of over-expressor lines

6.1 Introduction

As described previously in Results Chapter 5, screening of the loss and gain-of-function lines resulted in no observable altered phenotype(s) (under the conditions tested). The next step was therefore to test for a molecular phenotype via microarray analyses. Due to time and financial constraints, it was decided to perform the microarray experiments on the over-expression lines rather than the T-DNA mutants (which, in theory, were less likely to result in a molecular phenotype due to the possibility of redundancy or the requirement for a specific stimulus). Microarray analyses were performed on the *ERF5*, *ERF6* and *APK* over-expressing lines. In this way it was hoped that any changes in the Arabidopsis transcriptome caused by over-expression of these genes would provide a clue as to the role(s) ERF5, ERF6 and APK play *in vivo*.

The aim of this chapter was to:

- *Investigate gene expression changes in plants over-expressing ERF5, ERF6 or APK*
- *Compare the resulting differentially regulated gene lists with those identified from the previous H_2O_2 microarray experiment (Chapter 3)*
- *Analyse upstream promoter sequences of the differentially regulated genes in order to identify potential transcription factor binding sites*

6.2 Results

6.2.1 Indirect method of microarray labelling

An indirect microarray labelling method was used to assess transcriptomic changes in the over-expressing lines (for full details of the protocol followed please refer to Materials and Methods 2.17). (N.B. the NASC H_2O_2 microarray experiment described in Chapter 3 used a direct labelling method). Modified nucleotides were not used during cDNA synthesis with the indirect system. Instead a capture sequence was added to each cDNA molecule via the dT primer. The cDNA mixture was directly hybridised to the array slide, and the slide was subsequently hybridised with dendrimers pre-labelled with either Cy3 or Cy5 (dendrimers are spherical complexes of partially double-stranded oligomers that recognise the target sequence). One dendrimer hybridises to each cDNA molecule, and since each dendrimer contains approximately 850 CyDyes, each cDNA will have 850 dyes. Thus the signal is independent of transcript length and nucleotide composition, unlike direct labelling, in which the amount of fluorescent dNTPs incorporated into each cDNA is sequence dependent. Since dye biases are not an issue, complicated normalisation procedures are not required and global median normalisation is sufficient. The dendrimer system is also much more sensitive than direct incorporation, requiring only 2 µg of total RNA (due to the high number of dyes in each dendrimer) and has a much lower signal to noise ratio (10:1 compared to 2:1 of direct incorporation; Stears *et* al., 2000).

Prior to performing microarray analyses, total RNA was extracted from pooled untreated 10-day old seedlings from three independent over-expressing lines for each gene, as well as three empty vector control lines. cDNA was reverse transcribed from the total RNA extracts and PCR was performed using the northern probe primers to verify over-expression (data not shown).

6.2.2 Differentially regulated genes in the over-expressor lines

Table 6.1 overleaf summarises results from the three arrays for each of the over-expressing genes. The number of transcripts with a detectable signal for each array was between 85.7 to 95.7 %, indicating the high quality of the hybridisation (a poorer hybridisation leads to higher background and artefacts, and thus has fewer detectable spots). Over-expression of the *ERF* genes resulted in a considerably larger number of altered transcript levels compared to *APK* over-expression. However, despite this large number, when the three slide replicates are compared the number of differentially regulated transcripts common across all three was relatively low: 113 for *ERF5*, 72 for *ERF6* and 35 for *APK* at the 1.5-fold cut-off (considerably less at the 2-fold cut-off). In each case, more genes were up-regulated than down-regulated, and no genes were repressed at all in the APK arrays.

Table 6.1

Summary of the microarray analyses of the *APK*, *ERF5* and *ERF6* over-expressing lines.

35S	Slide rep	Fold change of 35S gene	Transcripts detected (Present)	>1.5 fold up	>2 fold up	>1.5 fold down	>2 fold down	Total >1.5 fold	Cy3 labelled line	Cy5 labelled line
APK	1	3.8	24877 (85.7%)	2017	550	2032	545	4049	8	EVi
	2	7.3	25453 (87.7%)	2453	836	2481	771	4934	EVii	11
	3	13.0	25789 (88.8%)	2418	897	2394	714	4812	12	EViii
	Across all 3		**23442 (80.7%)**	**35**	**16**	**0**	**0**	**35**		
ERF5	1	40.9	27782 (95.7%)	4998	1790	3482	771	8480	9	EVi
	2	2.3	27555 (94.9%)	5459	2264	7428	2560	12887	EVii	11
	3	12.4	25223 (86.9%)	6643	2719	5778	2337	12421	12	EViii
	Across all 3		**23879 (82.2%)**	**90**	**57**	**23**	**14**	**113**		
ERF6	1	2.1	26518 (91.3%)	4274	1329	3533	605	7807	1	EVi
	2	1.8	27503 (94.7%)	4682	1288	6456	2377	11138	EVii	4
	3	2.2	26426 (91.0%)	4268	1696	3286	689	7554	7	EViii
	Across all 3		**24290 (83.7%)**	**51**	**37**	**21**	**10**	**72**		

CyDye labelled lines refer to those depicted in Figure 4.13 of Results Chapter 4. EV stands for empty vector. Excluding blanks and controls, there are a total of 29,551 spots on the arrays used, of which 26,273 represent different genes.

Figure 6.1 below shows the extent of the overlap of differentially regulated genes in the three over-expressors. There is some overlap between the *ERF* over-expressors: 21 genes that are up-regulated and one that is down-regulated are common to both *ERF5* and *ERF6* arrays. None of the genes from the *APK* array were shared. Full lists of the up- and down-regulated genes for each over-expressor are listed in Appendices G, H and I for *APK*, *ERF5* and *ERF6* respectively. Notably, transcripts encoding plant defensins (PDFs) were most highly up-regulated in both the *ERF* over-expressor arrays (Appendices H1 and I1).

Figure 6.1

Venn diagram showing the overlap of regulated genes across all 3 biological replicates from the over-expressing *APK*, *ERF5* and *ERF6* microarrays

> 1.5 fold up-regulated genes:

> 1.5 fold down-regulated genes:

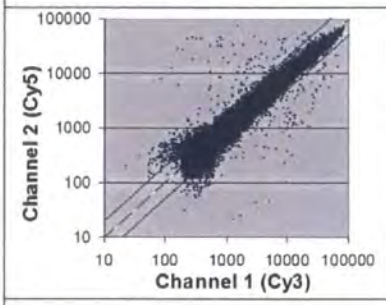
Figure 6.2 overleaf depicts the changes in expression of all the probe sets in each slide. The shape of the *ERF* over-expressor scatter plots (particularly *ERF5*) are "fatter" compared to those of *APK*, indicative of the larger number differentially regulated transcripts.

Figure 6.2

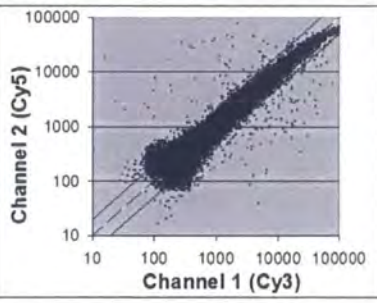
Scatter plots of normalised expression values for all present detected probe sets in the over-expressor microarrays. Dashed diagonal lines represent no change, whilst solid diagonal lines represent 2-fold up- and down-regulation ratio cut-offs.

***APK* over-expressor microarrays:**

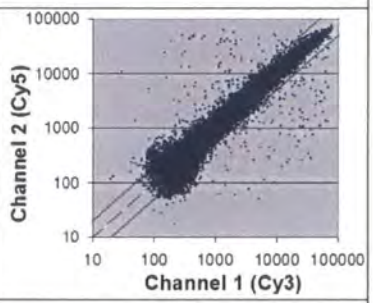
Slide 1



Slide 2

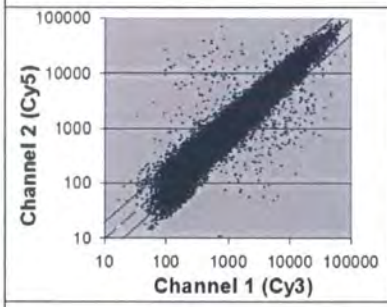


Slide 3

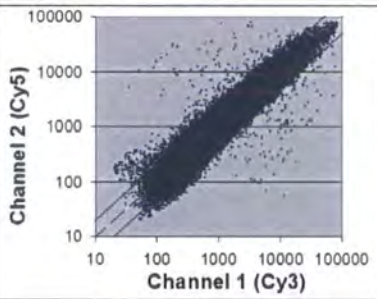


***ERF5* over-expressor microarrays:**

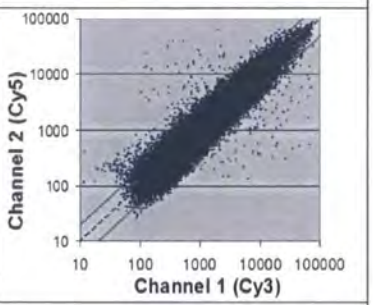
Slide 1



Slide 2

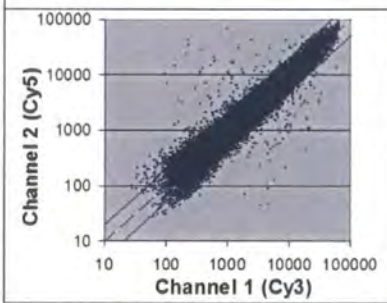


Slide 3

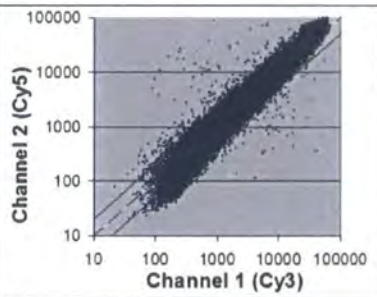


***ERF6* over-expressor microarrays:**

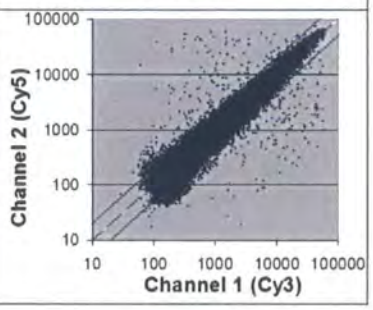
Slide 1



Slide 2



Slide 3



6.2.3 Gene ontology analysis

The next step was to couple the observed changes in transcript abundance with biological meaningful processes. The up- and down-regulated gene lists were classified into gene ontologies by using the "classify genes" function of the DChip microarray data analysis program. However, the results should be interpreted with a degree of caution, as DChip is designed for use with Affymetrix data (and thus can generally only analyse genes that are present on the Affymetrix gene chip). There are more genes present on the Operon slides than on the Affymetrix slide, so DChip was not able to analyse the complete number of genes on each list. However, there appeared to be no inherent bias in the ontology enrichment between the Affymetrix and the Operon gene chips when two randomly selected gene lists (of 50 genes each) were compared (data not shown).

The results are shown in the following three tables. *APK* had no significantly over-represented ontologies for its up-regulated genes. The most obvious ontologies for the *ERF5* up-regulated genes were starvation-related (Table 6.2), whilst those for *ERF6* showed over-representation in genes belonging to categories associated with ROS (e.g. oxygen and ROS metabolic processes, antioxidants; Table 6.3). Both *ERFs* showed a strong emphasis on pathogen defence. Very few ontologies were over-represented in the down-regulated gene lists (Table 6.4).

Table 6.2

Significantly over-represented gene ontologies of 1.5-fold **up**-regulated genes from the ***ERF5*** over-expression microarray experiments. Where "O/E" is the Observed/Expected ratio. Classified according to DChip ($p < 0.001$). (N.B. There were 90 genes in the list, but only 61 could be analysed by DChip).

Gene ontology	Within gene list		Within ATH1 array		O/E
	Occurrences within gene list	Occurrence %	Occurrence within ATH1 Array	Occurrence %	
cellular response to starvation	2/61	3.8	18/18499	0.10	33.70
cellular response to nutrient levels	2/61	3.28	18/18499	0.10	33.70
cellular response to extracellular stimulus	2/61	3.28	21/18499	0.11	28.88
cellular response to stimulus	2/61	3.28	21/18499	0.11	28.88
response to starvation	2/61	3.28	24/18499	0.13	25.27
response to nutrient levels	2/61	3.28	27/18499	0.15	22.46
toxin metabolic process	3/61	4.92	43/18499	0.23	21.16
toxin catabolic process	3/61	4.92	43/18499	0.23	21.16
glutathione transferase activity	3/61	4.92	47/18499	0.25	19.36
antibiotic biosynthetic process	2/61	3.28	36/18499	0.19	16.85
response to extracellular stimulus	2/61	3.28	37/18499	0.20	16.39
antibiotic metabolic process	2/61	3.28	37/18499	0.20	16.39
drug metabolic process	2/61	3.28	37/18499	0.20	16.39
defence response to fungus	3/61	4.92	56/18499	0.30	16.25
isopenicillin-N synthase activity	2/61	3.28	38/18499	0.21	15.96
oxidoreductase activity\ acting on X-H and Y-H to form an X-Y bond	2/61	3.28	38/18499	0.21	15.96
oxidoreductase activity\ acting on X-H and Y-H to form an X-Y bond\ with oxygen as acceptor	2/61	3.28	38/18499	0.21	15.96
response to toxin	3/61	4.92	58/18499	0.31	15.69
NAD binding	2/61	3.28	43/18499	0.23	14.11
aging	2/61	3.28	50/18499	0.27	12.13
response to fungus	3/61	4.92	92/18499	0.50	9.89
pepsin A activity	2/61	3.28	62/18499	0.33	9.78

(Table continues on the following page)

Table 6.2 *(Continued from the previous page)*

aspartic-type endopeptidase activity	2/61	3.28	63/18499	0.34	9.63
nutrient reservoir activity	2/61	3.28	65/18499	0.35	9.33
response to oxidative stress	5/61	8.20	171/18499	0.92	8.87
hydrogen peroxide metabolic process	2/61	3.28	72/18499	0.39	8.42
hydrogen peroxide catabolic process	2/61	3.28	72/18499	0.39	8.42
oxygen and reactive oxygen species metabolic process	5/61	8.20	187/18499	1.01	8.11
antioxidant activity	3/61	4.92	113/18499	0.61	8.05
transferase activity\ transferring alkyl or aryl (other than methyl groups	3/61	4.92	114/18499	0.62	7.980
response to hydrogen peroxide	2/61	3.28	81/18499	0.44	7.49
response to reactive oxygen species	2/61	3.28	87/18499	0.47	6.97
carbohydrate binding	3/61	4.92	139/18499	0.75	6.54
defence response	8/61	13.11	476/18499	2.57	5.10
catabolic process	10/61	16.39	659/18499	3.56	4.60
cellular catabolic process	9/61	14.75	610/18499	3.30	4.47
response to other organism	4/61	6.56	295/18499	1.59	4.11
response to chemical stimulus	14/61	22.95	1054/18499	5.70	4.03
response to stress	10/61	16.39	891/18499	4.82	3.40
response to stimulus	20/61	32.79	2193/18499	11.85	2.77

Table 6.3

Significantly over-represented gene ontologies of 1.5-fold **up**-regulated genes from the ***ERF6*** over-expression microarray experiments. Where "O/E" is the Observed/Expected ratio. Classified according to DChip ($p < 0.001$). (N.B. There were 51 on the list, but only 41 could be analysed by DChip).

Gene ontology	Within gene list		Within ATH1 array		O/E
	Occurrences within gene list	Occurrence %	Occurrence within ATH1 Array	Occurrences %	
defence response to fungus	5/41	12.20	56/18499	0.30	40.29
O-methyltransferase activity	2/41	4.88	28/18499	0.15	32.23
hydrogen peroxide metabolic process	4/41	9.76	72/18499	0.39	25.07
hydrogen peroxide catabolic process	4/41	9.76	72/18499	0.39	25.07
response to fungus	5/41	12.20	92/18499	0.50	24.52
response to hydrogen peroxide	4/41	9.76	81/18499	0.44	22.28
response to reactive oxygen species	4/41	9.76	87/18499	0.47	20.74
peroxidase activity	4/41	9.76	99/18499	0.54	18.23
oxidoreductase activity\ acting on peroxide as acceptor	4/41	9.76	99/18499	0.54	18.23
antioxidant activity	4/41	9.76	113/18499	0.61	15.97
innate immune response (sensu Viridiplantae	3/41	7.32	102/18499	0.55	13.27
response to oxidative stress	5/41	12.20	171/18499	0.92	13.19284
innate immune response	3/41	7.32	103/18499	0.56	13.14
immune system process	3/41	7.32	108/18499	0.58	12.53
immune response	3/41	7.32	108/18499	0.58	12.53
defence response\ incompatible interaction	2/41	4.88	74/18499	0.40	12.19
oxygen and reactive oxygen species metabolic process	5/41	12.20	187/18499	1.01	12.06
vacuole	3/41	7.32	126/18499	0.68	10.74
response to other organism	6/41	14.63	295/18499	1.59	9.18
regulation of developmental process	2/41	4.88	111/18499	0.60	8.13
defence response	8/41	19.51	476/18499	2.57	7.58
response to biotic stimulus	6/41	14.63	386/18499	2.09	7.01

(Table continues on the following page)

Table 6.3 *(Continued from the previous page)*					
response to ethylene stimulus	2/41	4.88	132/18499	0.71	6.84
calcium ion binding	5/41	12.20	377/18499	2.038	5.99
multicellular organismal process	3/41	7.32	228/18499	1.23	5.94
response to chemical stimulus	11/41	26.83	1054/18499	5.697605	4.708867
iron ion binding	6/41	14.63	586/18499	3.167739	4.619745
cellular catabolic process	5/41	12.20	610/18499	3.297476	3.698321
response to stimulus	15/41	36.59	2193/18499	11.85469	3.08615

Table 6.4

Significantly over-represented gene ontologies of 1.5-fold **down**-regulated genes from the ***ERF5*** and ***ERF6*** over-expression microarray experiments. Where "O/E" is the Observed/Expected ratio. Classified according to DChip ($p < 0.001$). (N.B. There were 23 genes on the *ERF5* down-regulated list and 21 on that of *ERF6*, but only 17 and 15 could be analysed respectively by DChip).

Gene ontology	**Within gene list**		**Within ATH1 array**		**O/E**
	Occurrences within gene list	**Occurrence %**	**Occurrence within ATH1 Array**	**Occurrences %**	
***ERF5* over-expressor microarray:**					
Response to osmotic stress	2/17	11.76	160/18499	0.86	13.60
***ERF6* over-expressor microarray:**					
aromatic compound metabolic process	2/15	13.33	312/18499	1.69	7.91
endopeptidase activity	2/15	13.33	327/18499	1.77	7.54
secondary metabolic process	2/15	13.33	354/18499	1.91	6.97

6.2.4 Comparison with the AtGenExpress microarray experiments

The differentially regulated genes lists were also compared to the microarray data from the AtGenExpress project, in order to look for treatments in which they were over-represented. The standard gene ontology file of DChip was replaced by gene expression from the AtGenExpress project (thanks to Richard Capper, University of Oxford, Oxford, UK) and analysis was performed by the "classify genes" function of the DChip program. Again, the results must be interpreted with caution for reasons already outlined in Section 6.2.3.

The AtGenExpress expression gene ontologies found to be significantly over-represented in the *ERF* over-expressor arrays are shown overleaf in Table 6.5. Both lists of gene are enriched in abiotic (salt and UV-B) and biotic stress gene ontologies.

Table 6.5

Significantly over-represented AtGenExpress experiments of 1.5-fold **up**-regulated genes from the ***ERF5*** and ***ERF6*** over-expression microarray experiments. Where "O/E" is the Observed/Expected ratio. Classified according to DChip ($p < 0.001$).

Gene ontology	Within gene list		Within ATH1 array		O/E
	Occurrences within gene list	Occurrence %	Occurrence within ATH1 array	Occurrence %	
***ERF5* over-expressor microarrays:**					
Wounding (shoot) 12-24 h (up-regulated)	4/43	9.30	38/10212	0.37	25.00
UV-B (shoot) 12-24 h (up-regulated)	6/43	13.95	80/10212	0.78	17.81
Phytophthora infection (up-regulated)	12/43	27.91	266/10212	2.60	10.71
Hairpinz (up-regulated)	6/43	13.95	277/10212	2.71	5.14
Salt (root) 6-24 h (up-regulated)	13/43	30.23	840/10212	8.23	3.68
***ERF6* over-expressor microarrays:**					
Wounding (shoot) 12-24 h (up-regulated)	4/26	15.38	38/10212	0.37	41.34
UV-B (shoot) 12-24 h (up-regulated)	5/26	19.23	80/10212	0.78	24.55
Phytophthora infection (up-regulated)	10/26	38.46	266/10212	2.60	14.77
Salt (root) 6-24 h (5-fold up-regulated)	5/26	19.23	264/10212	2.59	7.44
Salt (root) 6-24 h (up-regulated)	11/26	42.31	840/10212	8.23	5.14

6.2.5 Comparison with the H_2O_2 microarray

Next, both the 1.5-fold up- and down-regulated genes (together) from the over-expressor arrays were compared to those 2-fold differentially regulated from the H_2O_2 microarray experiment (described in Chapter 3). Figure 6.3 shows the overlap: 4 genes were common to all three arrays. Nine transcripts were common to both *ERF5* over-expression and H_2O_2 treatment (see Table 6.6 overleaf) and 9 for *ERF6* over-expression and H_2O_2 (Table 6.7), although the direction of the regulation (up or down) was not always the same. There was no overlap between the H_2O_2- and *APK*- regulated gene lists.

Figure 6.3

Comparison of the differentially regulated genes (both up- and down-regulated) between the *ERF5* and *ERF6* over-expression arrays and H_2O_2 treatment array.

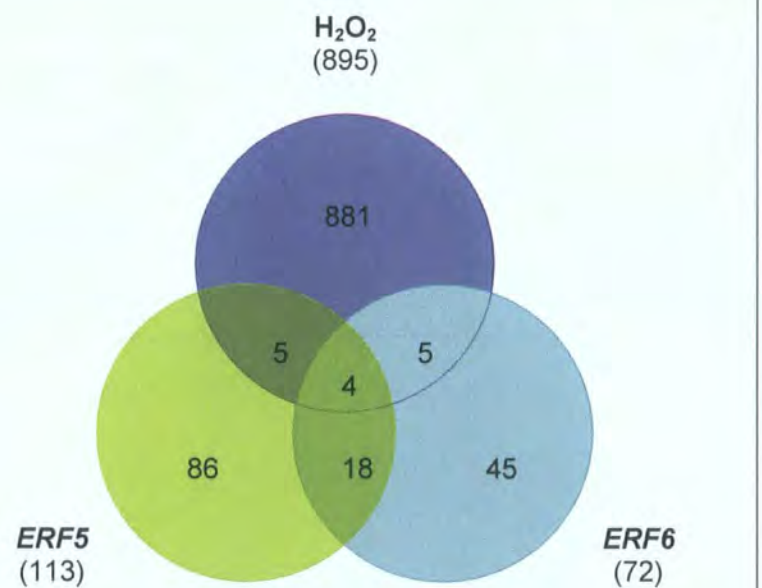


Table 6.6

Genes regulated by **ERF5** and H_2O_2. Asterisks indicate genes common to the *ERF6* over-expression arrays too.

AGI code	Putative ID	35S ERF5 fold	H_2O_2 fold
At1g02930	Gluathione-S-transferase *	+ 7.67	+ 3.58
At1g78860	Curculin-like (mannose binding) lectin family protein *	+ 4.13	+ 2.62
At2g25735	Expressed protein *	+ 2.71	+ 2.18
At2g26560	Patatin *	+ 2.58	+ 2.65
At5g57785	Expressed protein	+ 2.31	- 2.04
At1g55450	Embryo-abundant protein	+ 2.26	+ 4.19
At2g22010	Zinc finger (C3HC4-type RING finger) family protein	+ 1.61	- 2.81
At2g37770	Aldo/keto reductase family protein	- 1.81	+ 3.44
At1g77120	Alcohol dehydrogenase 1 (ADH1)	- 2.44	+ 2.46

The genes from 1.5-fold cut off of the over-expressor were compared to those with the 2-fold cut off from the H_2O_2 microarray experiment. Highlighting indicates the direction of the expression change: red for up-regulation and green for down-regulation.

Table 6.7

Genes regulated by **ERF6** and **H_2O_2**. Asterisks indicate genes common to the *ERF5* over-expression arrays too.

AGI code	Putative ID	35S ERF6 fold	H_2O_2 fold
At1g02930	Gluathione-S-transferase *	+ 5.49	+ 3.58
At2g18980	Peroxidase	+ 3.56	- 2.25
At2g26560	Patatin *	+ 2.77	+ 2.65
At1g78860	Curculin-like (mannose binding) lectin family protein *	+ 2.55	+ 2.62
At4g11650	Osmotin-like protein	+ 2.53	- 2.20
At5g47450	Arabidopsis thaliana intrinsic protein 2;3 (ATTIP2;3)	+2.10	- 2.98
At2g25735	Expressed protein *	+ 2.02	+ 2.18
At1g64710	Alcohol dehydrogenase	+ 1.60	+ 2.05
At1g02850	Glycosyl hydrolase family 1 protein	- 1.92	+ 4.08

The genes from 1.5-fold cut off of the over-expressor were compared to those with the 2-fold cut off from the H_2O_2 microarray experiment. Highlighting indicates the direction of the expression change: red for up-regulation and green for down-regulation.

6.2.6 Analysis of potential transcription factor binding sites

The upstream promoter sequences of the 1.5- and 2-fold regulated genes from the over-expressor lines were analysed in order to identify over-represented oligonucleotide motifs which may represent transcription factor binding sites or regulatory sites. Both 500 and 1000 bp of upstream promoter sequence were analysed as previously described in Results Chapter 3 (Section 3.2.3), using the "oligo analysis" tool available online at the Regulatory Sequence Analysis Tools (RSAT) site (*http://rsat.ulb.ac.be/rsat*). Only motifs with a *p* value less than 1e-04 were considered significant. All over-represented motifs were then compared to those listed in the PLACE database to check if they had been previously characterised in the literature. The over-represented motifs were also compared with those previously identified from the H_2O_2 microarray (Results Chapter 3). The results of the analyses are shown on the following pages in Tables 6.8 to 6.13.

For the *APK* up-regulated gene lists only 3 elements (a 7-mer and two 8-mers) were identified. None of these elements have been described in the literature nor matched any elements previously identified from the H_2O_2 up- or down-regulated gene lists.

Table 6.8

RSAT motif analysis of 1.5-fold and 2-fold **up**-regulated genes identified from the ***APK*** over-expression microarray experiments.

Column headings are as follows: "Seq" oligomer sequence; "Identifier" oligomer identifier; "Occ" observed occurrences; "Exp Occ" expected occurrences; "Occ P" occurrence probability (binomial); "Occ E" E-value for occurrences (binomial); "Z score" Z-score (Gaussian approximation; "O/E Ratio" observed/expected ratio.

Sequence	Identifier	Occ	Exp Occ	Occ P	Occ E	Z Score	O/E Ratio
>1.5-fold up-regulated (34 genes)							
500 bp of upstream sequence:							
8-mer:							
tcccaaaa	tcccaaaa|ttttggga	10	1.55	5.5e-06	1.8e-01	6.78	6.44
1000 bp of upstream sequence:							
8-mer:							
tcccaaaa	tcccaaaa|ttttggga	13	3.13	2.5e-05	8.2e-01	5.58	4.15
>2-fold up-regulated (15 genes):							
500 bp of upstream sequence:							
8-mer:							
attaaacg	attaaacg|cgtttaat	5	0.34	2.9e-05	9.7e-01	7.96	14.62
1000 bp upstream sequence:							
7-mer:							
cacaagg	cacaagg|ccttgtg	8	1.02	1.2e-05	9.7e-02	6.91	7.84

Table 6.9

RSAT motif analysis of 1.5-fold and 2-fold **up**-regulated genes identified from the ***ERF5*** over-expression microarray experiments. Grey highlighting shows promoters which have previously been described in the literature. Asterisks denote promoters also identified in the H_2O_2 regulated genes from Results Chapter 3 (one asterisk for those up-regulated and two for those down-regulated). Column headings are as detailed in Table 6.8.

Sequence	Identifier	Occ	Exp Occ	Occ P	Occ E	Z Score	O/E Ratio
>1.5-fold up-regulated (89 genes):							
500 bp of upstream sequence:							
5-mer:							
gtcaa *	gtcaa|ttgac	145	98.79	7.8e-06	4.0e-03	4.65	1.47
cagcc	cagcc|ggctg	46	23.15	1.8e-05	9.3e-03	4.75	1.99
6-mer:							
ggtcaa *	ggtcaa|ttgacc	40	19.74	4e-05	8.3e-02	4.56	2.03
cagcca	cagcca|tggctg	22	8.46	7.4e-05	1.5e-01	4.66	2.60
7-mer:							
ccgctta	ccgctta|taagcgg	9	1.25	6.9e-06	5.7e-02	6.91	7.17
8-mer:							
ccgcttag	ccgcttag|ctaagcgg	5	0.27	9.5e-06	3.1e-01	9.11	18.54
tagcgata	tagcgata|tatcgcta	6	0.53	1.9e-05	6.3e-01	7.53	11.37
aaccagcc	aaccagcc|ggctggtt	6	0.53	1.9e-05	6.4e-01	7.52	11.34
1000 bp of upstream sequence:							
5-mer:							
ccatc	ccatc|gatgg	157	104.19	8.6e-07	4.4e-04	5.17	1.51
6-mer:							
agccat	agccat|atggct	49	26.67	6.7e-05	1.4e-01	4.32	1.84
7-mer:							
ccgctta	ccgctta|taagcgg	12	2.52	1.4e-05	1.1e-01	5.97	4.76
atggctg	atggctg|cagccat	16	4.66	3.1e-05	2.5e-01	5.25	3.43
agccgcc	agccgcc|ggcggct	11	2.52	6.6e-05	5.4e-01	5.34	4.36
8-mer:							
cagccatc	cagccatc|gatggctg	7	0.79	1.9e-05	6.4e-01	6.98	8.85
ccgcttag	ccgcttag|ctaagcgg	6	0.54	2.2e-05	7.3e-01	7.41	11.06
aacttagc	aacttagc|gctaagtt	11	2.23	2.2e-05	7.4e-01	5.87	4.93
>2-fold up-regulated (56 genes):							
500 bp of upstream sequence:							
5-mer:							
gtcaa *	gtcaa|ttgac	98	61.89	1.4e-05	6.9e-03	4.59	1.58
7-mer:							
ccgctta	ccgctta|taagcgg	7	0.79	1.9e-05	1.5e-01	7.01	8.91
accagcc	accagcc|ggctggt	7	0.88	3.7e-05	3.1e-01	6.53	7.97
aacgatc	aacgatc|gatcgtt	10	2.13	7.6e-05	6.3e-01	5.40	4.70

(Table continues on the following page)

Table 6.9 *(Continued from the previous page)*							
8-mer:							
ccgcttag	ccgcttag|ctaagcgg	5	0.17	1e-06	3.3e-02	11.75	29.59
aaccagcc	aaccagcc|ggctggtt	6	0.33	1.4e-06	4.6e-02	9.85	18.10
aggttgat	aggttgat|atcaacct	8	0.94	6.5e-06	2.1e-01	7.29	8.52
1000 bp of upstream sequence:							
5-mer:							
ccgcc	ccgcc|ggcgg	45	22.44	1.8e-05	9.2e-03	4.76	2.01
ccatc	ccatc|gatgg	99	65.33	6.3e-05	3.2e-02	4.17	1.52
6-mer:							
cgcctc	cgcctc|gaggcg	18	5.58	2.3e-05	4.8e-02	5.25	3.22
7-mer:							
agccatc	agccatc|gatggct	13	3.07	2.1e-05	1.7e-01	5.66	4.23
agccgcc	agccgcc|ggcggct	9	1.58	4.1e-05	3.4e-01	5.90	5.69
agagggc	agagggc|gccctct	9	1.64	5.4e-05	4.4e-01	5.76	5.50
atggctg	atggctg|cagccat	12	2.92	5.6e-05	4.6e-01	5.31	4.11
ggaacac	ggaacac|gtgttcc	12	3.00	7.2e-05	5.9e-01	5.19	4.00
accagcc	accagcc|ggctggt	9	1.77	9.6e-05	7.8e-01	5.44	5.09
8-mer:							
cagccatc	cagccatc|gatggctg	7	0.50	9.5e-07	3.1e-02	9.23	14.11
atattagc	atattagc|gctaatat	11	2.05	1e-05	3.4e-01	6.25	5.37
attagcta	attagcta|tagctaat	12	2.46	1.1e-05	3.5e-01	6.09	4.89
aacgatcc	aacgatcc|ggatcgtt	7	0.78	1.8e-05	6.0e-01	7.02	8.92
catgtgaa	catgtgaa|ttcacatg	13	3.15	2.7e-05	8.8e-01	5.55	4.12
ccgcttag	ccgcttag|ctaagcgg	5	0.34	2.9e-05	9.4e-01	7.99	14.70
aggttgat	aggttgat|atcaacct	10	1.89	2.9e-05	9.6e-01	5.90	5.29

Table 6.10

RSAT motif analysis of 1.5-fold and 2-fold **down**-regulated genes identified from the ***ERF5*** over-expression microarray experiments. Grey highlighting shows promoters which have previously been described in the literature. Asterisks denote promoters also identified in the H_2O_2 regulated genes from Results Chapter 3 (one asterisk for those up-regulated and two for those down-regulated). Column headings are as detailed in Table 6.8.

Sequence	Identifier	Occ	Exp Occ	Occ P	Occ E	Z Score	O/E Ratio
> 1.5-fold down-regulated (23 genes):							
500 bp of upstream sequence:							
5-mer:							
acgtc *	acgtc|gacgt	26	8.21	5.7e-07	2.9e-04	6.21	3.17
aacgt	aacgt|acgtt	30	13.73	9.6e-05	4.9e-02	4.39	2.19
6-mer:							
aacgtc	aacgtc|gacgtt	14	2.78	1.4e-06	3.0e-03	6.73	5.03
acgtca *	acgtca|tgacgt	13	3.09	2.2e-05	4.5e-02	5.64	4.21
7-mer:							
aaacgtc	aaacgtc|gacgttt	10	1.05	1.7e-07	1.4e-03	8.76	9.56
1000 bp of upstream sequence:							
5-mer:							
aggag	aggag|ctcct	60	29.86	7.8e-07	4.0e-04	5.52	2.01
acgtc *	acgtc|gacgt	35	16.49	4.8e-05	2.5e-02	4.56	2.12
6-mer:							
aaggag	aaggag|ctcctt	31	11.87	2.7e-06	5.6e-03	5.55	2.61
aacgtc	aacgtc|gacgtt	17	5.59	7.6e-05	1.6e-01	4.83	3.04
aggaga	aggaga|tctcct	31	14.29	8.6e-05	1.8e-01	4.42	2.17
7-mer:							
aaacgtc	aaacgtc|gacgttt	12	2.10	2.3e-06	1.9e-02	6.82	5.70
gagggcc	gagggcc|ggccctc	5	0.39	5.4e-05	4.4e-01	7.39	12.84
> 2-fold down-regulated (14 genes):							
500 bp of upstream sequence:							
5-mer:							
acgtc *	acgtc|gacgt	20	5.00	3.4e-07	1.7e-04	6.71	4.00
cgtca *	cgtca|tgacg	18	6.03	6e-05	3.1e-02	4.87	2.98
6-mer:							
acgtca *	acgtca|tgacgt	10	1.88	2.8e-05	5.8e-02	5.92	5.32
aacgtc	aacgtc|gacgtt	9	1.69	6.9e-05	1.4e-01	5.62	5.32
1000 bp of upstream sequence:							
5-mer:							
aggag	aggag|ctcct	41	18.17	2.8e-06	1.5e-03	5.35	2.26
agcct	agcct|aggct	27	11.17	4.2e-05	2.2e-02	4.73	2.42
acgtc *	acgtc|gacgt	25	10.04	5e-05	2.5e-02	4.72	2.49
6-mer:							
aaggag	aaggag|ctcctt	21	7.22	2.3e-05	4.7e-02	5.13	2.91
agcttg	agcttg|caagct	16	5.01	7e-05	1.4e-01	4.91	3.20

Table 6.11

Published promoter elements identified from the analysis of 1.5 and 2-fold **up**- and **down**-regulated from the ***ERF5*** over-expression microarray. Asterisks denote promoters also identified in the H_2O_2 regulated genes from Results Chapter 3 (one asterisk for those up-regulated).

Promoter	O/E Ratio	Upstream (bp)	Description	References
Up-regulated:				
5-mer:				
TTGAC *	1.47	500 (1.5-fold)	**W-box** * Recognised by WRKY DNA binding proteins. Found in promoters of stress-tolerance genes e.g. Arabidopsis *NPR1* (*NON EXPRESSOR OF PR GENES 1*).	Eulgem *et al.* (2000), Yu *et al.* (2001), Xu *et al.* (2006)
	1.58	500 (2-fold)		
6-mer:				
TTGACC *	2.03	500 (1.5-fold)	**W-box related** * **EIRE (Elicitor Responsive Element) core** of parsley *PR1* genes; consensus sequence of elements W1 and W2 of parsley *PR1-1* and *PR1-2* promoters, which are the binding site of WRKY1 and WRKY2, respectively. Present in the Arabidopsis thioredoxin *h5* gene (involved in response to pathogens).	Rushton *et al.* (1996), Eulgem *et al.* (2000) Laloi *et al.* (2004)
7-mer:				
AGCCGCC	4.36	1000 (1.5-fold)	**GCC-box** Binding sequence of ERFs (stress signal-response factors). Present in most PR-protein genes.	Sato *et al.* (1996), Fujimoto *et al.* (2000), Takagi *et al* (2000), Cheong *et al.* (2003), Ohme- Zhang *et al.* (2004)
	5.69	1000 (2-fold)		
Down-regulated:				
6-mers:				
TGACG *	4.21	500 (1.5-fold)	**TGA1 motif/AS1 motif** * Biding site for basic domain/leucine zipper (bZIP) TGA factors e.g. Arabidopsis TGA1. Activation sequence-1 in GST genes.	Schindler *et al.* (1992), Xiang *et al.* (1997), Klinedinst *et al.* (2000)
	5.32	500 (2-fold)		
TGACGT *	2.98	500 (2-fold)	**TGACGT motif (similar to TGA1 motif)** * Binding site for the rice bZIP protein OsOBF1 (involved in cold-signalling). Binding site of the wheat histone DNA binding protein-1 (HBP-1). Present in promoter of the wheat histone genes H3 and H4. Present in the *Vigna mungo* alpha-Amylase (Amy) gene promoter.	Terada *et al.* (1995), Yamauchi (2001), Shimizu *et al.* (2005)

As expected, the GCC box was found to be over-represented in the promoters of the *ERF5* up-regulated genes. In addition, the W-box and a W-box related sequence were identified, both of which were also over-represented in the H_2O_2 up-regulated gene promoters. Three of the putative promoter elements identified from the *ERF5* down-regulated genes were also found in the promoters of the H_2O_2 up-regulated genes (GACGT, TGACG and TGAGCGT). All three overlap in sequence and include the two characterised motifs shown in Table 6.11.

Table 6.12

RSAT motif analysis of 1.5-fold and 2-fold **up**-regulated genes identified from the ***ERF6*** over-expression microarray experiments. Orange highlighting shows promoters which have previously been described in the literature. Asterisks denote promoters also identified in the H_2O_2 regulated genes from Results Chapter 3 (one asterisk for those up-regulated and two for those down-regulated). Column headings are as detailed in Table 6.8.

Sequence	Identifier	Occ	Exp Occ	Occ P	Occ E	Z Score	O/E Ratio
>1.5-fold up-regulated (50 genes):							
500 bp of upstream sequence:							
5-mer:							
ctata **	ctata|tatag	96	61.18	2.3e-05	1.2e-02	4.45	1.57
tataa **	tataa|ttata	172	124.58	3.2e-05	1.6e-02	4.25	1.38
cagcc	cagcc|ggctg	30	12.94	3.4e-05	1.8e-02	4.74	2.32
agcca	agcca|tggct	50	27.57	7.7e-05	4.0e-02	4.27	1.81
6-mer:							
ggttga	ggttga|tcaacc	25	9.92	4.1e-05	8.6e-02	4.79	2.52
ccagcc	ccagcc|ggctgg	11	2.52	6.7e-05	1.4e-01	5.34	4.36
7-mer:							
aaccagc	aaccagc|gctggtt	10	1.44	2.8e-06	2.3e-02	7.14	6.96
atcaacc	atcaacc|ggttgat	14	3.27	8.7e-06	7.2e-02	5.94	4.29
cagccgc	cagccgc|gcggctg	6	0.49	1.3e-05	1.1e-01	7.82	12.12
accagcc	accagcc|ggctggt	7	0.78	1.8e-05	1.5e-01	7.02	8.93
gccgccc	gccgccc|gggcggc	5	0.31	1.9e-05	1.5e-01	8.41	16.07
atctatc	atctatc|gatagat	15	4.33	4.9e-05	4.0e-01	5.12	3.46
agccatc	agccatc|gatggct	8	1.36	8.9e-05	7.3e-01	5.68	5.87
agccgcc	agccgcc|ggcggct	6	0.70	9.1e-05	7.4e-01	6.33	8.56
8-mer:							
aaccagcc	aaccagcc|ggctggtt	7	0.30	3e-08	1.0e-03	12.33	23.67
agccgccc	agccgccc|gggcggct	4	0.10	3.2e-06	1.1e-01	12.62	41.79
agtataat	agtataat|attatact	11	1.91	5.6e-06	1.8e-01	6.57	5.74
aagccatc	aagccatc|gatggctt	6	0.49	1.3e-05	4.1e-01	7.88	12.27
gccatcaa	gccatcaa|ttgatggc	6	0.51	1.5e-05	5.1e-01	7.71	11.82
gctggtta	gctggtta|taaccagc	5	0.31	1.7e-05	5.7e-01	8.48	16.33
agccatca	agccatca|tgatggct	6	0.52	1.8e-05	6.0e-01	7.57	11.45
ccgcttag	ccgcttag|ctaagcgg	4	0.15	1.9e-05	6.3e-01	9.92	26.54
1000 bp of upstream sequence:							
5-mer:							
cagcc	cagcc|ggctg	57	26.03	1e-07	5.4e-05	6.07	2.19
agcca	agcca|tggct	95	55.48	8.8e-07	4.5e-04	5.31	1.71
6-mer:							
cagcca	cagcca|tggctg	27	9.52	2.7e-06	5.6e-03	5.66	2.84
agcagc	agcagc|gctgct	24	9.46	5.3e-05	1.1e-01	4.72	2.54
accagc	accagc|gctggt	21	7.69	5.4e-05	1.1e-01	4.80	2.73
ccagcc	ccagcc|ggctgg	16	5.08	8.3e-05	1.7e-01	4.84	3.15
agccat	agccat|atggct	32	14.96	8.6e-05	1.8e-01	4.41	2.14

(Table continues on the following page)

Table 6.12 *(Continued from the previous page)*

7-mer:							
atggctg	atggctg|cagccat	15	2.61	1.2e-07	9.9e-04	7.66	5.74
agccatc	agccatc|gatggct	14	2.75	1.3e-06	1.0e-02	6.79	5.09
agccgcc	agccgcc|ggcggct	10	1.41	2.4e-06	2.0e-02	7.22	7.07
tctccaa	tctccaa|ttggaga	29	11.57	1.2e-05	9.6e-02	5.13	2.51
agcagcc	agcagcc|ggctgct	10	1.89	2.9e-05	2.4e-01	5.90	5.29
accagcc	accagcc|ggctggt	9	1.58	4.1e-05	3.4e-01	5.90	5.69
cagccgc	cagccgc|gcggctg	7	1.00	8.2e-05	6.7e-01	6.01	7.01
8-mer:							
aaccagcc	aaccagcc|ggctggtt	7	0.60	3.2e-06	1.0e-01	8.29	11.73
cagccatc	cagccatc|gatggctg	6	0.44	7.3e-06	2.4e-01	8.34	13.52
agccatca	agccatca|tgatggct	8	1.06	1.5e-05	5.0e-01	6.75	7.56
aaaaatgc	aaaaatgc|gcattttt	15	3.96	1.8e-05	5.9e-01	5.54	3.79
agcagccg	agcagccg|cggctgct	5	0.32	2.2e-05	7.1e-01	8.26	15.59
>2-fold up-regulated (36 genes):							
500 bp of upstream sequence:							
5-mer:							
cagcc	cagcc|ggctg	25	9.27	1.4e-05	7.1e-03	5.17	2.70
agcca	agcca|tggct	41	19.75	1.9e-05	9.8e-03	4.78	2.08
ctata **	ctata|tatag	71	43.83	9.7e-05	5.0e-02	4.10	1.62
6-mer:							
ggttga	ggttga|tcaacc	23	7.11	1.7e-06	3.6e-03	5.96	3.24
accagc	accagc|gctggt	12	2.73	3e-05	6.2e-02	5.61	4.39
agccgc	agccgc|gcggct	9	1.61	4.7e-05	9.9e-02	5.83	5.59
7-mer:							
aaccagc	aaccagc|gctggtt	10	1.03	1.4e-07	1.2e-03	8.84	9.72
cagccgc	cagccgc|gcggctg	6	0.35	2e-06	1.7e-02	9.48	16.92
accagcc	accagcc|ggctggt	7	0.56	2.1e-06	1.8e-02	8.59	12.47
gccgccc	gccgccc|gggcggc	5	0.22	3.8e-06	3.1e-02	10.12	22.44
atcaacc	atcaacc|ggttgat	12	2.34	6.6e-06	5.4e-02	6.32	5.13
agccgcc	agccgcc|ggcggct	6	0.50	1.5e-05	1.2e-01	7.76	11.95
atctatc	atctatc|gatagat	13	3.11	2.3e-05	1.9e-01	5.62	4.19
aagccaa	aagccaa|ttggctt	13	3.24	3.6e-05	2.9e-01	5.42	4.01
agcagcc	agcagcc|ggctgct	6	0.67	7.2e-05	5.9e-01	6.50	8.94
agccatc	agccatc|gatggct	7	0.98	7.2e-05	5.9e-01	6.10	7.17
8-mer:							
aaccagcc	aaccagcc|ggctggtt	7	0.21	3.1e-09	1.0e-04	14.75	33.05
agccgccc	agccgccc|gggcggct	4	0.07	8.7e-07	2.9e-02	15.02	58.35
gctggtta	gctggtta|taaccagc	5	0.22	3.5e-06	1.2e-01	10.21	22.80
ccgcttag	ccgcttag|ctaagcgg	4	0.11	5.2e-06	1.7e-01	11.85	37.05
ggtcagac	ggtcagac|gtctgacc	4	0.16	2.4e-05	7.8e-01	9.61	25.06
1000 bp of upstream sequence:							
5-mer:							
cagcc	cagcc|ggctg	44	18.69	4.3e-07	2.2e-04	5.86	2.35
agcca	agcca|tggct	72	39.83	2.9e-06	1.5e-03	5.10	1.81
6-mer:							
agccgc	agccgc|gcggct	14	3.25	8.3e-06	1.7e-02	5.96	4.31
cagcca	cagcca|tggctg	20	6.84	3.2e-05	6.7e-02	5.04	2.93
gcagcc	gcagcc|ggctgc	14	3.93	6.3e-05	1.3e-01	5.09	3.57

(Table continues on the following page)

Table 6.12 *(Continued from the previous page)*							
accagc	accagc|gctggt	17	5.52	6.6e-05	1.4e-01	4.89	3.08
gatggc	gatggc|gccatc	17	5.70	9.7e-05	2.0e-01	4.73	2.98
7-mer:							
agccgcc	agccgcc|ggcggct	10	1.02	1.3e-07	1.0e-03	8.92	9.85
atggctg	atggctg|cagccat	12	1.88	7.1e-07	5.8e-03	7.39	6.40
agccatc	agccatc|gatggct	12	1.97	1.2e-06	9.7e-03	7.14	6.08
agcagcc	agcagcc|ggctgct	10	1.36	1.7e-06	1.4e-02	7.42	7.37
accagcc	accagcc|ggctggt	9	1.13	3.1e-06	2.5e-02	7.38	7.93
cagccgc	cagccgc|gcggctg	7	0.72	1e-05	8.4e-02	7.42	9.77
aaccagc	aaccagc|gctggtt	10	2.08	6.4e-05	5.2e-01	5.49	4.81
8-mer:							
aaccagcc	aaccagcc|ggctggtt	7	0.43	3.6e-07	1.2e-02	10.04	16.34
agcagccg	agcagccg|cggctgct	5	0.23	4.5e-06	1.5e-01	9.94	21.72
agccgccc	agccgccc|gggcggct	4	0.14	1.4e-05	4.5e-01	10.37	28.84
agccatca	agccatca|tgatggct	7	0.76	1.5e-05	4.9e-01	7.16	9.22
cagccatc	cagccatc|gatggctg	5	0.32	2.1e-05	6.9e-01	8.29	15.69
ggtcagac	ggtcagac|gtctgacc	5	0.32	2.2e-05	7.4e-01	8.23	15.49

Table 6.13
RSAT motif analysis of 1.5 and 2-fold **down**-regulated genes identified from the ***ERF6*** over-expressor microarray experiments

Sequence	Identifier	Occ	Exp Occ	Occ P	Occ E	Z Score	Ratio
>1.5-fold up-regulated (21)							
1000 bp of upstream sequence:							
5-mer:							
agatg	agatg|catct	70	42.65	7.4e-05	3.8e-02	4.19	1.64

Table 6.14

Published promoter elements identified from the analysis of 1.5 and 2-fold **up**-regulated from the ***ERF6*** over-expression microarray.

Promoter	O/E Ratio	Upstream (bp)	Description	References
Up-regulated:				
6-mer:				
AGCAGC	2.54	1000 (1.5-fold)	Present in promoters of anaerobic genes involved in the fermentative pathway of monocots and dicots.	Mohanty *et al.* (2005)
7-mer:				
AGCCGCC	8.56	500 (1.5-fold)	**GCC-box** Binding sequence of ERFs (stress signal-response factors). Present in most PR-protein genes.	Sato *et al.* (1996), Fujimoto *et al.* (2000), Takagi *et al* (2000), Cheong *et al.* (2003), Ohme- Zhang *et al.* (2004)
	7.07	1000 (1.5-fold)		
	11.95	500 (2-fold)		
	9.85	1000 (2-fold)		

As with *ERF5*, the GCC box was also found to be over-represented in the promoters of the *ERF6* regulated genes, but it was more significantly over-represented here than in the *ERF5* regulated genes. Two of the motifs identified in the ERF6 up-regulated list were also present in the promoters of the down-regulated H_2O_2 genes, and have overlapping sequences (CTATA and TATAA).

6.3 Discussion

The most striking result of this study was the up-regulation of transcripts encoding pathogen defence genes in both the *ERF5* and *ERF6* over-expression arrays (please refer to Appendices H1 and I1 respectively). Six of the 13 Arabidopsis *PDF* genes (encoding defensin proteins that inhibit the growth of a broad range of fungi) were up-regulated in both the arrays, along with two *PR* genes (*PR4* and *PR1*-related). ERFs are known to modulate the expression of pathogenesis-related genes through the GCC box which is present in many promoter regions of pathogenesis-related genes (Ohme-Takagi *et al.*, 2000; Zhang *et al.*, 2004). Promoter analysis confirmed that this element was over-represented in the genes up-regulated by *ERF* over-expression, and more significantly in those regulated by ERF6. The fact that the GCC box was not over-represented in the H_2O_2-regulated gene list might indicate it has a ROS-independent regulation. Additionally, both arrays showed over-representation of genes associated with oxidative stress such as peroxidases and glutathione-*S*-transferases.

In comparison, relatively few genes were differentially regulated in the *APK* over-expression arrays. This is not surprising, since APK does not directly affect transcription unlike the ERFs. Promoter analysis revealed three motifs (previously uncharacterised), which may potentially be regulated by APK-controlled transcription factors (Table 6.8).

Few genes were found to be common to the over-expression arrays compared to the H_2O_2 microarray experiment analysed earlier in Chapter 3. For those that were, it is likely that ROS induce these elements via the ERFs. However, this comparison is somewhat limited due to differences between the two arrays, such as the Arabidopsis ecotype used, method of microarray (the NASC Affymetrix direct labelling method detects less transcripts) and lack of replication in the H_2O_2 experiment. Comparison with other publicly available ROS microarray experiments will also encounter such differences. It would have been preferable to perform 4 arrays in parallel: wild-type untreated, wild-type treated with H_2O_2, over-expressor untreated and over-expressor treated with H_2O_2 (and use three biological replicates for each treatment). However, the expense and time constraints involved limited the number of microarrays performed in this study.

6.3.1 Conclusion

It is important to bear in mind that because these genes are constitutively over-expressed by the 35*S* promoter, the observed effects could be due to other transcription activators or repressors. Other elements may not be directly controlled by the ERFs, for example the ERF could induce transcripts for other transcription factors. Further work might therefore include use of an inducible system (such as the Cre/loxP RNAi system; Guo *et al.*, 2003), and sampling at regular time points (e.g. 0.5 and 1 h to catch early genes) and later to capture late genes likely to be secondary effects of the over-expression. Additionally, the proteins may require another factor in order to be activated, such as post-translational modification or coupling elements. Future work could also include transformation of the over-expressor lines with concatamer reporter constructs of identified motifs, and yeast one-hybrid screens could be used to identify proteins that bind to the concatamer(s). Protein binding band shift assays will confirm whether the protein in question can bind to a putative target. Additionally the progressive deletion of promoter regions within known regulated genes could identify those regions within the promoter that are regulated.

Chapter 7
General discussion and future work

7.1 Introduction

As reviewed in Chapter 1, ROS have been implicated in signal transduction pathways involved in a variety of responses, from coping with environmental challenges to making developmental decisions. Most of the research to date has focused on the mechanisms for generating ROS and the ultimate end responses to ROS signals. Yet relatively little is known about how these signals are actually transduced. The work described in this thesis sought to identify protein signalling components acting downstream of H_2O_2 in Arabidopsis. This Chapter will briefly recap this work in context with the wider literature, and suggest further studies that could be done in the future to build upon the research presented herein.

7.2 Transcriptomic changes in response to ROS

Exogenous H_2O_2 treatment of wild-type seedlings (10 mM for 3 h) was able to both up-regulate and down-regulate gene expression, as gauged from the microarray experiment described in Results Chapter 3. Genes differentially regulated included those involved in protection against oxidative stress (e.g. antioxidants) and those involved in signalling events. These findings agree with a previous exogenous H_2O_2 microarray experiment in which Arabidopsis cell cultures were treated with 20 mM H_2O_2 for 1.5 or 3 h (samples were pooled from both time points [Desikan *et al.*, 2001]). General trends common to both microarray experiments were an up-regulated expression of genes whose products function in cell rescue and defence, as well as zinc finger proteins, calcium-/calmodulin-related genes, ERFs and a nitrite reductase. Specific genes that were up-regulated in both the microarray experiments included oxidative stress responsive *GST6* and *MDAR2*, the heat shock proteins HSP83, HSP17.4C and HSP17.6A, the transcription factors, *ZAT6*, *ZAT12*, *DREB2A*, *CCA1* and *WRKY6* as well as the sodium-inducible calcium-binding protein *ACP1*. The main difference observed between the two experiments was the down-regulation of chloroplast gene expression in the cell culture microarray, which in contrast appeared up-regulated on the array described in Results Chapter 3 (e.g. *psaA*).

More recently a comparative analysis of various ROS-related Arabidopsis microarray experiments has been published (Gadjev *et al.*, 2006). This report compared the gene expression levels from eight individual ROS microarray experiments, summarised below in Table 7.1. Only Affymetrix ATH1 or Agilent Arabidopsis2 arrays performed on leaf samples of plants at least 2 weeks old were analysed. ROS treatments of wild-type plants included the exogenous application of methyl viologen (MV), ozone or 3-aminotriazole (AT; a potent inhibitor of CAT [Gechev *et al.*, 2002]). Plants with reduced or abolished activity of a particular antioxidant enzyme (APX1, Cu/ZnSOD or CAT) were also examined for transcriptional changes. Additionally, the transcript profile of toxin sensitive LAG one homolog2 (LOH2) mutant plants treated with the fungal *Alternaria alternata* f.sp. *lycopersici* (AAL) toxin (which perturbs the sphingolipid metabolism and results in increased H_2O_2 levels) was also compared in this report (Gechev *et al.*, 2004). Lastly, microarray data obtained from the conditional *fluorescent* (*flu*) mutant (which accumulates the photosensitiser protochlorophyllide in the dark, and generates $^1O_2$ in plastids upon re-illumination [Meskauskiene *et al.*, 2001]) was also analysed.

Table 7.1

Summary of the ROS-related Arabidopsis microarray experiments that were comparatively analysed by Gadjev *et al.* (2006). Table reproduced from Gadjev *et al.* (2006).

Experiment	Major ROS	Localisation	Plant age (weeks)	Time points (h)	Replicates	Array	Reference
flu mutant	$^1O_2$	Plastids	3	0.5, 1, 2	1	ATH1	Op den Camp *et al.* (2003)
KD-SOD	$O_2^{\cdot -}$	Chloroplast	3	-	3	ATH1	Rizhsky *et al.* (2003)
KO-APX1 + HL	H_2O_2	Cytosol	3	0, 0.25, 0.5, 1.5, 3, 6, 24	2	ATH1	Davletova *et al.* (2005)
CAT2HP1 + HL	H_2O_2	Peroxisome	6	0, 3, 8	2	ATH1	Vanderauwera *et al.* (2005)
LOH2 + AAL	H_2O_2	-	4	7, 24,48,72	1	Arab2	Gechev *et al.* (2004)

(Table continues on the following page)

Table 7.1 *(Continued from the previous page)*

WT + AT	H_2O_2	Peroxisome	4	7	1	Arab2	Gechev *et al.* (2005)
WT + MV	$O_2^{\bullet-}$	Chloroplast, Mitochondria	2.5	0.5, 1, 3, 6, 12, 24	2	ATH1	Bartels *et al.* (AtGen-Express)
WT + Ozone	$O_2^{\bullet-}$, H_2O_2	Apoplast	2	6	3	ATH1	Short and Shirras (2002; NASCArray 26)

Where AAL, *Alternaria alternata* f.sp. *lycopersici* toxin; AS, antisense; AT, 3-aminotriazole; CAT2HP1, CAT-deficient plants; HL, high light; KD, knock-down; WT, wild-type. For full details of individual microarray experiments please refer to the corresponding quoted reference.

Examination of this report revealed that 4 of the 14 genes initially selected for further study in Results Chapter 3, were also 5-fold up-regulated in at least one of these eight arrays. These four genes were: *ERF5*, *ERF6*, a serine/threonine kinase (At4g23190) and the putative leucine-rich repeat transmembrane protein kinase (At1g09970). Specific details are given overleaf in Table 7.2. This table also shows all those genes that were up-regulated in both the H_2O_2 microarray experiment of Results Chapter 3 (by 2-fold) and in response to at least one of the eight ROS arrays (by 5-fold). Thus, these transcripts may therefore be considered as core genes in the general response to ROS, irrespective of the type of ROS or its subcellular production site.

Table 7.2

Transcripts up-regulated across nine ROS microarray experiments (8 analysed in the study by Gadjev *et al.* (2006) as well as the exogenous H_2O_2 array described in Results Chapter 3). Blue and yellow shading denote transcripts at least 5-fold or 2-fold up-regulated respectively. Those genes in bold and marked with asterisks were initially selected for further study in Results Chapter 3.

AGI code	Gene annotation	KD-SOD	APX1 -KO 1.5 h	flu 2 h	O_3	MV 24 h	CAT-AS 8 h	AAL 48 h	AT	H_2O_2 3 h
At1g19020	Expressed protein	0.65	5.36	78.53	60.06	110.59	7.86	6.45	11.16	18.29
At2g41380	Putative embryo-abundant protein	0.94	3.06	17.16	7.19	32.24	9.90	15.82	36.32	14.90
At1g62300	WRKY6	0.61	2.57	16.45	12.98	13.97	6.74	8.50	6.29	5.53
At4g01870	TolB protein related	0.78	2.92	55.96	13.96	47.95	115.82	21.27	22.65	3.66
At3g09350	Expressed protein	2.08	0.79	32.75	23.70	10.87	41.36	8.55	21.42	16.69
At2g43820	UDP-glucoronosyl / UDP-glucosyl transferase	0.45	1.28	11.60	25.51	13.03	224.68	12.14	30.00	5.27
At3g11340	UDP-glucoronosyl / UDP-glucosyl transferase	1.10	2.28	5.87	25.80	29.90	50.35	36.88	27.02	3.57
At3g53230	Cell division cycle protein 48 (CDC48)	2.04	1.30	15.61	33.92	12.53	10.73	8.85	5.59	2.79
At1g22400	UDP-glucose glucosyltransferase	1.62	2.52	30.72	42.88	18.44	26.75	14.87	47.45	6.93
At2g29460	Glutathione *S*-transferase	0.89	3.01	35.11	338.21	38.48	15.86	31.26	66.21	8.03
At2g32190	Unknown protein	0.99	1.21	154.16	418.95	19.70	118.71	19.92	5.89	7.96
At5g14760	L-aspartate oxidase-like protein	0.14	1.42	1.09	4.87	1.02	10.40	6.64	15.88	6.33
At5g63790	NAM transcription factor	0.70	1.12	40.06	9.15	16.65	6.95	2.42	3.13	9.41
At5g05410	DREB2A	1.94	0.81	183.22	24.00	23.17	17.73	1.35	1.61	13.18
At4g29780	Expressed protein	0.63	2.97	312.95	30.74	6.98	0.82	0.56	0.56	2.84
At1g80840	WRKY40	0.89	2.87	262.56	123.50	36.46	0.72	1.18	1.85	4.85
At1g77450	NAM transcription factor	0.38	1.11	175.37	38.64	18.33	3.98	0.84	2.29	8.88
At5g04340	C2H2 zinc finger transcription factor	2.04	1.96	147.10	23.30	44.81	1.71	0.48	0.92	6.88
At5g59820	ZAT12	1.36	1.35	130.86	65.98	46.33	4.88	4.03	3.06	5.48
At1g27730	ZAT10	1.51	2.61	126.29	72.22	85.18	0.86	0.61	0.81	8.20
At2g38470	WRKY33	1.29	3.59	90.32	23.93	21.25	2.87	1.62	1.34	5.59
At1g76600	Expressed protein	1.06	0.95	85.07	15.87	39.45	3.50	1.74	3.96	4.56
At2g26530	Expressed protein	0.92	1.51	81.85	21.65	26.49	0.95	0.47	0.96	5.37

At4g17230	Scarecrow-like 13 (SCL13)	1.68	1.56	67.07	10.06	9.02	0.17	0.14	0.33	2.19

(Table continues on the following page)

Table 7.2 *(Continued from the previous page)*

At4g17500	ERF1	0.99	0.92	46.66	13.31	19.26	0.59	1.56	4.05	2.96
At4g18880	HSF21	4.01	3.06	36.20	14.88	10.63	1.81	2.47	1.24	2.72
At4g23190	**Ser/thr kinase-like protein ***	**0.41**	**1.20**	**33.83**	**27.62**	**11.87**	**4.97**	**0.90**	**0.83**	**4.79**
At3g06500	Receptor-like protein kinase	0.42	2.47	32.21	11.13	6.92	0.70	0.83	2.07	2.49
At1g51760	Auxin conjugate hydrolase (ILL5)	0.38	1.29	31.98	58.30	7.48	0.62	3.80	3.25	2.20
At1g23710	Expressed protein	1.34	1.35	26.92	7.26	5.02	3.01	0.78	1.96	2.93
At3g08720	Ser/thr protein kinase (PK19)	1.38	1.95	26.45	11.09	7.61	0.80	1.31	1.23	2.02
At4g27280	Calcium-binding EF-hand family protein	0.69	2.20	25.58	10.82	5.09	0.61	0.80	0.89	2.51
At1g09970	**Leucine rich repeat transmembrane protein kinase ***	**1.07**	**1.55**	**22.77**	**11.98**	**6.30**	**1.56**	**0.46**	**1.80**	**2.53**
At1g32920	Expressed protein	1.03	3.22	18.91	6.16	7.12	1.22	2.65	4.40	2.27
At3g55980	Zinc finger transcription factor	0.94	1.64	16.94	9.44	6.14	0.36	0.27	0.41	4.01
At1g72900	Disease resistance protein	1.04	2.77	14.32	26.19	15.07	2.19	0.71	1.27	5.78
At1g18570	MyB51	2.81	1.35	13.79	23.65	5.19	4.72	1.76	1.31	2.51
At2g40140	CCCH-type zinc finger	2.27	2.05	13.67	12.30	15.42	2.23	0.81	0.97	4.46
At4g11280	ACC synthase (ACS6)	0.75	1.53	12.97	6.54	8.05	2.42	0.59	0.66	7.03
At2g04040	MATE efflux protein	1.17	0.93	12.06	22.11	30.80	3.40	0.97	1.00	3.83
At4g18010	Inositol polyphosphate 5-phosphatase	0.84	0.54	11.90	11.01	7.45	0.95	1.05	4.38	2.64
At3g22370	AOX1a	1.35	2.50	10.43	7.45	5.74	4.69	3.83	3.50	5.73
At3g11820	Syntaxin 121	1.01	2.11	9.85	11.77	6.26	2.50	0.60	1.50	2.24
At5g52750	Heavy metal associate domain-containing protein	1.53	4.01	9.23	66.00	15.04	1.04	0.91	1.26	2.07
At1g73500	MKK9	1.16	2.58	9.16	7.41	7.74	0.71	0.36	0.66	2.41
At2g37940	Expressed protein	1.11	1.17	8.54	6.14	5.44	2.61	2.60	4.96	2.26
At4g33050	Calmodulin binding family protein	1.00	4.39	7.92	28.92	5.87	1.51	1.67	4.81	4.66
At4g17490	**ERF6 ***	**1.98**	**0.98**	**6.40**	**18.54**	**7.21**	**0.35**	**0.70**	**0.71**	**6.32**
At5g47230	**ERF5 ***	**1.96**	**1.01**	**8.85**	**3.37**	**1.64**	**2.21**	**1.14**	**1.46**	**2.27**

Collectively, these observations validate that, despite the lack of slide replication, the H_2O_2 microarray experiment of Results Chapter 3 exhibited similar gene expression changes as seen in response to other ROS treatments. Therefore it can be used with confidence as a platform for selection of genes encoding candidate ROS signalling proteins. What makes this

specific microarray experiment unique from those others described in the literature, is that it utilised a 3 h exogenous H_2O_2 treatment and was performed on whole wild-type plants.

However, comparison between separate microarray experiments should be interpreted with a high degree of caution, since numerous factors can contribute to differences in microarray results. These include the method of ROS treatment/generation, the time points sampled, the plant ecotype and age, the plant system used (whole plants or cell culture, wild-type or mutants), the number of replicate slides as well as the array chip used (hence the number of represented genes). For instance, approximately 30 % of the Arabidopsis genome performed by Desikan and colleagues (2001) was represented on the cell culture H_2O_2 array compared to 82 % on the array described in this thesis.

The TGA1/AS1 motif (TGACG [the binding site for bZIP TGA factors]) was identified as significantly over-represented in the H_2O_2-regulated gene lists from both the H_2O_2 cell culture microarray experiment performed by Desikan and colleagues, and that of Results Chapter 3. This observation highlights the possibility of this motif as a potential binding site for redox-sensitive transcription factors. The TGA1/AS1 motif shares high homology to the redox-sensitive mammalian AP-1 *cis*-element, and is present in many *GSTs* and SA- and auxin-inducible genes (Qin *et al.*, 1994; Karin *et al.*, 1997; Xiang *et al.*, 1997).

7.3 Assigning a biological role

Three genes encoding candidate signalling proteins were identified and selected for further study (*APK*, *ERF5* and *ERF6*). Expression profiling, revealed that all three were responsive to a wide range of stress treatments (including UV-B, cold and response to pathogens [Results Chapter 5]). *ERF5* was most responsive to the lowest H_2O_2 concentration tested and reacted rapidly (within 1 h) to many stress stimuli. Therefore (assuming that transcript levels were representative of the corresponding protein and the three proteins have equal activity), this suggests a potential role of ERF5 as a sensitive responder to ROS. Thus ERF5 may be involved in response to lower H_2O_2 levels (e.g. for ROS signalling) whilst ERF6 and APK respond to higher ROS levels (e.g. during oxidative stress).

The accumulation of mRNA for all three genes following cycloheximide (CHX) treatment, an agent which inhibits protein translation, indicates that *de novo* protein synthesis may not be required for their expression, as is often the case with primary rapid response genes. Since CHX treatment alone was examined, further experiments with CHX treatment in conjunction with stress treatments are necessary. However, these genes may be responding to a general proteotoxic stress exerted by CHX, and it is tempting to speculate that their induction following other stresses may not require *de novo* protein synthesis.

7.4 Loss and gain-of-function studies

Loss- and gain-of-function lines were identified in order to investigate if these three genes were necessary and/or sufficient to confer a particular phenotype (Results Chapter 4). However, under the conditions tested, no abnormal development or enhanced sensitivity/tolerance to stress/hormone treatments was observed.

Functional redundancy may explain the lack of observed phenotype in the *erf5* and *erf6* mutants, since the Arabidopsis ERF family contains 65 closely related members, many of which are regulated by the same stimuli and potentially bind the same promoter elements (Nakano *et al.*, 2006). However, some loss-of-function phenotypes have been described for *aterf* mutants. For example, T-DNA insertion mutants of the transcriptional activator *AtERF14* displayed increased susceptibility to infection by the necrotrophic pathogen *Fusarium oxysporum* and impaired induction of defence gene expression (*PDF1.2* and *ChiB*) whilst mutant plants of the transcriptional repressor *ERF4* exhibited increased resistance to *F. oxysporum* and increased *PDF1.2* levels (McGrath *et al.*, 2005; Onate-Sanchez *et al.*, 2007). Additionally, RNAi lines of another repressor, *AtERF7*, were more sensitive to ABA as demonstrated by the enhanced stomatal closure in these lines (Song *et al.*, 2005).

Although work within this thesis did not detect any abnormal phenotypes for the over-expression lines tested, reports of phenotypes with *AtERF* over-expressors are relatively common (as summarised in Table 5.1 of Results Chapter 5 [page 128]). For example, over-expression of *AtERF14* had dramatic effects on plant development (growth retardation and loss of seed set) as well as increased expression of *PDF1.2* and *ChiB* genes. Over-expression of the transcriptional activators *ERF1* and *ERF2* genes increased resistance to

necrotrophic pathogens (*F. oxysporum*) and up-regulated defence gene transcript levels (*PDF1.2* and *CHIB*) (Berrocal-Lobo *et al.*, 2002; McGrath *et al.*, 2005). Additionally, transgenic lines over-expressing *ERF4* were more susceptible to *F. oxysporum* infection and over-expressors of the transcriptional repressor *AtERF7* show reduced stomatal closure following ABA treatments and increased transpirational water loss (McGrath *et al.*, 2005; Song *et al.*, 2005).

7.5 Determining a molecular phenotype

Since no altered phenotype was observed, microarray analyses were performed on the *APK*, *ERF5* and *ERF6* over-expression lines in order to reveal gene regulation to gain clues as to the biological role of these genes. However, genes that were differentially regulated in the *APK* over-expression lines gave no indication of its function. In contrast, *ERF5* and *ERF6* over-expression induced the expression of pathogenesis genes (six *PDFs* and two *PR* genes). One Arabidopsis microarray experiment performed on an *ERF* over-expressor has been reported in the literature. The Affymetrix system was used to monitor transcript levels of 8000 genes in 4-week old 35*S* lines of the of the transcription activator *AtERF1* (Lorenzo *et al.*, 2003). In agreement with the findings in this thesis, those genes differentially regulated in the *AtERF1* over-expression lines were mainly defence and oxidative stress related. However, the specific types of genes induced differed from those up-regulated in the *ERF5* or *ERF6* over-expression arrays: *ERF1* over-expression resulted in induction of chitinase and endochitin genes (7), β-glucosidase/glucanase (3), *PR1*-related (4), glutathione-*S*-transferases (4) and peroxidases (2). Specific genes up-regulated by *ERF1* over-expression included *WRKY6*, *WRKY53* and *AtRbohD*, No *PDF* genes were up-regulated in the 35*S* *ERF1* lines. The over-expression of *ERF1* gives a disease resistance phenotype to necrotrophic pathogens (Berrocal-Lobo *et al.*, 2002).

ERFs are known to bind to a short *cis*-acting element known as the GCC box (AGCCGCC in the promoters of JA-/ethylene-inducible pathogenesis related genes (e.g. *PDF 1.2*), and either induce or repress the expression of these genes (Ohme-Takagi and Shinshi, 1995; Fujimoto *et al.*, 2000; Ohata *et al.*, 2000). For example (as previously mentioned) AtERF1 and AtERF2 are able to function as GCC-box specific trans-activators in Arabidopsis, whilst AtERF3 and AtERF4 transcriptional repressors (Fujimoto *et al.*, 2000). The promoter motif

analysis presented in this thesis demonstrates that ERF5 and ERF6 are both activators of endogenous genes that have the GCC box in their promoter region (e.g. the *PDFs*; Results Chapter 6). The GCC box element was more significantly over-represented in genes differentially regulated by *ERF6* over-expression compared to those of *ERF5*. It is unlikely that this is due to higher levels of ERF6 protein being produced, as *ERF6* was on average 2-fold over-expressed compared to 18-fold over-expression of *ERF5*. Since ERFs appear to bind the same target GCC box sequence, one point of regulation of transactivation (or repression) may lie in DNA binding affinity, which may allow for a degree of target specificity. ERF6 may therefore be more "potent" compared to ERF5, i.e. a "stronger" transcriptional activator. The observation that over-expression lines with more than 2-fold expression of *ERF6* were not recovered adds weight to this potency theory. It is possible that ERF6 may require a more profound stress treatment in order for it to be transcriptionally induced (as compared to *ERF5* which is rapidly responsive to low levels of various stress treatments). Interestingly, the GCC box was not over-represented on the H_2O_2 regulated microarray gene lists, which suggests its regulation is ROS-independent.

A few promoter elements were common to the ERF(s) and H_2O_2 regulated genes, such as the W-box for ERF5 and H_2O_2. It is unlikely that this observation is a result of the direct binding of ERFs to this promoter, as the W-box is very different from the GCC box. Furthermore, the transcription factors which bind the W-box are WRKYs, and unlike the H_2O_2 microarray, these did not appear up-regulated by *ERF5* over-expression. Although the lack of induction of WRKYs on the *ERF5* microarrays may be due to the time point sampled, the most likely explanation is that the over-expression of *ERF5* does not specifically induce the expression of genes containing W-box, but that they are "guilty by association", as perhaps a significant subset of genes containing the GCC box also possess the W-box. Therefore the W-box may appear enriched. Future work could verify if this is the case.

7.6 Future work

A number of experiments that could be carried out to place the results presented in this thesis into a broader context have already been suggested in the relevant Discussion sections within each Results Chapter. This section will not repeat the suggestions made, but will propose further general experiments.

7.6.1 Coupling transcript levels with protein levels

The body of work described herein has focused on gene expression. However, the level of encoded protein may not necessarily correspond to that of the transcript. Therefore, an important extension is to determine protein abundance by Western blot analysis before any firm conclusions can be drawn. Western blot analysis of T-DNA insertion and over-expression lines should be performed in order to gauge ERF/APK levels. Additionally, the levels of these proteins in wild-type plants following ROS and stress treatments should be determined, as well as confirming that *ERF* over-expression enhanced the pathogen defence protein levels.

7.6.2 Pathogenesis studies

As already mentioned, various members of the *ERF* gene family have previously been shown to be involved in plant defence (summarised in Table 5.1 of Results Chapter 5 [page 128]). This thesis only examined response to *P. syringae* challenge (and no altered phenotype was observed). The *ERF5* and *ERF6* over-expression lines should be tested for enhanced tolerance against a wide range of pathogens, particularly necrotrophic fungal pathogens (e.g. *Fusarium oxysporum* and *Botrytis cinerea*).

7.6.3 Dissecting the signalling pathways

Analysis of proteins interacting with APK, ERF5 and ERF6 could reveal further signalling components of the H_2O_2 transduction chain and place APK, ERF5 and ERF6 into a wider signalling context. For example, a yeast 2-hybrid screen using these proteins as bait could be used to detect interacting proteins. Additionally, immunoprecipitation of these three proteins under native conditions followed by electrophoreses under denaturing conditions could also

be used to detect proteins associated with them, which could subsequently be identified via mass spectrometry.

The initial observation that H_2O_2 induced the expression of *ERFs*, points to a possible role for ROS to work in conjunction with ethylene in a variety of different processes. Ethylene is a fundamental plant hormone involved in a vast range of processes, such as root and shoot growth, fruit ripening and PCD. This raises the possibility of ROS involvement in such numerous ethylene-mediated responses and consequent cross-talk between these signalling pathways. The ethylene receptor ETR1 can function as a ROS sensor, mediating stomatal closure in response to H_2O_2 (Desikan *et* al.,2005) and thus this protein may constitute a node mediating cross-talk between ethylene and H_2O_2. Further work could address whether H_2O_2-triggered *ERF5* and *ERF6* gene induction is independent or dependent of ethylene signalling, for example by examining their transcriptional activation in ethylene signalling mutant backgrounds (e.g. *ein2* or *etr1*).

Hormones are intimately linked with the regulation of defence signalling pathways in pathogen-challenged plants. It has been proposed that the SA pathways primarily regulate resistance to biotrophic pathogens, as mutants defective in SA biosynthesis or signalling show increased susceptibility to pathogens such as *Pseudomonas syringae*, *Peronospora parasitica*, and *Erysiphe cichoracearum* (Thomma *et al.*, 1998). Conversely JA and ethylene signalling have been proposed to be more effective against necrotrophic pathogens such as *Fusarium oxysporum*, *Botrytis cinerea*, *Pythium irregulare* and *Plectosphaerella cucumerina*. For example, plants that over-express transcription factors involved in the ethylene and JA pathways show an increased resistance to several necrotrophs (Berrocal-Lobo *et al.*, 2002), whilst Arabidopsis mutants that are unable to activate JA-dependent defence gene expression showed compromised resistance to necrotrophic fungal pathogens (Staswick *et al.*, 1998; Thomma *et al.*, 1998). The observation of increased expression of *PDF* genes in the *ERF5* and *ERF6* over-expressing lines, suggests the JA/ethylene signalling pathways are likely involved, as JA and ethylene act together to activate gene expression of defence-related genes such as *PDF1.2* and *ChiB*, while SA is primarily involved in the *PR* gene expression pathway. Therefore, to determine if these proteins work in hormone signalling pathways (and if so whether they are positioned upstream or downstream relative to each other) crossing experiments could be conducted between over-expression lines and hormone insensitive mutants (e.g. *coi1*, *jar1*, *etr1*, *ein2* and/or *nahG*).

7.6.4 Microarray analysis

Additional microarray analyses may also be performed to further characterise the molecular phenotype of these genes and help to identify their biological role. For example, examination of the T-DNA insertion and over-expression lines in response to stress treatments (e.g. following pathogen challenge/elicitation) may reveal the roles the genes play *in vivo*.

7.6.5 Multiple stressors

The identification of genes and proteins regulated by ROS is an important step towards the development of treatments that might confer tolerance to multiple stresses. In the field crops and other plants are routinely subjected to a combination of different stresses, however, the co-occurrence of different stresses has rarely been addressed in the literature. In drought stricken areas for example many crops encounter a combination of drought and other stresses such as heat or salinity. The molecular and metabolic response of plants to a combination of drought and heat is unique and cannot be directly extrapolated from the response of plants to each of these different stresses applied individually (Rizhsky *et al.*, 2004), thus future work should also include the study of stresses in combination.

7.7 The benefit of hindsight

On reviewing the body of work presented herein, and with the benefit of hindsight, the research may have proved more fruitful by several alterations in the approach utilised. Firstly, an initial H_2O_2 microarray performed on the wild-type plants of the Columbia ecotype using an array chip representing a larger number of genes would have provided a more solid basis for candidate gene selection. Secondly, combining transcriptomic data with proteomic data would have ensured that any selected proteins were responsive to ROS/stress treatments and increased confidence in the candidate signalling components short-listed. Thirdly, a modification of the criteria used for candidate gene selection, to strictly exclude genes that are part of large gene families or with close relatives may have reduced the possibility of functional redundancy. Finally, it would have been more beneficial to perform microarray analyses first, prior to the functional characterisation, in order to direct and focus the types of experimental screens performed.

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Appendix A: Solution Recipes

A.1 Nucleic acid extraction buffers

A.1.1 Edwards' extraction buffer

25 mM EDTA
250 mM NaCl
0.5 % (w/v) SDS
200 mM Tris-HCl, pH 7.5

A.1.2 TE buffer

10 mM EDTA
25 mM Tris-HCl, pH 8.0

A.1.3 STET buffer

50 mM EDTA
8 % (w/v) Sucrose
50 mM Tris-HCl, pH 8.0
5 % (w/v) Triton X-100

A.2 Agarose gel electrophoresis of DNA

A.2.1 0.5 x TBE buffer

1.0 mM EDTA
45mM Tris-boric acid, pH 8.0

A.2.2 DNA sample loading buffer

0.25 % (w/v) Bromophenol blue
40 % (w/v) Sucrose
0.25 % (w/v) Xylene cyanole FF

A.3 Formaldehyde agarose gel electrophoresis of RNA

A.3.1 1 x MOPS buffer

1 mM EDTA, pH 7.0
20 mM MOPS
5 mM Na acetate

A.3.2 RNA sample loading buffer

200 µg/ml Bromophenol blue
50 µg/ml Ethidium bromide
1.14 M Formaldehyde
62.5 % (v/v) Formamide (deionised)
1.25 x MOPS buffer
200 µg/ml Xylene cyanole FF

A.4 cDNA synthesis

A.4.1 5 x first strand buffer

250 mM Tris-HCl, pH 8.3
375 mM KCl
15 mM MgCl2

A.4.2 10 mM RNase-free dNTPs

2.5 mM dATP
2.5 mM dCTP
2.5 mM dGTP
2.5 mM dTTP

A.5 DNA sequencing

A.5.1 2.5 x sequencing buffer

200 mM Tris-HCl, pH 9.0
5 mM MgCl2

A.6 Northern blot analysis

A.6.1 20 x SSC

0.3 M Na citrate, pH 7.0
3 M NaCl

A.6.2 Pre-hybridisation solution

0.5 % (w/v) SDS, 5 x Denhardt's reagent (A4.3)
50 % (v/v) Formamide
5 x SSC
100 µg/ml salmon sperm DNA (Sigma-Aldrich) denatured

A.6.3 5 x Denhardt's reagent

0.1 % Ficoll 400
0.1 % (w/v) polyvinylpyrrolidone (PVP)
0.1 % bovine serum albumin (BSA) (fraction V)

A.6.4 Wash solution 1

2 x SSC
0.1 % (w/v) SDS

A.6.5 Wash solution 2

1 x SSC
0.1 % SDS

A.6.6 Wash solution 3

x SSC
% (w/v) SDS

A.6.7 Strip solution

50 % (v/v) formamide
50 mM Tris-HCl pH 8.0
1 % (w/v) SDS

Appendix B: Primers

All primers used are listed with their direction relative to the direction of the amplified sequence, sequences and annealing temperatures.

All primers listed in Table 2.1 are specific for regions within their respective cDNAs or genomic DNA. All hybridisation probes for Northern analysis were amplified from cDNA synthesised from seedlings treated for 1 h with 10 mM H_2O_2. Direct sequencing was used to verify the identity of the PCR product before use in Northern analysis.

Primer names, sequences and annealing temperatures for primers used are listed. The primer direction relative to the direction of the amplified sequence is indicated (forward or reverse). The exact name under which the primer was ordered from MWG-Biotech AG, Ebersberg, Germany, is also given.

B.1 Primers for sequencing

Table B.1
Primers for sequencing

Primer	Direction	Sequence (5' to 3')	Annealing temp (°C)	Comments / labelled as
Standard sequencing primers:				
T3	For	AATTAACCCTCACTAAAGGG	53.2	For sequencing from pBluescript II SK (+)
T7	Rev	TAATACGACTCACTATAGGG	53.2	For sequencing from pBluescript II SK (+)
M13	For Rev	TGTAAAACGACGGCCAGT CAGGAAACAGCTATGACC	53.7 53.7	For sequencing from pENTR/D-TOPO
SP6		CATTTAGGTGACACTATAG	50.2	For sequencing from pCMV sport 6
pUni51_seq_R T35S_R	Rev Rev	TGGCAACTAGAAGGCAC CTACTCACACATTATTCTGG	52.8 53.2	For sequencing from pUni51
Gene-specific sequencing primers:				
Kinases:				
APK (At4g18950)	For	GATTCTTGAGATACATGGAG	53.2	APK_Internal_F
At5g25930	For For For For	CGGCTAAGATTCCGATAGAG CCTTCTCGGATCTGGAATGC GTGTTGTATCTCAAGGGAAG CGCGGTTCTGCTTCTCACC	57.3 59.4 55.3 61.0	RPK1_Internal_1F RPK1_Internal_2F RPK1_Internal_3F RPK1_Internal_4F
At3g2200	For	GTCCGAACAACAAGGCGGG	61.0	RPK2_Internal_F
Transcription factors:				
ERF5 (At5g47230)	For	CCGTGGGGGAAATTCGCGGC	65.5	ERF5_Internal_F
At3g18290	For	CGTGGCTTCGTGTCGCATTG	61.4	ZnF_Internal_1F
	For	GGCCAGTTGCTACAATATTC	55.3	ZnF_Internal_2F
	For	GGCTCTTGCGTTTGCTACTGG	59.8	ZnF_Internal_3F
	For	CCGCATGAACCAGAATGAAC	57.3	ZnF_Internal_4F
	Rev	GGAAGATGCACACTTCAGC	56.7	ZnF_Internal_1R

B.2 Probe primers for northern blot analysis

Table B.2
Probe primers for northern blot analysis

Primer	Direction	Sequence (5' to 3')	Annealing temp (°C)	Labelled as
Kinases:				
APK (At4g18950)	For	CAGCCGAGGTTTACGATTGG	59.4	At4g18950_F (27.05.04)
	Rev	GCTTCCCCAACGATCTTTAG	57.3	At4g18950_R (27.05.04)
At5g25930	For	CGTTCTTTGTGGTTAGGGAC	57.3	pRPK_F_101003
	Rev	CGGGAGTACAATCATGATGC	57.3	pRPK_R_101003
At3g22060	For	GCATCGTTTGGTTCCCTATC	57.3	At3g22060_F (27.05.04)
	Rev	TGGCAGTCTCAAGACAAGAG	57.3	At3g22060_R (27.05.04)
At4g23190	For	CACAAACATGCACGACGGAC	59.4	pPK2_F_170304
	Rev	GGGTTGTCCTGATCACCTGC	61.4	pPK2_R_110204
At1g09970	For	GGGTATGAGAGACCGGTGATTCACC	66.3	At1g09970_F
	Rev	CATCAATCTGCAAGGTTCCGCGTCC	66.3	At1g09970_R
At2g39660	For	CCAAACCCAAAACTCCCTTC	57.3	At2g39660_F
	Rev	GGTCGAACTCAATATCTCCC	57.3	At2g39660_R
Phosphatases:				
At2g30020	For	CGTCGCCGTATGTAATTCTC	57.3	At2g30020_F
	Rev	CTGGAAACGGAGACGAAATG	57.3	At2g30020_R
At4g31860	For	GAACTAAAACCAGAAGCAAG	53.2	At4g31860_F
	Rev	CTAAGTGAAGCTTTATGTCC	53.2	At4g31860_R
At1g08420	For	GTCTATGGTGGCTGACAACG	59.4	pPhos_F_110204
	Rev	GCTGCTCTTGTCCAACAACC	59.4	pPhos_R_110204
At2g33700	For	GCAAATGCTGGTGATTGCCG	59.4	pPPC2_F_110204
	Rev	CGTCTTCACTCAGGTCTGTC	59.4	pPP2C_R_110204
Transcription factors:				
ERF5 (At5g47230)	For	CATCGAGAAACATCTACTCG	55.3	ERF5_F_200204
	Rev	GTTTAGTAACTTCCGGTTTG	53.2	ERF5_R_200204
ERF6 (At4g17490)	For	GTCTCCGTTGCCTACTACTG	59.4	ERF6_F_200204
	Rev	CGATTGGATTGAACAGTAAC	53.2	ERF6_R_200204
At3g18290	For	TGCACAACACCTTCATGCGACGGAT	64.6	ZnF(Affy)_F_200204
	Rev	ACCCGGGTATTGTAAGAACCACAGC	64.6	ZnF(Affy)_R_200204
	For	CGGGAATGGTTTATGCCCTG	59.4	pZnF_F_101003
	Rev	CGAACAGTGTCAGTTTGAGC	57.3	pZn_F_R_101003
At1g32240	For	CCAATTTCCGACACAGCAGC	59.4	pMybTF_F_101003
	Rev	GGCCACCGAGTAATTCAACG	59.4	pMybTF_R_101003

B.3 Primers for cloning coding regions into Gateway entry vector (pENTR/D-TOPO)

Table B.3
Primers for cloning coding regions into the Gateway entry vector pENTR/D-TOPO

Primer	Direction	Sequence (5' to 3')	Annealing temp (°C)	Labelled as
Kinase:				
APK (At4g18950)	For	CACCATAACAATGGAAGAGGATT-ATCAACA	62.7	At4g18950_F (03.07.04)
	Rev	TCACAAATGTGAACCGGATG	55.3	At4g18950_R (03.07.04)
Transcription factors:				
ERF5 (At5g47230)	For	CACCATAACAATGGCGACTCCTA-ACGAAGT	66.8	At5g47230_F
	Rev	TCAAACAACGGTCAACGTGG	57.3	At5g47230_R
ERF6 (At4g17490)	For	CACCATAACAATGGCTACACCAA-ACGAAGT	65.4	At4g17490_F
	Rev	TCAAACAACGGTCAATTGTG	53.2	At4g17490_R

B.4 Primers for screening loss-of-function lines for T-DNA insertions

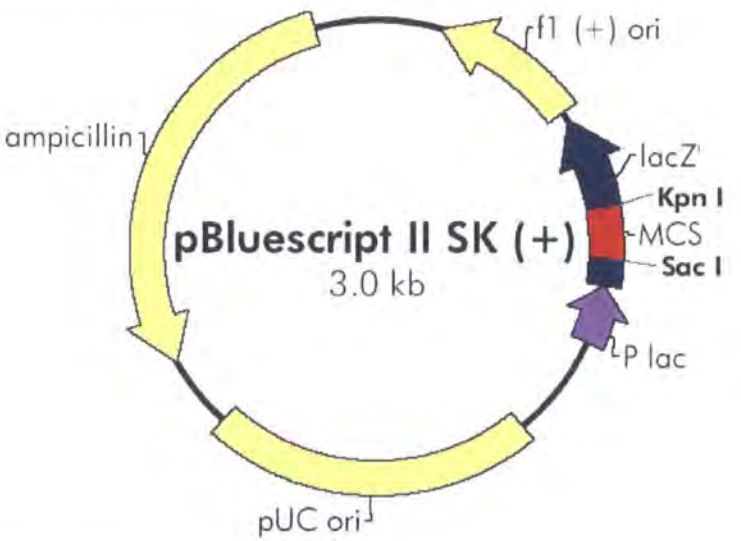
Table B.4
Primers for screening loss-of-function lines for T-DNA insertions

Primer	Direction	Sequence (5' to 3')	Annealing temp (°C)	Label
T-DNA left border primers:				
SALK_LBA	For	TGGTTCACGTAGTGGGCCATCG	55.3	SALK_LBA
GABI-Kat LB	For	ATATTGACCATCATACTCATTGC	64.0	GK_8409
Kinases:				
APK (At4g18950)	For	CTACTGCTGTTAGATATGCC	55.3	APK_S_050024/32_F
	Rev	CTTCACTCGTGCAAACCTCG	55.3	APK_S_050024/32_R
		GTAACCCAATGATGATTGTGACTG	59.3	APK_GK_626D02_GSp
At5g25930	For	GTTATAAGCCCTCGTCATGG	57.3	RPK1_S_091274_F
	Rev	CGTGCAATTGTAAAGAACCG	55.3	RPK1_S_091274_R
	For	CGTCAAAGGTAGATGAGAAG	55.3	RPK1_GK_751D04_F
	Rev	CTTCGACCCGATAAACTAAC	55.3	RPK1_GK_751D04_R
	Rev	CGCTGCGTTCTTCATAACCG	59.4	RPK1_GK_751D04_R2
		AAACCAAGAACCTCATACCATGTC	59.3	RPK1_GK_751D04_GSp
At3g2200	Rev	CTGCGAGTTGAATGTTGATG	55.3	RPK2_S_151902_R
Phosphatases:				
At2g30020	For	CATGGAGGAGTTAAAGCGGC	59.4	PP2CA-S-065126_F
	Rev	GACGAGCCTCGTGAAGCAG	61.0	PP2CA-S-065126_R
	For	CAGGGATCTTTAGCTGTGTC	57.3	PP2CA_GK_109H08_F
	Rev	GTCGATTTCTTTCACGTTGC	55.3	PP2CA_GK_109H08_R
		CGTGTATTCTAAACGTTGTTTTGA	57.6	PP2CA_GK_109H08_GSp
Transcription factors:				
ERF5 (At5g47230)	For	GAGAAGAAGCATTACAGAGG	55.3	ERF5_GK_681E07_F
	Rev	CAACTGGGAATAACCAAACG	55.3	ERF5_GK_681E07_R
	Rev	GAACAACTTCACATAACGCC	55.3	ERF5_GK_681E07_R2
		GGAATTTCTATCGATTCCATTTGA	55.9	ERF5_GK_681E07_GSp
ERF6 (At4g17490)	For	CGACAAAGAAGCGTTTAGAC	55.3	ERF6_S_087356/7_F
	Rev	GTGTTATGTGTTCTCTGTTC	53.2	ERF6_S_087356/7_R
	For	CCAAAGAAACCCAATATTAG	51.2	ERF6_GK_080F09_F
	For	CGATTCAGATGGAATTTCAGATTT	55.9	ERF6_GK_080F09_F2
		CCAGCTGCCATTGATGAACC	59.4	ERF6_GK_080F09_GSp
At3g18290	For	CACGTGCTACAAAACCTATG	55.3	ZnF_S_036012_F
	Rev	GTTGAAGAAGACGCCACTGC	59.4	ZnF_S_036012_F

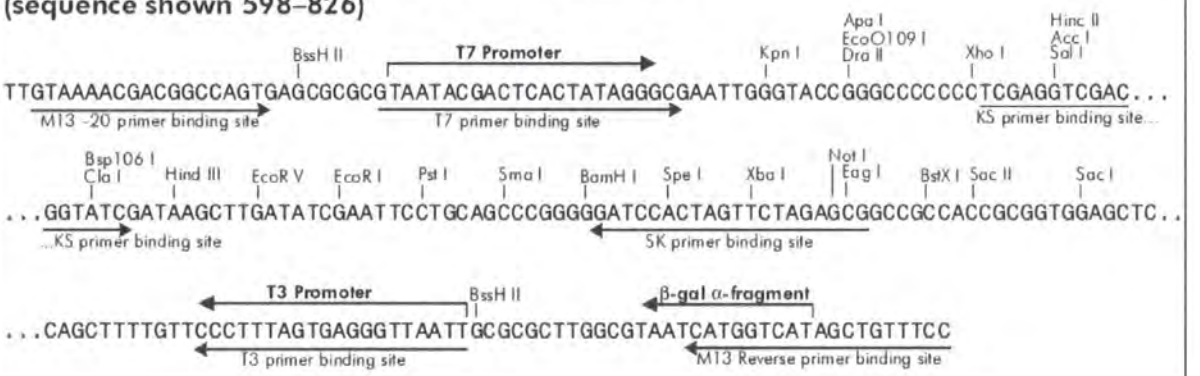
Appendix C: Plasmid Vectors

C.1 pBluescript SK +

Figure C.1
pBluescript SK+ vector map as shown at *http://www.stratagene.com*

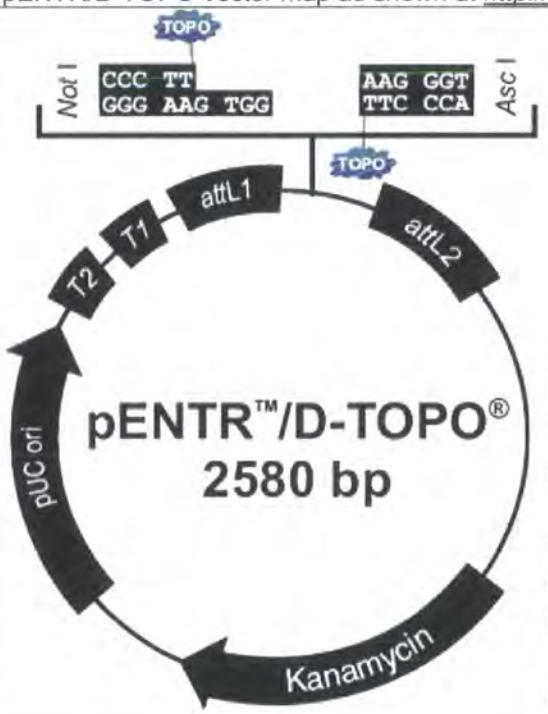


pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)



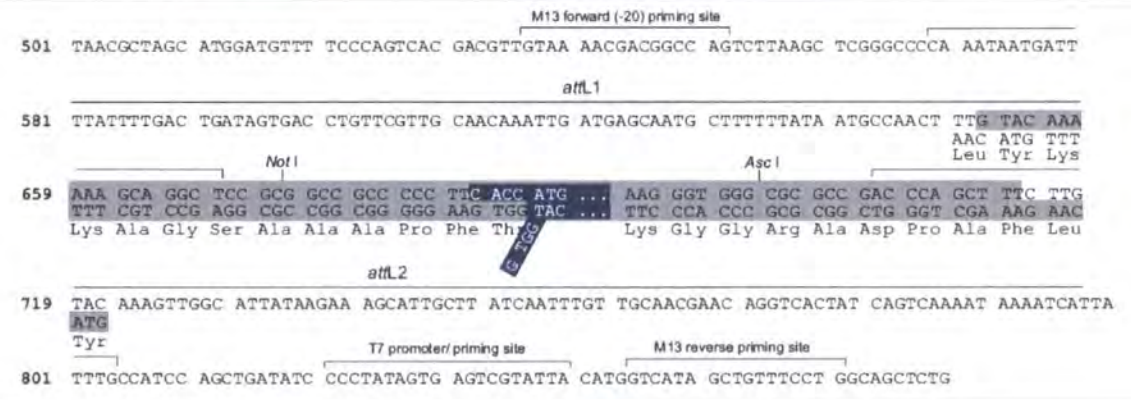
C.2 pENTR/D-TOPO

Figure C.2
pENTR/D-TOPO vector map as shown at http://www.invitrogen.com



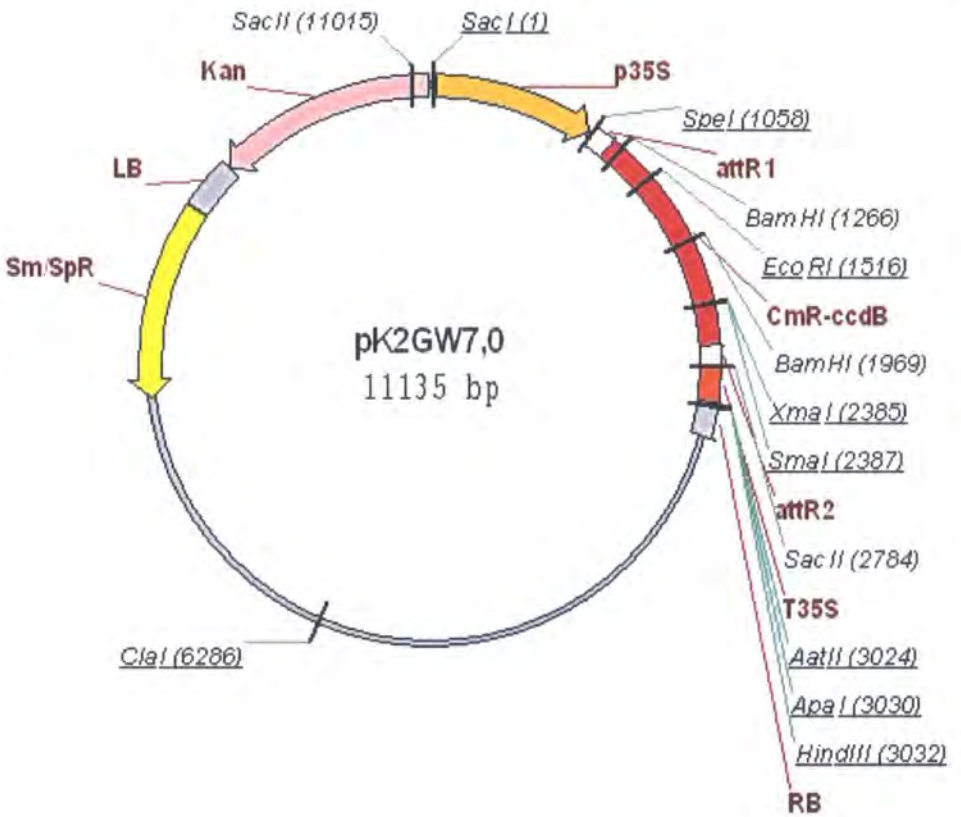
*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*attL*1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*attL*2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence



C.3 pK2GW7

Figure C.3
pK2GW7 vector map as shown at the makers' website (Karimi *et al.*, 2002)
http://www.psb.ugent.be/gateway/index.php?NAME=pK2GW7&_app=vector&_act=construct_show&



Appendix D:

Transcriptomic Changes in Response to H_2O_2 Treatment

D.1 Transcript induction by H_2O_2 treatment

Only transcripts with at least a 2-fold induction and present only calls in both slides are shown. Annotations are according to the TAIR version 6 genome release (2007). Those shown in bold were selected as candidate genes for northern blot analysis (Results Chapter 3.2.4). For full details of the microarray experiment please refer to Results Chapter 3.2.1

AGI code	Probe set	Gene annotation	10 mM H_2O_2	Control	Fold change
Kinases:					
At5g14470	250182_at	GHMP kinase-related	942.69	91.61	10.29
At4g23190	**254241_at**	**putative receptor-like protein kinase**	**186.00**	**38.80**	**4.79**
At5g25930	**246858_at**	**protein kinase family protein**	**457.90**	**104.72**	**4.37**
At1g02970	262106_at	protein kinase	226.08	53.92	4.19
At4g28350	253819_at	lectin protein kinase family protein	87.19	26.82	3.25
At1g70530	260362_at	protein kinase family protein	1083.70	396.36	2.73
At4g18950	**254605_at**	**putative ankyrin protein kinase**	**1028.54**	**383.18**	**2.68**
At3g46930	252470_at	protein kinase family protein	261.31	102.77	2.54
At1g09970	**264663_at**	**putative leucine-rich repeat transmembrane protein kinase**	**1095.01**	**433.27**	**2.53**
At1g73500	245731_at	mitogen-activated protein kinase kinase (MKK9)	692.09	286.58	2.41
At2g39660	**267624_at**	**Botrytis-induced kinase 1 (BIK1)**	**1217.98**	**507.72**	**2.40**
At5g24100	249768_at	putative leucine-rich repeat transmembrane protein kinase	174.19	73.57	2.37
At5g61560	247532_at	protein kinase family protein	185.03	80.57	2.30
At5g58350	247819_at	WNK protein kinase	1439.20	635.86	2.26
At2g40500	255875_s_at	protein kinase family protein	47.54	21.06	2.26
At1g30270	245775_at	CBL-interacting protein kinase 23 (CIPK23)	318.77	145.30	2.19
At5g53450	248270_at	protein kinase family protein	1305.06	611.01	2.14
At3g45420	252569_at	lectin protein kinase family protein	117.57	57.27	2.05
At2g03890	263333_at	phosphatidylinositol 3- and 4-kinase family protein	534.96	261.23	2.05
At5g35980	249678_at	protein kinase family protein	300.80	147.35	2.04
At3g08720	258682_at	serine/threonine protein kinase	128.55	63.51	2.02
At4g23260	254247_at	protein kinase family protein	79.00	39.44	2.00
Phosphatases:					
At3g02800	257536_at	tyrosine specific protein phosphatase family protein	166.10	30.47	5.45
At4g31860	**253453_at**	**putative protein phosphatase 2C**	**810.17**	**249.01**	**3.25**

At1g05000	265214_at	tyrosine specific protein phosphatase family protein	641.01	205.85	3.11
At4g32950	253408_at	putative protein phosphatase 2C	1590.88	534.46	2.98
At4g23570	254211_at	phosphatase-related (SGT1A)	553.74	187.47	2.95
At2g33700	**267448_at**	**putative protein phosphatase 2C**	**284.33**	**98.94**	**2.87**
At1g08420	**261743_s_at**	**serine/threonine phosphoesterase family protein**	**114.15**	**40.75**	**2.80**
At2g30020	**266834_s_at**	**putative protein phosphatase 2C**	**336.84**	**138.70**	**2.43**
Transcription factors:					
At5g05410	250781_at	DRE-binding protein (DREB2A)	1183.87	89.80	13.18
At3g24500	258133_at	transcriptional coactivator, multiprotein bridging factor 1 (MBF1C)	1611.01	140.88	11.44
At5g63790	247351_at	no apical meristem (NAM) family protein	4454.07	473.21	9.41
At1g77450	259705_at	no apical meristem (NAM) family protein	715.73	80.63	8.88
At1g27730	261648_at	salt-tolerance zinc finger protein (STZ) / zinc finger (C2H2 type) family protein (ZAT10)	1813.88	221.16	8.20
At3g56400	251705_at	WRKY family transcription factor (WRKY70)	584.49	82.94	7.05
At5g04340	245711_at	zinc finger (C2H2 type) family protein	1241.87	180.53	6.88
At4g17490	**245250_at**	**ethylene responsive element binding factor 6 (AtERF6)**	**795.90**	**125.87**	**6.32**
At1g01720	261564_at	no apical meristem (NAM) family protein (ATAF1)	712.64	122.78	5.80
At2g38470	267028_at	WRKY family transcription factor (WRKY33)	1664.48	297.90	5.59
At1g62300	264746_at	WRKY family transcription factor (WRKY6)	642.35	116.19	5.53
At5g59820	247655_at	ZAT12	1096.06	199.85	5.48
At1g80840	261892_at	WRKY family transcription factor (WRKY40)	1291.44	266.51	4.85
At2g24500	265662_at	zinc finger (C2H2 type) family protein (FZF)	1540.25	332.21	4.64
At2g40140	263379_at	zinc finger (CCCH-type) family protein	851.79	190.85	4.46
At3g55980	251745_at	zinc finger (CCCH-type) family protein	856.06	213.75	4.01
At1g32240	**245758_at**	**KANADI family of putative transcription factors (KAN2)**	**173.75**	**45.93**	**3.78**
At1g10170	264460_at	NF-X1 type zinc finger family protein	606.77	183.52	3.31
At1g22985	264726_at	ERF (ethylene response factor) subfamily B-5 of ERF/AP2 transcription factor family	438.88	133.13	3.30
At3g10500	258921_at	no apical meristem (NAM) family protein	648.10	200.32	3.24
At5g58340	247803_at	similar to myb family transcription factor	84.05	27.65	3.04
At2g46830	266719_at	myb-related transcription factor (CCA1)	355.55	119.73	2.97
At4g17500	245252_at	ethylene responsive element binding protein 1 (ERF1)	998.24	337.77	2.96
At5g37260	249606_at	myb family transcription factor	220.28	80.42	2.74
At4g18880	254592_at	heat shock transcription factor 21 (HSF21)	320.97	117.83	2.72
At3g18290	**257062_at**	**zinc finger protein-related / embryo defective 2454 (EMB2454)**	**1004.57**	**378.02**	**2.66**
At2g27580	266261_at	zinc finger (AN1-like) family protein	997.13	377.58	2.64

At2g20180	265584_at	basic helix-loop-helix (bHLH) family of transcription factors / PHY-INTERACTING FACTOR 1 (PIF1)	662.34	252.23	2.63
At4g31800	253485_at	WRKY family transcription factor (WRKY18)	403.75	154.88	2.61
At1g77920	262137_at	bZIP family transcription factor	333.15	128.11	2.60
At2g40350	263823_s_at	DREB subfamily A-2 of ERF/AP2 transcription factor family	422.27	166.40	2.54
At1g18570	255753_at	myb family transcription factor (MYB51)	248.19	98.93	2.51
At4g31550	253535_at	WRKY family transcription factor (WRKY11)	801.29	327.35	2.45
At2g23320	245051_at	WRKY family transcription factor (WRKY15)	1931.14	817.64	2.36
At1g53160	261375_at	squamosa promoter-binding protein-like 4 (SPL4)	82.10	34.86	2.36
At5g47230	**248799_at**	**ethylene responsive element binding factor 5 (AtERF5)**	**294.09**	**129.27**	**2.27**
At4g18170	254652_at	WRKY family transcription factor (WRKY28)	250.47	110.36	2.27
At1g31290	262549_at	PAZ domain-containing protein / piwi domain-containing protein	152.36	67.61	2.25
At3g46600	252483_at	scarecrow transcription factor family protein	920.70	419.00	2.20
At4g17230	245247_at	scarecrow-like transcription factor 13 (SCL13)	457.10	208.30	2.19
At5g15850	246523_at	zinc finger protein CONSTANS (CO)	613.92	283.61	2.16
At5g62020	247509_at	heat shock transcription factor B2A (HSFB2A)	660.68	308.05	2.14
At1g69570	259834_at	Dof-type zinc finger domain-containing protein	327.97	154.85	2.12
At1g42990	259626_at	bZIP transcription factor family protein (AtbZIP60)	1128.19	533.30	2.12
At4g24660	254132_at	zinc finger homeobox family protein	26.42	12.58	2.10
At5g58620	247795_at	zinc finger (CCCH-type) family protein	138.56	66.70	2.08
At4g24240	254159_at	WRKY family transcription factor (WRKY7)	216.50	104.22	2.08
At1g01060	261569_at	myb-related putative transcription factor (LHY)	319.99	156.32	2.05
At5g39660	249415_at	Dof-type zinc finger domain-containing protein (CDF2)	251.19	122.78	2.05
At5g46910	248814_at	transcription factor jumonji (jmj) family protein	190.39	93.37	2.04
At5g59450	247707_at	scarecrow-like transcription factor 11 (SCL11)	280.22	139.03	2.02
Calcium:					
At5g49480	248607_at	sodium-inducible calcium-binding protein (ACP1)	3360.07	197.49	17.01
At3g50770	252136_at	putative calmodulin-related protein	1419.90	254.38	5.58
At3g63380	251176_at	calcium-transporting ATPase	572.04	105.36	5.43
At4g34150	253284_at	C2 domain-containing protein	1773.01	358.05	4.95
At1g21550	260881_at	putative calcium-binding protein	216.66	46.38	4.67
At4g33050	253414_at	calmodulin-binding family protein	829.24	178.00	4.66
At1g76650	259879_at	calcium-binding EF hand family protein	551.01	162.11	3.40
At2g41410	266371_at	putative calmodulin	1879.65	583.04	3.22
At5g39670	249417_at	calcium-binding EF hand family protein	243.19	92.95	2.62

At4g27280	253915_at	calcium-binding EF hand family protein	1479.21	588.15	2.51
At5g54490	248164_at	putative calcium-binding EF-hand protein (PBP1)	275.28	113.13	2.43
At2g41090	267076_at	calmodulin-like calcium-binding protein (CaBP-22)	458.99	192.61	2.38
At5g62070	247464_at	calmodulin-binding family protein	266.58	117.07	2.28
At3g59820	251432_at	calcium-binding mitochondrial protein-related	140.14	67.35	2.08
At1g27770	261650_at	calcium-transporting ATPase 1 (ACA1)	451.81	219.80	2.06
Hormones:					
At1g76690	259875_s_at	12-oxophytodienoate reductase (OPR2)	7135.02	287.46	24.82
At4g11280	254926_at	1-aminocyclopropane-1-carboxylate synthase 6 / ACC synthase 6 (ACS6)	731.13	103.94	7.03
At5g62000	247508_at	auxin-responsive factor (ARF2) / transcriptional factor B3 family protein	471.76	84.17	5.60
At5g35735	249719_at	auxin-responsive family protein	1317.37	314.24	4.19
At3g25760	257641_s_at	allene oxide cyclase (AOC1) / early-responsive to dehydration stress protein (ERD12)	1325.32	317.96	4.17
At2g23170	245076_at	IAA-amido synthase (GH3.3)	869.83	297.07	2.93
At2g17500	263073_at	auxin efflux carrier family protein	1434.14	501.52	2.86
At5g13360	250293_s_at	auxin-responsive GH3 family protein	238.23	87.85	2.71
At1g76520	259980_at	auxin efflux carrier family protein	1960.87	807.49	2.43
At1g51780	256178_s_at	IAA-amino acid hydrolase 5 / auxin conjugate hydrolase (ILL5)	427.23	194.43	2.20
At1g02400	259445_at	gibberellin 2-oxidase	628.10	293.83	2.14
At4g16110	245477_at	two-component responsive regulator family protein (ARR2)	337.02	168.38	2.00
At3g07390	259018_at	auxin-responsive protein / auxin-induced protein (AIR12)	137.43	68.85	2.00
Response to stress:					
Heat:					
At5g12030	250351_at	17.7 kDa class II heat shock protein 17.6A (HSP17.6A)	4709.87	32.50	144.90
At2g29500	266294_at	17.6 kDa class I small heat shock protein (HSP17.6B-CI)	4118.98	36.78	111.99
At3g46230	252515_at	17.4 kDa class I heat shock protein (HSP17.4-CI)	3184.35	33.78	94.28
At1g53540	260978_at	17.6 kDa class I small heat shock protein (HSP17.6C-CI) (AA 1-156)	4253.29	54.89	77.48
At3g12580	256245_at	putative heat shock protein 70 / putative HSP70	4918.35	137.24	35.84
At5g52640	248332_at	heat shock protein 81-1 (HSP81-1) / heat shock protein 83 (HSP83)	4149.21	160.30	25.88
At1g16030	261838_at	putative heat shock protein 70 / putative HSP70	5118.29	216.73	23.62
At2g32120	265675_at	heat shock protein 70 family protein / HSP70 family protein	1549.63	138.21	11.21
At3g09440	258979_at	heat shock cognate 70 kDa protein 3 (HSC70-3) (HSP70-3)	3637.26	331.49	10.97
At4g21320	254414_at	heat stress associated 32 (HSA32)	1314.46	230.67	5.70
At5g09590	250502_at	heat shock protein 70 / HSP70	596.83	150.26	3.97

		(HSC70-5)			
At5g03030	250934_at	DNAJ heat shock N-terminal domain-containing protein	1237.87	366.28	3.38
At5g56030	248045_at	heat shock protein 81-2 (HSP81-2)	1585.13	498.83	3.18
At3g23990	256905_at	chaperonin (HSP60)	554.37	186.31	2.98
At2g25140	264402_at	putative heat shock protein 100 / HSP100 / HSP98.7	258.69	87.98	2.94
At5g22060	245686_at	DNAJ heat shock protein, putative, strong similarity to SP:O60884 DnaJ homolog subfamily A member 2 (Dnj3) Homo sapiens, several plant DnaJ proteins from PGR; contains Pfam profiles PF00226 DnaJ domain, PF00684 DnaJ central domain (4 repeats), PF0155	1045.83	368.59	2.84
At1g79920	262054_s_at	putative heat shock protein 70 / HSP70	580.01	214.83	2.70
At5g15450	246554_at	chloroplast-targeted Hsp101 homologue (APG6)	723.21	285.11	2.54
At5g56000	248043_s_at	heat shock protein 81-4 (HSP81-4)	2031.14	1003.78	2.02
Biotic stress:					
At4g10270	255807_at	wound-responsive family protein	140.72	24.11	5.84
At1g72900	262381_at	putative disease resistance protein (TIR-NBS class)	915.51	158.37	5.78
At3g51660	252076_at	macrophage migration inhibitory factor family protein / MIF family protein	2327.06	438.88	5.30
At1g66090	256526_at	putative disease resistance protein (TIR-NBS class)	136.80	30.04	4.55
At1g72930	262374_s_at	Toll/interleukin-1 receptor-like protein (TIR)	359.33	83.63	4.30
At2g38870	266168_at	putative protease inhibitor	1651.92	614.10	2.69
At4g16990	245460_at	putative disease resistance protein (TIR-NBS-LRR class)	262.43	102.02	2.57
At1g11310	262455_at	seven transmembrane MLO family protein / mildew resistance lous O 2, MLO-like protein 2 (MLO2)	1138.72	467.77	2.43
At1g65390	264153_at	putative disease resistance protein (TIR class)	179.30	81.73	2.19
At3g04220	258577_at	putative disease resistance protein (TIR-NBS-LRR class)	86.76	42.46	2.04
At5g46450	248873_at	putative disease resistance protein (TIR-NBS-LRR class)	221.99	109.71	2.02
Other stresses:					
At2g46240	266590_at	Bcl-2-associated athanogene 6 (BAG6)	1282.57	54.58	23.50
At4g12400	254839_at	putative stress-inducible protein, putative	616.38	69.80	8.83
At5g27760	246744_at	hypoxia-responsive family protein	2515.63	490.02	5.13
At2g21620	263517_at	responsive to dessication protein (RD2)	2374.39	995.57	2.38
Protective enzymes:					
At2g29420	266296_at	putative glutathione S-transferase (GST25)	3684.62	236.75	15.56
At1g75270	256453_at	dehydroascorbate reductase (DHAR3)	1969.29	202.70	9.72
At2g29460	266267_at	putative glutathione S-transferase (GST22/GSTF8)	134.17	16.71	8.03
At4g19880	254549_at	glutathione S-transferase-related	3024.51	755.99	4.00

At4g11600	254890_at	glutathione peroxidase (GPX6)	3874.60	1009.19	3.84
At5g03630	250916_at	monodehydroascorbate reductase (MDAR2)	2084.54	544.91	3.83
At2g47730	266461_at	glutathione S-transferase 6 (GST6)	8978.47	2371.76	3.79
At2g29450	266299_at	glutathione S-transferase (GSTU5)	1353.02	374.05	3.62
At1g02930	262119_s_at	putative glutathione S-transferase (GST1/GSTF6)	4997.07	1396.55	3.58
At1g78380	260746_at	putative glutathione S-transferase (GST8)	8700.54	2950.18	2.95
At4g08390	255142_at	L-ascorbate peroxidase, stromal (sAPX)	495.77	181.39	2.73
At1g02940	262103_at	putative glutathione S-transferase (GSTF5)	98.48	44.36	2.22
At1g07890	261412_at	L-ascorbate peroxidase 1, cytosolic (APX1)	6184.06	2979.85	2.08
At1g03850	265067_at	glutaredoxin family protein	1113.49	554.65	2.01
Electron transport:					
At3g28740	256589_at	cytochrome P450 family protein	3341.35	325.65	10.26
At5g57220	247949_at	putative cytochrome P450	443.08	59.27	7.48
At1g64950	266155_at	putative cytochrome P450	93.99	13.04	7.21
At3g44190	252671_at	pyridine nucleotide-disulphide oxidoreductase family protein	1939.48	294.36	6.59
At5g16980	246464_at	putative NADP-dependent oxidoreductase	1027.71	162.46	6.33
At3g22370	258452_at	alternative oxidase 1a, mitochondrial (AOX1A)	1318.37	230.18	5.73
At4g13180	254759_at	short-chain dehydrogenase/reductase (SDR) family protein	1415.73	255.39	5.54
At5g64250	247283_at	2-nitropropane dioxygenase family / NPD family	2304.29	427.10	5.40
At5g25450	246944_at	putative ubiquinol-cytochrome C reductase complex 14 kDa protein	1469.38	275.06	5.34
At4g10040	255011_at	putative cytochrome c	642.20	127.59	5.03
At5g16970	246463_at	2-alkenal reductase	6560.05	1373.27	4.78
At4g05390	255230_at	root-type ferredoxin:NADP(H) oxidoreductase (RFNR1)	358.28	101.23	3.54
At3g04000	258815_at	short-chain dehydrogenase/reductase (SDR) family protein	532.63	154.22	3.45
At3g14660	258114_at	putative cytochrome P450	1117.56	357.67	3.12
At3g03080	258870_at	putative NADP-dependent oxidoreductase	80.66	27.61	2.92
At4g05020	255259_at	NADH dehydrogenase-related	258.73	102.71	2.52
At5g16960	246418_at	putative NADP-dependent oxidoreductase	92.31	38.35	2.41
At3g14690	258094_at	putative cytochrome P450	1907.98	864.24	2.21
At4g38540	252993_at	monooxygenase, putative (MO2)	1050.82	484.27	2.17
At1g65840	262933_at	amine oxidase family protein	305.41	147.90	2.07
At5g20400	246098_at	oxidoreductase, 2OG-Fe(II) oxygenase family protein	463.23	225.90	2.05
At2g34500	266995_at	cytochrome P450 family protein	643.82	317.90	2.03
Photosynthesis:					
psaA Atcg00350	245007_at	psaA protein	4234.38	1110.04	3.81
ndhF Atcg01010	244994_at	chloroplast encoded NADH dehydrogenase unit	316.07	111.26	2.84

psbD Atcg00270	245002_at	photosystem II D2 protein	3733.05	1452.90	2.57
ndhH Atcg01110	244937_at	plastid NAD(P)H dehydrogenase subunit H 49KDa protein	208.56	81.96	2.54
Nucleic acid binding/processing:					
At2g13330	265364_at	gypsy-like retrotransposon family	412.26	28.65	14.39
At2g18820	266943_at	non-LTR retrotransposon family (LINE)	346.16	88.57	3.91
At3g20670	256666_at	putative histone H2A	1084.43	507.73	2.14
At1g20880	262804_at	RNA recognition motif (RRM)-containing protein	549.73	261.67	2.10
At5g63330	247368_at	DNA-binding bromodomain-containing protein	667.48	323.14	2.07
Protein metabolism:					
Translation:					
At1g26910	263686_at	60S ribosomal protein L10 (RPL10B)	1149.89	435.40	2.64
At1g67360	264968_at	rubber elongation factor (REF) family protein	659.34	270.23	2.44
At1g66580	256385_at	60S ribosomal protein L10 (RPL10C)	7099.45	2977.70	2.38
Protein degradation:					
At1g17870	255891_at	S2P-like putative metalloprotease	593.82	108.64	5.47
At1g65350	264181_at	putative polyubiquitin	27.58	10.41	2.65
At5g20620	245989_s_at	polyubiquitin (UBQ4)	1751.87	733.61	2.39
At3g11840	258787_at	U-box domain-containing protein	174.08	73.97	2.35
At3g61710	251286_at	autophagy protein Apg6 family	591.27	273.38	2.16
At3g49340	252309_at	putative cysteine proteinase	65.99	31.14	2.12
At4g17895	254708_at	ubiquitin-specific protease 20, putative (UBP20)	706.63	339.25	2.08
At5g51070	248487_at	ATP-dependent Clp protease ATP-binding subunit (ClpD) / (ERD1)	2435.25	1171.63	2.08
At1g66220	259821_at	subtilase family protein	53.35	26.14	2.04
Protein binding:					
At1g14200	262656_at	zinc finger (C3HC4-type RING finger) family protein	1551.03	271.15	5.72
At3g56710	246293_at	sig1-binding protein (SIB1)	206.78	41.91	4.93
At5g20910	246189_at	zinc finger (C3HC4-type RING finger) family protein	322.26	77.29	4.17
At3g46620	252474_at	zinc finger (C3HC4-type RING finger) family protein	1548.26	424.41	3.65
At3g16720	258436_at	zinc finger (C3HC4-type RING finger) family protein	425.02	150.49	2.82
At1g26800	261265_at	zinc finger (C3HC4-type RING finger) family protein	1124.91	429.96	2.62
At5g47610	248759_at	zinc finger (C3HC4-type RING finger) family protein	954.68	396.34	2.41
At1g55530	265077_at	zinc finger (C3HC4-type RING finger) family protein	1195.12	551.51	2.17
At5g10380	250435_at	zinc finger (C3HC4-type RING finger) family protein	383.06	186.24	2.06
N-terminal protein myristoylation					
At3g01650	259178_at	copine-related, low similarity to SP:Q99829 Copine I {Homo sapiens}	517.94	184.63	2.81
At4g21510	254424_at	F-box family protein	636.69	300.89	2.12

At5g27750	246743_at	F-box family protein	72.43	34.94	2.07
At3g60040	251451_s_at	F-box family protein	83.24	40.18	2.07
Protein folding:					
At5g48570	248657_at	putative peptidyl-prolyl cis-trans isomerase / putative FK506-binding protein	684.54	19.47	35.16
At5g45110	248981_at	ankyrin repeat family protein / BTB/POZ domain-containing protein	573.79	164.37	3.49
At5g55200	248101_at	co-chaperone grpE protein, putative	396.39	170.84	2.32
Protein glycosylation:					
At1g73740	260047_at	glycosyl transferase family 28 protein	272.64	55.20	4.94
At1g08280	261813_at	glycosyl transferase family 29 protein / sialyltransferase family protein	347.40	107.69	3.23
At2g04560	263861_at	glycotransferase family protein 19	87.83	43.53	2.02
Protein targeting:					
At4g03320	255430_at	chloroplast protein import component-related	460.36	157.73	2.92
At2g34940	267412_at	putative vacuolar sorting receptor	183.56	67.88	2.70
At1g60970	259728_at	clathrin adaptor complex small chain family protein	30.32	12.22	2.48
At5g40930	249322_at	mitochondrial import receptor subunit (TOM20-4)	371.79	182.49	2.04
Primary metabolism:					
Glycolysis:					
At1g79550	262944_at	putative phosphoglycerate kinase	3063.63	1426.06	2.15
At3g08590	258679_at	putative phosphoglyceromutase	921.95	454.90	2.03
Fermentation:					
At1g77120	264953_at	alcohol dehydrogenase 1 (ADH1)	161.15	65.39	2.46
At5g17380	250094_at	pyruvate decarboxylase family protein	1203.35	522.66	2.30
At1g64710	262870_at	putative alcohol dehydrogenase	297.75	145.34	2.05
Carbohydrate metabolism:					
At3g04010	258805_at	glycosyl hydrolase family 17 protein	636.58	113.20	5.62
At1g02850	262118_at	glycosyl hydrolase family 1 protein	1245.11	305.34	4.08
At1g60730	264929_at	aldo/keto reductase family protein	79.92	20.18	3.96
At2g37770	267168_at	aldo/keto reductase family protein	186.88	54.33	3.44
At3g23920	256861_at	putative beta-amylase / putative 1,4-alpha-D-glucan maltohydrolase	1129.27	377.41	2.99
At4g31140	253559_at	glycosyl hydrolase family 17 protein	664.45	226.67	2.93
At5g15870	246532_at	glycosyl hydrolase family 81 protein	374.11	139.36	2.68
At4g18010	254707_at	inositol polyphosphate 5-phosphatase II (IP5PII)	537.00	203.41	2.64
At2g37760	267181_at	aldo/keto reductase family protein	683.91	263.55	2.60
At3g06500	258507_at	putative beta-fructofuranosidase / putative invertase / putative saccharase / putative beta-fructosidase	398.62	160.16	2.49
At3g55430	251804_at	glycosyl hydrolase family 17 protein / putative beta-1,3-glucanase	1165.15	546.66	2.13
Amino acid metabolism:					
At1g55920	260602_at	putative serine O-acetyltransferase	4453.31	447.04	9.96
At2g47180	263320_at	putative galactinol synthase	1107.15	247.52	4.47
At4g34710	253203_at	arginine decarboxylase 2 (SPE2)	7445.47	2100.12	3.55

At3g44720	252652_at	prephenate dehydratase family protein	459.63	168.88	2.72
At3g10050	258884_at	threonine ammonia-lyase / threonine dehydratase / threonine deaminase (OMR1)	372.99	159.28	2.34
At2g35390	266624_s_at	ribose-phosphate pyrophosphokinase 2 / phosphoribosyl diphosphate synthetase 2 (PRS2)	1248.42	542.27	2.30
At5g38530	249515_at	tryptophan synthase-related	265.24	123.87	2.14
At4g27070	253898_s_at	tryptophan synthase, beta subunit 2 (TSB2)	1518.77	730.57	2.08
At5g38710	249527_at	putative proline oxidase / putative osmotic stress-responsive proline dehydrogenase	288.88	143.57	2.01
At2g27820	266257_at	prephenate dehydratase family protein	630.96	314.58	2.01
Lipid metabolism:					
At2g19450	267280_at	diacylglycerol O-acyltransferase / acyl CoA:diacylglycerol acyltransferase (DGAT)	774.48	204.02	3.80
At1g28600	262745_at	putative lipase	418.59	168.92	2.48
At5g37690	249576_at	GDSL-motif lipase/hydrolase family protein	192.70	93.11	2.07
N-metabolism:					
At1g77760	259681_at	nitrate reductase 1 (NR1)	7175.28	3591.70	2.00
UDP glucosyl and glucoronyl transferases:					
At2g15480	265499_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1165.28	37.67	30.93
At4g34135	253268_s_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1767.07	62.91	28.09
At1g05560	263184_at	UDP-glucose transferase (UGT75B2)	801.37	40.80	19.64
At4g01070	255622_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	550.10	66.06	8.33
At1g22400	261934_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1378.34	199.02	6.93
At3g21560	258167_at	putative UDP-glucosyltransferase	425.02	62.35	6.82
At2g36790	265200_s_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	968.50	156.91	6.17
At2g43820	260567_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1172.69	222.33	5.27
At4g15490	245352_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1264.82	258.91	4.89
At3g11340	256252_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	500.98	140.29	3.57
At4g15550	245277_at	UDP-glucose:indole-3-acetate beta-D-glucosyltransferase (IAGLU)	1029.32	323.52	3.18
At3g46670	252482_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	312.11	105.42	2.96
At2g30140	267300_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	915.53	424.05	2.16
Secondary metabolism:					
At1g75280	256454_at	putative isoflavone reductase	2374.43	485.03	4.90
At4g20830	254432_at	FAD-binding domain-containing protein	1242.16	292.20	4.25
At1g72680	259911_at	putative cinnamyl-alcohol dehydrogenase	1390.37	345.14	4.03
At5g19440	246042_at	putative cinnamyl-alcohol	1357.92	348.87	3.89

		dehydrogenase			
At5g07870	250550_at	transferase family protein	425.22	111.12	3.83
At4g30210	253664_at	putative NADPH-cytochrome p450 reductase / putative NADPH-ferrihemoprotein reductase	1417.18	537.13	2.64
At2g33590	255787_at	cinnamoyl-CoA reductase family	619.26	234.84	2.64
At5g01210	251144_at	transferase family protein	1524.21	671.45	2.27
Transport:					
At1g79410	262935_at	transporter-related	564.06	81.01	6.96
At5g17860	250054_at	putative cation exchanger (CAX7)	1041.57	203.38	5.12
At5g13750	250252_at	transporter-related	648.82	131.25	4.94
At2g23150	267266_at	NRAMP metal ion transporter 3 (NRAMP3)	1633.08	340.23	4.80
At1g05030	265212_at	putative hexose transporter	1088.29	260.65	4.18
At1g33110	261618_at	MATE efflux family protein	1313.66	333.59	3.94
At2g38290	267142_at	ammonium transporter 2 (AMT2)	571.76	149.08	3.84
At2g04040	263403_at	MATE efflux family protein	428.98	112.11	3.83
At5g26340	246831_at	putative hexose transporter	1264.88	345.05	3.67
At1g71880	260143_at	sucrose transporter / sucrose-proton symporter (SUC1)	1389.41	409.01	3.40
At5g13490	245854_at	ADP, ATP carrier protein 2, mitochondrial / ADP/ATP translocase 2 / adenine nucleotide translocator 2 (ANT2)	911.41	271.73	3.35
At3g21690	258179_at	MATE efflux family protein	1213.54	393.99	3.08
At2g34660	267319_at	glutathione S-conjugate ABC transporter (MRP2)	567.71	187.23	3.03
At5g01340	251090_at	mitochondrial substrate carrier family protein	211.78	70.60	3.00
At3g62770	251187_at	transport protein-related	554.01	186.17	2.98
At2g39190	266990_at	ABC1 family protein	131.90	46.25	2.85
At1g67300	264992_at	putative hexose transporter	229.77	82.77	2.78
At4g28390	253776_at	putative ADP, ATP carrier protein, mitochondrial / putative ADP/ATP translocase / putative adenine nucleotide translocator	404.16	147.77	2.74
At4g35180	253181_at	amino acid transporter family protein	109.62	40.71	2.69
At1g69870	260410_at	proton-dependent oligopeptide transport (POT) family protein	574.34	214.50	2.68
At5g14570	250151_at	putative transporter	981.46	388.14	2.53
At3g55640	251757_at	mitochondrial substrate carrier family protein	200.67	82.15	2.44
At1g16780	255760_at	putative vacuolar-type H+-translocating inorganic pyrophosphatase	239.04	97.87	2.44
At3g21250	258033_at	ABC transporter family protein	292.78	123.16	2.38
At3g11820	258786_at	syntaxin 121 (SYP121) / syntaxin-related protein (SYR1)	662.58	296.12	2.24
At1g30410	256308_s_at	putative ATP-binding cassette transport protein	166.26	76.15	2.18
At4g25640	254077_at	MATE efflux family protein	626.87	290.97	2.15
At4g05110	255261_s_at	equilibrative nucleoside transporter, putative (ENT6)	96.72	45.15	2.14
At5g04930	250818_at	phospholipid-transporting ATPase 1 / aminophospholipid flippase 1 / magnesium-ATPase 1 (ALA1)	1194.86	559.04	2.14

At5g54860	248134_at	integral membrane transporter family protein	311.83	147.92	2.11
At3g47420	252414_at	putative glycerol-3-phosphate transporter / putative glycerol 3-phosphate permease	178.90	85.12	2.10
At4g16143	245216_at	importin alpha-2, putative (IMPA-2)	567.55	271.65	2.09
At5g60790	247593_at	ABC transporter family protein	927.17	448.54	2.07
At5g52750	248327_at	heavy-metal-associated domain-containing protein	116.32	56.29	2.07
At3g11130	256437_s_at	putative clathrin heavy chain	1450.10	702.14	2.07
Co-factor and vitamin metabolism:					
At5g14760	246597_at	L-aspartate oxidase family protein	344.75	54.50	6.33
At5g64300	247272_at	putative riboflavin biosynthesis protein (RIBA)	1255.81	363.00	3.46
At5g50210	248550_at	quinolinate synthetase A-related	715.22	227.62	3.14
At2g44750	266888_s_at	putative thiamin pyrophosphokinase	256.82	125.99	2.04
S-assimilation:					
At4g21990	254343_at	5'-adenylylsulfate reductase (APR3) / PAPS reductase homolog (PRH26)	3390.28	125.11	27.10
At4g04610	255284_at	5'-adenylylsulfate reductase (APR1) / PAPS reductase homolog (PRH19)	573.46	57.58	9.96
At1g62180	264745_at	5'-adenylylsulfate reductase 2, chloroplast (APR2) (APSR) / adenosine 5'-phosphosulfate 5'-adenylylsulfate (APS) sulfotransferase 2 / 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase homolog 43 (PRH-43)	1408.07	400.68	3.51
At5g04590	250846_at	sulfite reductase / ferredoxin (SIR)	1844.58	610.91	3.02
At3g22890	256835_at	sulfate adenylyltransferase 1 / ATP-sulfurylase 1 (APS1)	2098.34	715.03	2.93
Metal handling:					
At5g44070	249078_at	phytochelatin synthase 1 (PCS1)	1275.24	450.25	2.83
At3g15352	257058_at	cytochrome c oxidase copper chaperone-related	437.75	182.40	2.40
Development:					
At1g04770	261177_at	male sterility MS5 family protein	285.85	102.09	2.80
At3g53230	251975_at	putative cell division cycle protein 48 (CDC48)	261.69	93.64	2.79
At2g26560	245038_at	putative patatin	556.77	210.22	2.65
At4g28520	253767_at	putative 12S seed storage protein / putative cruciferin	388.10	169.06	2.30
At5g12990	250271_at	CLAVATA3/ESR-Related 40 (CLE40)	27.65	12.54	2.21
At1g01470	259426_at	putative late embryogenesis abundant protein / putative LEA protein	1008.02	471.32	2.14
Cell wall:					
At5g64310	247279_at	arabinogalactan-protein (AGP1)	1766.50	225.59	7.83
At1g55850	260592_at	cellulose synthase family protein	1448.73	309.07	4.69
At1g78570	263134_at	NAD-dependent epimerase/dehydratase family protein	581.53	246.84	2.36

At5g06860	250670_at	polygalacturonase inhibiting protein 1 (PGIP1)	4153.31	1885.46	2.20
At4g28300	253808_at	hydroxyproline-rich glycoprotein family protein	419.20	195.34	2.15
At2g17120	263582_at	peptidoglycan-binding LysM domain-containing protein	921.80	442.03	2.09
At3g62110	251261_at	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	749.63	365.67	2.05
At4g16790	245384_at	hydroxyproline-rich glycoprotein family protein	164.44	81.67	2.01
Miscellaneous:					
At3g09350	259037_at	armadillo/beta-catenin repeat family protein	3521.63	210.94	16.69
At2g41380	266368_at	embryo-abundant protein-related	1878.90	132.38	14.19
At3g16530	257206_at	legume lectin family protein	5175.54	494.10	10.47
At1g30070	260025_at	SGS domain-containing protein	916.73	99.97	9.17
At1g67810	245193_at	Fe-S metabolism associated domain-containing protein	1334.34	158.25	8.43
At4g24160	254204_at	hydrolase, alpha/beta fold family protein	1876.70	235.33	7.97
At2g22880	266800_at	VQ motif-containing protein	813.94	128.55	6.33
At3g50620	252164_at	nodulation protein-related	374.87	65.52	5.72
At4g02940	255462_at	oxidoreductase, 2OG-Fe(II) oxygenase family protein	661.51	141.11	4.69
At1g27760	261651_at	interferon-related developmental regulator family protein / IFRD protein family	747.03	165.62	4.51
At4g12130	254854_at	glycine cleavage T family protein / aminomethyl transferase family protein	125.51	28.41	4.42
At4g15420	245313_at	PRLI-interacting factor K	995.92	234.80	4.24
At1g55450	265075_at	embryo-abundant protein-related	2554.35	610.17	4.19
At4g15520	245562_at	tRNA/rRNA methyltransferase (SpoU) family protein	753.39	182.27	4.13
At3g62330	251267_at	zinc knuckle (CCHC-type) family protein	629.83	153.73	4.10
At5g58770	247780_at	putative dehydrodolichyl diphosphate synthase / DEDOL-PP synthase	222.82	55.11	4.04
At3g25230	257822_at	FK506-binding protein (ROF1)	806.35	202.17	3.99
At3g52720	252011_at	carbonic anhydrase family protein	499.90	130.06	3.84
At1g09070	264655_at	SRC2	5234.41	1376.25	3.80
At1g03220	264365_s_at	putative extracellular dermal glycoprotein EDGP	3669.00	984.49	3.73
At4g01870	255543_at	tolB protein-related	7360.40	2012.65	3.66
At4g33540	253343_at	metallo-beta-lactamase family protein	1031.11	282.51	3.65
At3g04640	258792_at	glycine-rich protein	593.76	170.17	3.49
At2g32020	265668_at	GCN5-related N-acetyltransferase (GNAT) family protein	95.75	28.68	3.34
At3g26910	258282_at	hydroxyproline-rich glycoprotein family protein	363.63	114.95	3.16
At1g36370	260126_at	putative serine hydroxymethyltransferase (SHM7)	292.78	98.54	2.97
At3g51790	246309_at	cytochrome c biogenesis protein CcmE family	514.30	177.03	2.91
At5g58070	247851_at	putative lipocalin	5076.94	1799.36	2.82

At3g43740	252703_at	leucine-rich repeat family protein	20.58	7.31	2.82
At5g59730	247693_at	exocyst subunit EXO70 family protein	787.36	288.14	2.73
At2g03760	264042_at	putative steroid sulfotransferase	266.23	99.83	2.67
At1g78860	264299_s_at	curculin-like (mannose-binding) lectin family protein	1286.12	490.83	2.62
At3g18690	257751_at	VQ motif-containing protein	394.97	152.27	2.59
At3g22060	**257264_at**	**Domain of unknown function that is usually associated with protein kinase domain**	**647.02**	**250.85**	**2.58**
At4g23100	254270_at	glutamate-cysteine ligase / gamma-glutamylcysteine synthetase (GSH1)	2113.22	829.93	2.55
At4g30490	253630_at	AFG1-like ATPase family protein	585.92	238.38	2.46
At1g66510	256363_at	AAR2 protein family	102.10	41.80	2.44
At5g26030	246870_at	ferrochelatase I	593.52	243.57	2.44
At1g09940	264660_at	glutamyl-tRNA reductase 2 / GluTR (HEMA2)	88.99	36.65	2.43
At5g42080	249232_at	dynamin-like protein 1 (ADL1)	1144.98	479.95	2.39
At5g58210	247855_at	hydroxyproline-rich glycoprotein family protein	184.95	79.42	2.33
At1g07350	261081_at	putative transformer serine/arginine-rich ribonucleoprotein	610.85	264.97	2.31
At3g22160	256793_at	VQ motif-containing protein	292.76	127.48	2.30
At4g30530	253606_at	putative defense-related protein	883.69	388.34	2.28
At4g09150	255077_at	T-complex protein 11	1381.21	608.99	2.27
At4g07360	255190_x_at	gypsy-like retrotransposon family (Athila)	140.91	64.70	2.18
At3g12050	256663_at	Aha1 domain-containing protein	952.71	437.67	2.18
At3g12740	257700_at	LEM3 (ligand-effect modulator 3) family protein / CDC50 family protein	1647.34	766.53	2.15
At4g01990	255557_at	pentatricopeptide (PPR) repeat-containing protein	140.24	65.31	2.15
At1g69750	260418_s_at	cox19 family protein	543.22	254.31	2.14
At1g11520	261872_s_at	pliceosome associated protein-related	304.25	145.37	2.09
At4g17650	245390_at	aromatic-rich family protein	458.82	219.41	2.09
At5g49700	248564_at	DNA-binding protein-related	562.05	269.99	2.08
At5g62640	247445_at	proline-rich family protein	542.66	261.82	2.07
At3g26690	257830_at	MutT/nudix family protein	569.69	277.38	2.05
At4g34180	253273_at	cyclase family protein	3014.34	1472.74	2.05
At4g15130	245533_at	putative cholinephosphate cytidylyltransferase / putative phosphorylcholine transferase	203.15	100.73	2.02
At4g17900	254694_at	zinc-binding family protein	1056.65	524.65	2.01
At1g33600	245765_at	leucine-rich repeat family protein	720.27	360.83	2.00
Unknown:					
At1g19020	259479_at	expressed protein	2201.80	120.41	18.29
At3g32260	256640_at	expressed protein	1645.57	99.83	16.48
At1g05575	263182_at	expressed protein	1513.71	96.55	15.68
At2g16900	266536_at	expressed protein	1031.56	102.28	10.09
At5g10695	246018_at	expressed protein	2665.64	290.24	9.18
At5g14730	246584_at	expressed protein	706.56	81.43	8.68
At2g32190	265674_at	expressed protein	297.43	37.39	7.96
At2g28400	265276_at	expressed protein	333.51	46.91	7.11
At2g31110	266474_at	expressed protein	1242.61	178.10	6.98
At2g32210	265670_s_at	expressed protein	526.18	78.77	6.68

At5g64510	247293_at	expressed protein	509.02	90.58	5.62
At2g26530	245041_at	expressed protein	951.90	177.30	5.37
At3g07090	258830_at	expressed protein	1615.16	318.94	5.06
At2g20240	265312_at	expressed protein	471.16	95.53	4.93
At1g76070	261748_at	expressed protein	575.75	120.11	4.79
At5g61820	247488_at	expressed protein	2254.24	476.03	4.74
At4g36500	246270_at	expressed protein	1207.17	261.03	4.62
At3g50910	252134_at	expressed protein	678.64	148.16	4.58
At1g76600	259979_at	expressed protein	3116.03	683.17	4.56
At3g49210	252303_at	expressed protein	553.72	137.14	4.04
At3g21700	257951_at	expressed protein	184.63	45.87	4.02
At5g47830	248774_at	expressed protein	440.19	111.15	3.96
At1g03070	263164_at	expressed protein	782.41	208.88	3.75
At1g67920	260005_at	expressed protein	70.26	19.17	3.66
At1g28135	259589_at	expressed protein	102.37	28.41	3.60
At4g27657	253859_at	expressed protein	152.06	44.99	3.38
At1g55500	265078_at	expressed protein	202.86	62.21	3.26
At5g18400	249984_at	expressed protein	689.81	224.97	3.07
At3g22530	256934_at	expressed protein	888.30	291.38	3.05
At1g73120	262373_at	expressed protein	4776.47	1583.30	3.02
At3g27350	257710_at	expressed protein	248.96	83.58	2.98
At1g18380	261719_at	expressed protein	246.25	83.39	2.95
At1g23710	265184_at	expressed protein	371.98	126.91	2.93
At2g19310	267336_at	expressed protein	2226.71	759.85	2.93
At5g19240	249918_at	expressed protein	809.21	276.90	2.92
At3g20340	257670_at	expressed protein	1085.98	372.56	2.91
At4g29780	253643_at	expressed protein	339.87	119.60	2.84
At5g11270	250421_at	expressed protein	193.62	68.85	2.81
At4g38550	252976_s_at	expressed protein	686.57	244.26	2.81
At5g54300	248205_at	expressed protein	98.31	35.10	2.80
At1g56060	262085_at	expressed protein	138.50	49.84	2.78
At1g62045	264288_at	expressed protein	55.46	19.98	2.78
At1g72060	256337_at	expressed protein	877.04	316.61	2.77
At4g24380	254158_at	expressed protein	426.70	154.24	2.77
At2g05030	263341_at	expressed protein	66.39	24.21	2.74
At4g30390	253637_at	expressed protein	871.54	318.99	2.73
At3g15760	258275_at	expressed protein	327.65	120.82	2.71
At5g47410	248786_at	expressed protein	343.30	129.93	2.64
At5g67600	247009_at	expressed protein	2294.13	877.59	2.61
At5g02020	251039_at	expressed protein	1184.77	463.04	2.56
At3g12320	256266_at	expressed protein	954.83	373.29	2.56
At1g63720	260243_at	expressed protein	451.53	180.78	2.50
At1g15430	262571_at	expressed protein	552.10	226.46	2.44
At3g63310	251163_at	expressed protein	1400.63	586.38	2.39
At2g36220	263931_at	expressed protein	1288.59	539.75	2.39
At1g73350	245727_at	expressed protein	217.88	91.77	2.37
At1g07090	256062_at	expressed protein	405.33	174.84	2.32
At5g14710	250150_at	expressed protein	179.57	77.69	2.31
At2g01300	265732_at	expressed protein	171.08	74.10	2.31
At3g23170	257925_at	expressed protein	434.22	189.91	2.29
At4g25670	254050_s_at	expressed protein	1071.66	470.96	2.28
At1g32920	261193_at	expressed protein	3888.84	1714.93	2.27
At2g37940	266101_at	expressed protein	863.04	381.12	2.26
At1g28380	261445_at	expressed protein	819.77	363.98	2.25
At4g35240	253185_at	expressed protein	86.80	39.10	2.22

At2g23120	267261_at	expressed protein	2938.10	1325.57	2.22
At5g12010	250350_at	expressed protein	814.96	369.23	2.21
At1g23550	257401_at	expressed protein	351.39	159.82	2.20
At2g25735	266658_at	expressed protein	401.99	184.46	2.18
At2g27830	266259_at	expressed protein	2095.49	968.26	2.16
At4g28085	253827_at	expressed protein	130.57	60.64	2.15
At2g03010	266771_s_at	expressed protein	370.02	171.97	2.15
At3g51890	246305_at	expressed protein	913.24	429.97	2.12
At5g64230	247287_at	expressed protein	412.09	194.50	2.12
At5g05190	250821_at	expressed protein	374.32	181.35	2.06
At3g18295	257728_at	expressed protein	190.59	92.84	2.05
At2g34070	256725_at	expressed protein	787.02	385.40	2.04
At2g07719	265235_s_at	expressed protein	229.50	114.34	2.01
At4g20300	254491_at	expressed protein	148.06	73.93	2.00
At1g19380	260656_at	expressed protein	1145.65	572.20	2.00
Obsolete:					
At3g28090	257556_at	nodulin MtN21 family protein	3.94	1.00	3.94
At3g17610	258349_at	bZIP transcription factor family protein / HY5-like protein (HYH)	178.95	46.78	3.83
At3g19730	257045_at	dynamin family protein	12.61	3.64	3.47
At5g24700	246959_at	expressed protein	440.27	165.81	2.66
At3g09610	258724_at	myb family transcription factor	46.28	15.64	2.96
At4g32740	253417_at	myb family transcription factor	96.65	38.82	2.49
At1g65400	264213_at	putative disease resistance protein (TIR class)	582.46	236.62	2.46
At4g23480	254262_at	putative protein	2031.48	897.13	2.26
At3g17615	258406_at	hypothetical protein	558.06	261.06	2.14
At1g77900	262183_at	expressed protein	228.90	112.53	2.03

D.2 Transcript repression by H_2O_2 treatment

Only transcripts with at least a 2-fold repression and present only calls in both slides are shown. Annotations are according to TAIR version 6 genome release (2007). For full details of the microarray experiment please refer to Results Chapter 3.2.1

AGI code	Probe set	Gene annotation	10 mM H_2O_2	Control	Fold change
Kinases:					
At1g51830	246375_at	putative leucine-rich repeat protein kinase	24.54	272.93	11.12
At3g56100	251718_at	meristematic receptor-like kinase (MRLK)	13.10	75.52	5.77
At3g09780	258704_at	protein kinase family protein	71.61	241.56	3.37
At3g45920	252554_s_at	receptor protein kinase-related	30.43	86.95	2.86
At1g07150	256045_at	mitogen-activated kinase kinase kinase 13 (MAPKKK13)	26.73	73.01	2.73
At3g46920	252469_at	protein kinase family protein	57.48	148.84	2.59
At1g78290	260774_at	SNF1-related protein kinase (SNRK2-8)	285.48	729.81	2.56
At2g23030	267254_at	SNF1-related protein kinases (SNRK2-9)	87.14	215.96	2.48
At3g46280	252511_at	protein kinase-related	63.11	150.71	2.39
At1g07560	261090_at	putative leucine-rich repeat protein kinase	45.59	107.04	2.35
At4g29380	253714_at	protein kinase family protein	95.56	219.59	2.30
At3g21510	258184_at	histidine phosphotransfer protein (APH1)	139.05	314.38	2.26
At2g28960	266784_at	putative leucine-rich repeat protein kinase	30.07	65.52	2.18
At2g45340	245130_at	putative leucine-rich repeat transmembrane protein kinase	75.62	162.14	2.14
At1g21920	260855_at	phosphatidylinositol-4-phosphate 5-kinase-related	366.68	759.89	2.07
At1g53730	259958_at	putative leucine-rich repeat transmembrane protein kinase	213.38	440.59	2.06
At5g49780	248570_at	putative leucine-rich repeat transmembrane protein kinase	31.53	64.49	2.05
At5g57630	247867_at	putative CBL-interacting protein kinase 2 (CIPK21)	82.29	168.16	2.04
Phosphatases:					
At1g04040	265042_at	acid phosphatase class B family protein	301.24	994.94	3.30
At4g01480	255587_at	putative inorganic pyrophosphatase / putative pyrophosphate phospho-hydrolase	85.04	218.50	2.57
At1g14700	262830_at	putative purple acid phosphatase 3 (PAP3)	201.32	456.53	2.27
At2g01890	263595_at	putative purple acid phosphatase 8 (PAP8)	171.59	373.87	2.18

Transcription factors:					
At5g62470	247455_at	myb family transcription factor (MYB96)	73.49	319.00	4.34
At4g37730	253064_at	bZIP transcription factor family protein	13.84	54.31	3.92
At1g69310	260337_at	WRKY family transcription factor (WRKY57)	58.36	195.81	3.36
At4g29190	253722_at	zinc finger (CCCH-type) family protein	312.55	916.92	2.93
At1g29280	260882_at	WRKY family transcription factor (WRKY65)	190.01	547.66	2.88
At4g38620	252958_at	myb family transcription factor (MYB4)	21.07	60.07	2.85
At4g16780	245276_at	homeobox-leucine zipper protein 4 (HAT4)	151.37	422.50	2.79
At1g17920	255907_at	homeobox-leucine zipper family protein / homeodomain glabrous 12 (HDG12)	49.03	136.14	2.78
At3g11090	256427_at	LOB domain family protein / lateral organ boundaries domain family protein (LBD21)	102.41	267.51	2.61
At3g57040	251665_at	two-component responsive regulator (ARR9)	85.26	204.06	2.39
At1g73830	260070_at	basic helix-loop-helix (bHLH) family protein	317.02	753.02	2.38
At5g06500	250731_at	MADS-box family protein	22.46	49.89	2.22
At2g31370	263253_at	bZIP transcription factor	85.70	187.74	2.19
At5g25160	246933_at	zinc finger (C2H2 type) family protein	49.95	108.46	2.17
At5g18240	250031_at	myb-related protein 1	54.52	116.35	2.13
At1g08000	260680_s_at	zinc finger (GATA type) family protein	36.52	77.01	2.11
At3g14740	258089_at	PHD finger family protein	30.64	64.46	2.10
At5g59780	247696_at	myb family transcription factor (MYB59)	630.89	1316.96	2.09
At1g65360	264182_at	MADS-box protein (AGL23)	11.51	23.88	2.07
At1g18330	261663_at	myb family transcription factor / EPR1	819.60	1676.86	2.05
At2g33810	267460_at	squamosa promoter-binding protein-like 3 (SPL3)	36.94	75.49	2.04
Calcium:					
At5g65930	247115_at	kinesin-like calmodulin-binding protein (ZWICHEL)	47.08	250.95	5.33
At4g37010	246197_at	putative caltractin / putative centrin	33.85	119.90	3.54
At2g43680	260610_at	calmodulin-binding family protein	268.68	868.02	3.23
At1g70790	262291_at	C2 domain-containing protein	70.04	171.75	2.45
At5g65020	247210_at	annexin 2 (ANN2)	237.05	546.24	2.30
At4g14750	245574_at	calmodulin-binding family protein	58.66	134.61	2.29
At2g38800	263296_at	calmodulin-binding protein-related	353.66	800.42	2.26
Hormones:					
At4g38860	252965_at	putative auxin-responsive protein / auxin-induced protein 10A	266.25	542.40	2.04
At5g14920	246550_at	gibberellin-regulated family protein	679.43	2879.89	4.24
Response to stress:					

Biotic stress:					
At5g45490	248943_s_at	disease resistance protein-related	12.01	42.53	3.54
At2g43535	260549_at	defensin-like (DEFL) family protein.	148.27	394.65	2.66
At1g33870	260115_at	putative avirulence-responsive protein	15.85	35.65	2.25
At1g65690	262930_at	harpin-induced protein-related / HIN1-related / harpin-responsive protein-related	71.62	159.02	2.22
At2g43530	260541_at	defensin-like (DEFL) family protein.	91.70	203.04	2.21
At4g11650	254889_at	osmotin-like protein (OSM34)	109.78	241.96	2.20
At4g19530	254553_at	putative disease resistance protein (TIR-NBS-LRR class)	190.87	410.82	2.15
At2g28670	263437_at	disease resistance-responsive family protein	371.05	781.14	2.11
Other stresses:					
At4g39960	252828_at	DNAJ heat shock family protein	77.69	308.05	3.97
At4g37220	246251_at	putative stress-responsive protein	650.73	1612.47	2.48
At1g06460	262629_at	31.2 kDa small heat shock family protein / hsp20 family protein (ACD32.1)	555.40	1128.77	2.03
Protective enzymes:					
At1g05250	264567_s_at	putative peroxidase	51.17	710.48	13.88
At3g01190	259276_at	peroxidase 27 (PER27) (P27)	44.10	395.74	8.97
At4g30170	253667_at	putative peroxidase	313.71	1473.18	4.70
At3g49120	252291_s_at	putative peroxidase	89.59	345.65	3.86
At5g19890	246149_at	putative peroxidase	79.40	253.94	3.20
At5g42180	249227_at	peroxidase 64 (PER64)	102.95	311.62	3.03
At1g17190	262516_at	putative glutathione S-transferase	89.53	253.02	2.83
At3g01420	258957_at	alpha-dioxygenase 1	238.52	655.74	2.75
At2g37130	265471_at	peroxidase 21 (PER21)	398.40	1095.55	2.75
At5g64100	247297_at	putative peroxidase	1278.48	3437.27	2.69
At5g66390	247091_at	peroxidase 72 (PER72)	84.90	223.84	2.64
At3g21770	257952_at	peroxidase 30 (PER30) (P30)	118.18	269.49	2.28
At2g18980	266941_at	putative peroxidase	130.93	294.33	2.25
At3g62950	251196_at	glutaredoxin family protein	197.79	417.37	2.11
At2g38390	267053_s_at	putative peroxidase	320.60	657.72	2.05
Electron transport:					
At5g44380	249045_at	FAD-binding domain-containing protein	152.18	753.91	4.95
At2g21260	263758_s_at	putative mannose 6-phosphate reductase (NADPH-dependent)	108.04	397.79	3.68
At2g14100	263276_at	cytochrome P450 family protein	44.74	148.50	3.32
At1g76150	261771_at	maoC-like dehydratase domain-containing protein	294.21	896.91	3.05
At5g49730	248566_s_at	ferric reduction oxidase 6 (FRO6)	378.37	1077.58	2.85
At1g78550	263135_at	oxidoreductase, 2OG-Fe(II) oxygenase family protein	60.97	172.41	2.83
At5g22500	249895_at	putative acyl CoA reductase / putative male-sterility protein	553.11	1491.50	2.70
At4g20840	254431_at	FAD-binding domain-containing protein	56.30	137.80	2.45
At5g24910	246978_at	cytochrome P450 family protein	41.14	99.25	2.41
At1g68540	260260_at	oxidoreductase family protein	19.20	46.33	2.41
At3g44540	252638_at	putative acyl CoA reductase / putative	26.29	62.27	2.37

		male-sterility protein			
At1g30720	263216_s_at	FAD-binding domain-containing protein	342.11	796.90	2.33
At2g46740	266711_at	FAD-binding domain-containing protein	76.52	176.27	2.30
At3g20100	257129_at	cytochrome P450 family protein	28.67	63.64	2.22
At2g46750	266712_at	FAD-binding domain-containing protein	132.94	286.53	2.16
At4g39330	252943_at	putative mannitol dehydrogenase	336.18	722.90	2.15
At3g16250	258055_at	ferredoxin-related	195.48	409.38	2.09
At2g29290	266279_at	putative tropinone reductase / putative tropine dehydrogenase	710.85	1478.58	2.08
At5g58660	247774_at	oxidoreductase, 2OG-Fe(II) oxygenase family protein	53.58	111.08	2.07
Nucleic acid binding/processing:					
At1g18800	261406_at	nucleosome assembly protein (NAP) family protein	83.12	483.16	5.81
At5g46920	248815_at	intron maturase, type II family protein	54.60	285.23	5.22
At1g53690	259962_at	putative DNA-directed RNA polymerases I, II, and III 7 kDa subunit	42.78	120.60	2.82
At3g10010	258931_at	HhH-GPD base excision DNA repair family protein	40.70	113.39	2.79
At1g79650	261352_at	putative DNA repair protein RAD23	93.11	251.23	2.70
At4g31210	253566_at	DNA topoisomerase family protein	89.91	234.21	2.61
At4g12080	254853_at	DNA-binding family protein	169.42	409.93	2.42
At4g21600	254392_at	putative bifunctional nuclease	132.01	282.37	2.14
At1g70200	264698_at	RNA recognition motif (RRM)-containing protein	123.87	263.94	2.13
At1g19480	260672_at	HhH-GPD base excision DNA repair family protein	67.65	137.50	2.03
At3g15950	257798_at	DNA topoisomerase-related	164.84	333.82	2.03
Protein metabolism:					
Protein synthesis:					
At5g13650	250256_at	elongation factor family protein	534.57	1105.87	2.07
Protein degradation:					
At3g16550	257231_at	putative DegP protease	2.91	20.09	6.89
At5g59090	247755_at	subtilase family protein	95.84	360.93	3.77
At2g18330	265340_at	AAA-type ATPase family protein	53.70	199.12	3.71
At3g16290	258048_at	putative FtsH protease	62.05	228.50	3.68
At2g22980	267265_at	serine carboxypeptidase S10 family protein	265.60	945.76	3.56
At5g65760	247156_at	serine carboxypeptidase S28 family protein	106.89	378.63	3.54
At3g02110	258857_at	serine carboxypeptidase S10 family protein	278.85	872.01	3.13
At5g17140	246473_at	cysteine proteinase-related	41.86	110.58	2.64
At5g43600	249103_at	putative N-carbamyl-L-amino acid hydrolase	114.15	283.66	2.49
At5g53350	248255_at	ATP-dependent Clp protease ATP-binding subunit ClpX1 (CLPX)	491.08	1206.25	2.46
At2g22970	267264_at	serine carboxypeptidase S10 family protein	28.99	70.04	2.42
At5g23210	249847_at	serine carboxypeptidase S10 family protein	419.73	955.87	2.28

At3g19400	258006_at	putative cysteine proteinase	72.02	151.66	2.11
At3g43960	252692_at	putative cysteine proteinase	55.24	112.94	2.04
At5g43580	249101_at	putative protease inhibitor, putative	407.75	823.90	2.02
Protein binding:					
At1g73760	260065_at	zinc finger (C3HC4-type RING finger) family protein	51.05	230.68	4.52
At3g61550	251330_at	zinc finger (C3HC4-type RING finger) family protein	94.45	393.00	4.16
At2g22010	263870_at	zinc finger (C3HC4-type RING finger) family protein	149.34	420.25	2.81
At2g20650	265432_at	zinc finger (C3HC4-type RING finger) family protein	51.12	126.70	2.48
At1g22040	255947_at	kelch repeat-containing F-box family protein	78.11	179.34	2.30
At5g63780	247347_at	zinc finger (C3HC4-type RING finger) family protein	205.83	471.81	2.29
At1g76410	259982_at	zinc finger (C3HC4-type RING finger) family protein	442.72	992.66	2.24
At1g01640	261586_at	speckle-type POZ protein-related	59.28	126.05	2.13
Protein glycosylation:					
At3g14960	257560_at	galactosyltransferase family protein	139.10	339.30	2.44
At1g76400	259883_at	ribophorin I family protein	120.96	243.08	2.01
Protein targeting:					
At1g29260	260844_at	peroxisomal targeting signal type 2 receptor (PEX7)	99.85	524.00	5.25
At4g20110	254500_at	vacuolar sorting receptor, putative	110.31	252.40	2.29
At5g52280	248344_at	protein transport protein-related	27.14	58.61	2.16
Post-translational modification:					
At1g73260	260101_at	trypsin and protease inhibitor family protein	1681.31	5120.21	3.05
At2g18390	265337_at	ADP-ribosylation factor-like protein 2 (ARL2)	121.61	249.07	2.05
Protein folding:					
At2g47320	260530_at	peptidyl-prolyl cis-trans isomerase cyclophilin-type family protein	620.75	1272.17	2.05
Primary metabolism:					
Glycolysis:					
At4g26530	253971_at	putative fructose-bisphosphate aldolase	233.76	1177.87	5.04
At3g14940	257217_at	putative phosphoenolpyruvate carboxylase / putative PEP carboxylase	89.06	318.92	3.58
At1g70820	262309_at	putative phosphoglucomutase / putative glucose phosphomutase	455.30	1077.56	2.37
Fermentation:					
At4g22110	254344_at	putative alcohol dehydrogenase	2.95	19.93	6.76
At1g22440	261930_at	putative alcohol dehydrogenase	98.19	270.47	2.75
Carbohydrate metabolism:					
At1g05590	263199_at	glycosyl hydrolase family 20 protein	43.43	150.52	3.47
At1g66280	260130_s_at	glycosyl hydrolase family 1 protein	352.28	1212.46	3.44
At3g01260	259264_at	aldose 1-epimerase family protein	21.80	72.42	3.32
At4g30290	253608_at	putative xyloglucan endotransglycosylase/hydrolase 19	38.10	117.91	3.09

At4g20460	254468_at	putative UDP-D-xylose 4-epimerase	26.56	76.72	2.89
At1g47840	261729_s_at	putative hexokinase	18.88	52.97	2.81
At2g43610	260557_at	glycoside hydrolase family 19 protein	161.91	440.59	2.72
At4g23820	254221_at	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	478.49	1275.41	2.67
At5g10560	250444_at	glycosyl hydrolase family 3 protein	220.21	581.05	2.64
At5g54570	248168_at	glycosyl hydrolase family 1 protein	54.04	135.56	2.51
At1g61820	264280_at	glycosyl hydrolase family 1 protein	228.48	557.23	2.44
At2g41850	260492_at	putative endo-polygalacturonase	81.27	191.77	2.36
At1g10760	262784_at	starch excess protein (SEX1)	377.58	879.18	2.33
At2g28470	264078_at	putative beta-galactosidase (BGAL8 gene)	227.80	505.37	2.22
C-1 metabolism:					
At3g10160	258927_at	dihydrofolate synthetase/folylpolyglutamate synthetase (DHFS/FPGS3)	101.91	287.59	2.82
Amino acid metabolism:					
At4g02610	255487_at	putative tryptophan synthase, alpha subunit	72.31	241.17	3.34
At2g38400	267035_at	putative alanine--glyoxylate aminotransferase / putative beta-alanine-pyruvate aminotransferase	926.20	2428.30	2.62
At5g17330	250090_at	glutamate decarboxylase 1 (GAD 1)	522.23	1363.29	2.61
At5g21060	246027_at	homoserine dehydrogenase family protein	72.28	146.42	2.03
Lipid metabolism:					
At5g45670	248912_at	GDSL-motif lipase/hydrolase family protein	49.52	199.85	4.04
At5g18630	250008_at	lipase class 3 family protein	231.34	797.79	3.45
At5g58560	247801_at	phosphatidate cytidylyltransferase family protein	74.92	222.62	2.97
At4g14070	245621_at	acyl activating enzyme 15	91.37	253.13	2.77
At1g27480	264442_at	lecithin:cholesterol acyltransferase family protein / LACT family protein	126.65	326.35	2.58
At2g27360	265646_at	putative lipase	135.60	344.75	2.54
At4g38690	252950_at	1-phosphatidylinositol phosphodiesterase-related	412.89	998.82	2.42
At4g18970	254609_at	GDSL-motif lipase/hydrolase family protein	182.11	407.75	2.24
At1g06080	260957_at	delta 9 desaturase (ADS1)	255.73	568.67	2.22
At5g57240	247951_at	oxysterol-binding family protein	113.66	242.71	2.14
At1g29670	259788_at	GDSL-motif lipase/hydrolase family protein	844.21	1787.97	2.12
UDP glucosyl and glucoronyl transferases:					
At3g21790	257940_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	48.97	114.97	2.35
At5g05870	250750_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein	76.70	157.99	2.06
Secondary metabolism:					
At1g32100	245792_at	putative pinoresinol-lariciresinol reductase	66.20	333.08	5.03
At5g63600	247333_at	putative flavonol synthase	169.51	811.60	4.79
At5g57030	247936_at	lycopene epsilon cyclase	216.42	561.44	2.59
At4g36220	253088_at	ferulate 5-hydroxylase (F5H)	204.60	475.30	2.32

At2g16280	263606_at	putative very-long-chain fatty acid condensing enzyme	576.28	1285.42	2.23
Transport:					
At4g23700	254215_at	putative cation/hydrogen exchanger (CHX17)	88.14	432.96	4.91
At1g32450	260693_at	proton-dependent oligopeptide transport (POT) family protein	196.84	838.02	4.26
At4g32650	253392_at	putative inward rectifying potassium channel (KAT3) (AKT4) (KC1)	40.57	151.93	3.75
At4g17340	245399_at	major intrinsic family protein / MIP family protein	881.03	3202.36	3.63
At1g80830	261895_at	NRAMP metal ion transporter 1 (NRAMP1)	204.12	707.16	3.46
At1g11670	262813_at	MATE efflux family protein	101.81	306.24	3.01
At5g47450	248790_at	major intrinsic family protein / MIP family protein	585.03	1745.99	2.98
At5g43370	249152_s_at	inorganic phosphate transporter (PHT1)	274.29	736.82	2.69
At1g77210	264482_at	putative sugar transporter	548.48	1421.09	2.59
At3g17440	257269_at	novel plant SNARE 13 (NPSN13)	246.47	620.07	2.52
At1g60960	259723_at	putative metal transporter (IRT3)	178.86	440.26	2.46
At4g19030	254606_at	major intrinsic family protein / MIP family protein	44.96	102.80	2.29
At3g23430	258293_at	putative phosphate transporter (PHO1)	143.89	325.90	2.26
At1g25500	255728_at	choline transporter-related	65.80	148.98	2.26
At5g03570	250952_at	iron-responsive transporter-related	109.74	247.13	2.25
At4g10380	254971_at	major intrinsic family protein / MIP family protein	185.38	413.70	2.23
At3g62270	251254_at	anion exchange family protein	196.02	428.61	2.19
At5g46050	248932_at	proton-dependent oligopeptide transport (POT) family protein	69.57	149.06	2.14
At2g47160	263319_at	anion exchange family protein	76.37	162.98	2.13
At1g79360	264124_at	transporter-related	236.45	503.72	2.13
At2g39350	267008_at	ABC transporter family protein	200.78	418.70	2.09
At5g40780	249346_at	putative lysine and histidine specific transporter	808.92	1684.37	2.08
Co-factor and vitamin metabolism:					
At2g29630	266673_at	thiamine biosynthesis family protein / thiC family protein	277.69	633.14	2.28
Metal handling:					
At5g52710	248319_at	heavy-metal-associated domain-containing protein	22.85	91.81	4.02
At3g23800	257197_at	selenium-binding family protein	68.63	153.07	2.23
Development:					
At3g16690	258421_at	nodulin MtN3 family protein	148.21	388.61	2.62
At5g66170	247136_at	senescence-associated family protein	394.80	944.58	2.39
At1g58250	256199_at	putative SABRE	47.74	111.74	2.34
At4g28040	253829_at	nodulin MtN21 family protein	860.91	1817.65	2.11
At3g25190	257823_at	putative nodulin	109.61	223.25	2.04
Cell organisation:					
At1g52250	257504_at	dynein light chain type 1 family protein	53.72	151.63	2.82

At2g29550	266295_at	tubulin beta-7 chain (TUB7)	288.34	813.83	2.82
At3g50240	252215_at	kinesin motor protein-related	104.51	246.71	2.36
At1g80260	262062_s_at	tubulin family protein	97.23	204.04	2.10
Cell division:					
At4g35620	253148_at	cyclin 2b protein (CYC2b)	33.76	160.59	4.76
Cell cycle:					
At4g37630	253055_at	cyclin family protein	49.55	253.58	5.12
At3g19650	257073_at	cyclin-related	36.73	75.91	2.07
Cell wall:					
At1g26250	245874_at	putative proline-rich extensin	34.62	479.61	13.85
At2g21770	263872_at	putative cellulose synthase, catalytic subunit	13.23	61.62	4.66
At3g54590	251843_x_at	proline-rich extensin-like family protein	288.58	1061.43	3.68
At5g04960	250801_at	pectinesterase family protein	33.41	104.90	3.14
At3g45970	252563_at	expansin family protein (EXPL1)	419.38	1049.36	2.50
At5g56540	247965_at	arabinogalactan-protein (AGP14)	103.94	257.52	2.48
At3g24670	256900_at	pectate lyase family protein	109.76	247.76	2.26
At4g40090	252833_at	arabinogalactan-protein (AGP3)	99.76	223.37	2.24
At5g53250	248252_at	putative arabinogalactan-protein (AGP22)	229.06	493.82	2.16
At1g54970	256352_at	proline-rich family protein	96.87	203.84	2.10
At5g65390	247189_at	arabinogalactan-protein (AGP7)	531.89	1073.30	2.02
Miscellaneous:					
At3g20270	257666_at	lipid-binding serum glycoprotein family protein	49.62	489.03	9.85
At5g38930	249477_s_at	putative germin-like protein	25.64	244.31	9.53
At1g23720	265169_x_at	proline-rich extensin-like family protein	237.11	1686.11	7.11
At4g38080	253024_at	hydroxyproline-rich glycoprotein family protein	110.07	748.01	6.80
At4g12550	254828_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	357.40	2301.14	6.44
At4g18975	254596_at	pentatricopeptide (PPR) repeat-containing protein	64.87	416.71	6.42
At5g60320	247623_at	lectin protein kinase family protein	23.49	141.85	6.04
At1g13590	256158_at	phytosulfokine-alpha (PSK) precursor	13.56	70.08	5.17
At5g62290	247449_at	nucleotide-sensitive chloride conductance regulator (ICln) family protein	100.04	445.31	4.45
At1g21100	261459_at	putaive O-methyltransferase	93.32	408.72	4.38
At2g02700	267478_at	DC1 domain-containing protein	10.97	43.36	3.95
At5g49750	248567_at	leucine-rich repeat family protein	21.60	78.85	3.65
At2g01530	266330_at	major latex protein-related / MLP-related	543.36	1926.97	3.55
At5g08490	250521_at	pentatricopeptide (PPR) repeat-containing protein	10.68	37.10	3.47
At4g23680	254234_at	major latex protein-related / MLP-related	131.43	433.51	3.30
At5g23840	249814_at	MD-2-related lipid recognition domain-containing protein / ML domain-containing protein	47.43	155.14	3.27

At2g36100	263284_at	integral membrane family protein	233.64	759.35	3.25
At5g26280	246855_at	meprin and TRAF homology domain-containing protein / MATH domain-containing protein	437.97	1413.27	3.23
At5g12200	250318_at	dihydropyrimidinase / DHPase / dihydropyrimidine amidohydrolase / hydantoinase (PYD2)	188.24	598.63	3.18
At5g09530	250500_at	hydroxyproline-rich glycoprotein family protein	115.18	349.83	3.04
At2g28630	263443_at	beta-ketoacyl-CoA synthase family protein	127.16	384.00	3.02
At4g29610	253679_at	putative cytidine deaminase / putative cytidine aminohydrolase	26.83	79.23	2.95
At1g74720	262209_at	C2 domain-containing protein	74.00	217.57	2.94
At5g64730	247256_at	transducin family protein / WD-40 repeat family protein	78.77	229.37	2.91
At1g52050	265048_at	jacalin lectin family protein	85.93	247.90	2.88
At1g48750	256145_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	310.10	889.32	2.87
At4g21830	254385_s_at	methionine sulfoxide reductase domain-containing protein / SelR domain-containing protein	49.47	138.48	2.80
At4g04840	255298_at	methionine sulfoxide reductase domain-containing protein	179.03	500.04	2.79
At2g17440	264908_at	leucine-rich repeat family protein	102.83	284.76	2.77
At3g62040	251298_at	haloacid dehalogenase-like hydrolase family protein	143.73	390.44	2.72
At4g03120	255431_at	proline-rich family protein	273.17	734.62	2.69
At5g35940	249675_at	jacalin lectin family protein	28.35	76.18	2.69
At4g28010	253847_at	pentatricopeptide (PPR) repeat-containing protein	15.55	41.29	2.66
At2g05510	265561_s_at	glycine-rich protein	55.55	146.54	2.64
At2g44380	267385_at	DC1 domain-containing protein	181.12	476.48	2.63
At1g78660	263137_at	putative gamma-glutamyl hydrolase / putative gamma-Glu-X carboxypeptidase / putative conjugase	259.97	684.93	2.63
At4g25910	254038_at	putative nitrogen fixation protein	256.35	670.92	2.62
At1g23040	264894_at	hydroxyproline-rich glycoprotein family protein	223.66	579.25	2.59
At1g55540	265074_at	proline-rich family protein	75.60	195.62	2.59
At3g06390	258905_at	integral membrane family protein	159.68	410.16	2.57
At1g22660	264212_at	putative tRNA-nucleotidyltransferase / putative tRNA adenylyltransferase	80.79	207.83	2.57
At2g45330	245131_s_at	putative tRNA 2'phosphotransferase	168.57	429.34	2.55
At4g17800	245382_at	DNA-binding protein-related	129.00	326.74	2.53
At1g74680	262223_at	exostosin family protein	132.20	333.97	2.53
At3g09220	259036_at	putative laccase (LAC7)	237.52	596.79	2.51
At4g01780	255566_s_at	XH/XS domain-containing protein / XS zinc finger domain-containing protein /// XH/XS domain-containing protein / XS zinc finger domain-containing protein	10.39	25.70	2.47

At4g10840	254951_at	kinesin light chain-related	810.58	1970.37	2.43
At5g36120	249685_at	YGGT family protein	78.69	191.11	2.43
At5g55740	248075_at	pentatricopeptide (PPR) repeat-containing protein	28.63	68.98	2.41
At5g47240	248793_at	MutT/nudix family protein	242.83	578.25	2.38
At1g33290	256530_at	sporulation protein-related	151.11	358.02	2.37
At5g24710	246960_at	similar to protein kinase family protein	253.54	599.03	2.36
At2g30210	267307_at	putative laccase (LAC3)	169.43	396.88	2.34
At2g01520	266353_at	major latex protein-related / MLP-related	284.40	665.73	2.34
At2g19430	267333_at	transducin family protein	74.82	173.56	2.32
At4g19840	254551_at	lectin-related protein	229.93	523.67	2.28
At5g43310	249146_at	COP1-interacting protein-related	78.35	176.01	2.25
At4g29210	253708_at	gamma-glutamyltranspeptidase family protein	70.42	157.50	2.24
At2g47470	245175_at	protein disulfide isomerase-like (PDIL) protein	452.91	1015.70	2.24
At4g14630	245567_at	germin-like protein (GLP9)	52.91	117.83	2.23
At3g16460	259327_at	jacalin lectin family protein	1281.88	2838.76	2.21
At1g07720	261420_at	beta-ketoacyl-CoA synthase family protein	103.34	228.49	2.21
At3g48680	252326_at	mitochondrial gamma carbonic anhydrase-like protein 2	370.42	816.72	2.20
At1g50240	262467_at	armadillo/beta-catenin repeat family protein	45.27	98.40	2.17
rpl14	244982_at	ribosomal protein L14	606.71	1314.06	2.17
At5g24290	249790_at	integral membrane family protein	103.25	223.18	2.16
At5g15290	250165_at	integral membrane family protein	76.74	164.64	2.15
At4g22490	254327_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	590.17	1266.24	2.15
At1g04640	264613_at	biotin/lipoate A/B protein ligase family protein	132.81	284.00	2.14
At4g39530	252899_at	pentatricopeptide (PPR) repeat-containing protein	22.82	48.69	2.13
At3g47860	252391_at	apolipoprotein D-related	268.55	570.31	2.12
At3g19850	257964_at	phototropic-responsive NPH3 family protein	202.78	429.05	2.12
At5g02540	251013_at	short-chain dehydrogenase/reductase (SDR) family protein	990.60	2103.62	2.12
At3g18000	258218_at	phosphoethanolamine N-methyltransferase 1 / PEAMT 1 (NMT1)	57.30	120.97	2.11
At5g26260	246825_at	meprin and TRAF homology domain-containing protein / MATH domain-containing protein	124.52	261.62	2.10
At5g13770	250257_at	pentatricopeptide (PPR) repeat-containing protein	441.84	926.92	2.10
At4g14440	245612_at	enoyl-CoA hydratase/isomerase family protein	152.79	316.07	2.07
At1g72030	256336_at	GCN5-related N-acetyltransferase (GNAT) family protein	260.73	540.67	2.07
At3g06170	256387_at	TMS membrane family protein / tumour differentially expressed (TDE) family protein	408.71	847.63	2.07
At5g28010	246727_at	Bet v I allergen family protein	27.87	57.55	2.06
At1g54010	263153_s_at	putative myrosinase-associated protein	180.84	371.47	2.05

At5g04530	250891_at	beta-ketoacyl-CoA synthase family protein	646.91	1326.66	2.05
At3g07940	258689_at	putative zinc finger and C2 domain protein	100.84	206.45	2.05
At1g55260	259660_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	297.63	606.97	2.04
At5g34940	255860_at	glycosyl hydrolase family 79 N-terminal domain-containing protein	54.39	110.77	2.04
At1g21090	261454_at	hydroxyproline-rich glycoprotein family protein	102.03	207.76	2.04
At5g12350	245210_at	regulator of chromosome condensation (RCC1) family protein	88.61	180.19	2.03
At3g48720	252317_at	transferase family protein	189.97	382.44	2.01
At2g25980	266838_at	jacalin lectin family protein	87.95	176.42	2.01
At3g06530	258505_at	BAP28-related	70.08	140.46	2.00
At2g28110	266156_at	exostosin family protein	116.17	232.26	2.00
At2g22170	263431_at	lipid-associated family protein	1120.95	2239.43	2.00
At1g17100	262536_at	SOUL heme-binding family protein	304.95	609.73	2.00
Unknown:					
At1g17620	260686_at	expressed protein	49.14	483.18	9.83
At2g18270	265322_at	expressed protein	25.93	214.75	8.28
At1g70550	260363_at	expressed protein	41.71	194.94	4.67
At3g63160	251155_at	expressed protein	44.99	209.98	4.67
At5g01790	251058_at	expressed protein	44.95	195.00	4.34
At3g22240	256617_at	expressed protein	485.50	2006.68	4.13
At5g64770	247252_at	expressed protein	44.22	180.32	4.08
At5g22970	249855_at	expressed protein	34.31	137.65	4.01
At1g10000	264519_at	expressed protein	26.89	107.43	3.99
At1g26920	263688_at	expressed protein	237.07	925.41	3.90
At1g67330	264998_at	expressed protein	81.88	315.39	3.85
At3g52110	252089_at	expressed protein	39.98	150.94	3.78
At5g10320	250482_at	expressed protein	27.30	96.94	3.55
At1g68380	260437_at	expressed protein	22.21	78.10	3.52
At3g48200	252353_at	expressed protein	148.22	512.50	3.46
At5g03230	250937_at	expressed protein	107.67	370.73	3.44
At5g04860	246982_s_at	expressed protein	39.81	137.03	3.44
At5g14330	250172_at	expressed protein	92.83	303.54	3.27
At3g44370	252682_at	expressed protein	3.65	11.53	3.16
At4g04330	255331_at	expressed protein	240.36	758.42	3.16
At2g21560	263545_at	expressed protein	37.68	116.04	3.08
At2g40435	263829_at	expressed protein	19.21	58.37	3.04
At3g11100	256413_at	expressed protein	70.52	213.06	3.02
At1g48580	261302_at	expressed protein	16.47	49.52	3.01
At5g08240	250575_at	expressed protein	72.96	216.53	2.97
At3g52040	252034_at	expressed protein	189.78	556.96	2.93
At3g60850	251389_at	expressed protein	114.78	333.08	2.90
At1g80240	262045_at	expressed protein	119.62	341.38	2.85
At1g08180	261817_at	expressed protein	39.74	112.55	2.83
At4g30670	253582_at	expressed protein	411.26	1137.90	2.77
At5g19970	246142_at	expressed protein	30.32	83.25	2.75
At2g47270	260527_at	expressed protein	60.80	165.82	2.73
At2g28780	266222_at	expressed protein	52.15	131.59	2.52
At2g29670	266617_at	expressed protein	503.31	1261.41	2.51
At2g15830	265539_at	expressed protein	51.81	129.51	2.50
At1g22630	264201_at	expressed protein	62.29	155.52	2.50

At5g48175	248717_at	expressed protein	22.32	54.87	2.46
At1g56260	256219_at	expressed protein	55.48	135.95	2.45
At3g61780	251278_at	expressed protein	47.43	116.00	2.45
At2g19950	266683_at	expressed protein	62.57	152.92	2.44
At3g16660	258418_at	expressed protein	395.18	956.39	2.42
At5g19300	246094_at	expressed protein	15.54	37.60	2.42
At5g60840	247596_at	expressed protein	216.24	521.79	2.41
At5g04790	250857_at	expressed protein	100.74	242.30	2.41
At5g12340	245209_at	expressed protein	47.14	113.29	2.40
At5g01400	251115_at	expressed protein	127.18	304.92	2.40
At2g31090	266476_at	expressed protein	323.94	764.11	2.36
At1g30130	256191_at	expressed protein	336.00	792.45	2.36
At2g37330	266004_at	expressed protein	180.00	416.53	2.31
At5g12950	250268_s_at	expressed protein	259.54	597.89	2.30
At2g14800	267110_at	expressed protein	20.78	47.74	2.30
At5g28500	245952_at	expressed protein	50.61	116.25	2.30
At2g04800	263673_at	expressed protein	64.18	147.12	2.29
At3g07470	259020_at	expressed protein	705.48	1607.80	2.28
At1g55960	260603_at	expressed protein	308.75	691.44	2.24
At3g22210	256796_at	expressed protein	221.64	496.22	2.24
At3g61380	251368_at	expressed protein	47.04	104.45	2.22
At5g50335	248509_at	expressed protein	38.00	84.13	2.21
At1g72640	259914_at	expressed protein	122.99	270.98	2.20
At2g29995	266808_at	expressed protein	463.22	1017.81	2.20
At2g44270	267396_at	expressed protein	80.82	177.27	2.19
At5g64180	247295_at	expressed protein	109.26	237.40	2.17
At1g15860	259497_at	expressed protein	189.21	409.80	2.17
At3g04550	258800_at	expressed protein	74.27	160.83	2.17
At2g17710	264590_at	expressed protein	723.79	1554.16	2.15
At2g38370	267054_at	expressed protein	94.26	200.88	2.13
At1g13930	262609_at	expressed protein	2684.05	5708.98	2.13
At5g46500	248846_at	expressed protein	8.85	18.72	2.11
At1g55370	259658_at	expressed protein	104.51	220.68	2.11
At1g56180	262065_at	expressed protein	87.62	184.04	2.10
At4g16146	245319_at	expressed protein	47.91	99.97	2.09
At2g44760	266874_at	expressed protein	93.41	193.40	2.07
At4g03180	255434_at	expressed protein	87.30	180.26	2.06
At4g34600	253246_at	expressed protein	245.04	505.96	2.06
At1g70100	264700_at	expressed protein	132.83	272.09	2.05
At4g32870	253401_at	expressed protein	188.70	386.38	2.05
At1g10660	257477_at	expressed protein	210.03	429.33	2.04
At3g51610	252065_at	expressed protein	303.93	619.32	2.04
At5g57785	247882_at	expressed protein	149.12	303.74	2.04
At4g33980	253322_at	expressed protein	82.03	167.02	2.04
At1g48360	262242_at	expressed protein	148.97	302.89	2.03
At2g41120	267063_at	expressed protein	95.17	193.36	2.03
At4g30790	253588_at	expressed protein	214.96	436.63	2.03
At4g17430	245421_at	expressed protein	67.31	136.57	2.03
At4g35760	253160_at	expressed protein	449.01	910.64	2.03
At5g16280	246501_at	expressed protein	106.66	215.30	2.02
At3g24870	257590_s_at	expressed protein	139.04	279.88	2.01
At3g04270	258572_at	expressed protein	72.12	144.80	2.01

Appendix E: T-DNA insertions

Precise location of T-DNA insertions in homozygous lines, as determined by DNA sequencing using T-DNA left border primers. Genomic nucleotide sequences were obtained from the TAIR database. The forward strand is shown in the 5' to 3' direction.

E.1 At5g42730: Ethylene-responsive element-binding factor 5

Figure E.1
GABI-Kat_681E07 (ERF5)

Uppercase and lowercase represent exons and untranslated regions respectively. Underlined regions indicate the gene specific probe used in northern blot analyses. Blue and green sections represent primers used in PCR screening of T-DNA lines, where LB stands for left border primer. Yellow areas denote primers used for amplification of northern blot probes. Arrows indicate the primer orientation

E.2 At4g17490: Ethylene-responsive element-binding factor 6

Figure E.2
SALK_087356 (ERF6)

agttgatacggccgctaaattttgatattgcaaatgcatgcatgcgaagaattctcacaacgagtagtaaatt
aatcaaatattaacacgaaaggtactttataataactattactaaaccgtactaatattttgataaacatgaa
tcgataaattcttgtctaatcatctgccaaagaaacccaatattagagcgcagcctaattaaaatatttgtgt
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aaaaaatcctgaggatgttgccttgaaagagtctctcattgacttgactctttcctataaatacacctttcca
ctctctcttctccactgtcaaatcaaatccctcttcctcttcaattctctgtttcctccaaaATGGCTACACC
AAACGAAGTATCAGCTCTTTTCCTCATCAAGAAGTATCTCCTCGACGAATTGTCTCCGTTGCCTACTACTG→
CCACCACCAATCGATGGATGAACGATTTCACGTCATTTGATCAAACCGGTTTCGAGTTTTCTGAATTTGAAAC
CAAACCGGAAATAATCGATCTCGTCACTCCCAAACCGGAGATTTTTGATTTCGATGTGAAATCTGAAATTCCA
TCTGAATCGAACGATTCCTTCACGTTCCAATCGAATCCTCCTCGC←GTTACTGTTCAATCCAATCGAAAACC
GCCGTTGAAGATCGCACCACCGAACCGAACCAAGTGGATTCAATTCGCAACCGGAAATCCTAAACCGGAACTT
CCCGTACCGGTTGTAGCAGCAGAGGAGAAGAGGCATTACAGAGGAGTGAGGATGAGGCCGTGGGGGAAATTCG
CGGCGGAGATTCGAGACCCGACTCGTCGTGGAACTCGTGTTTGGCTCGGGACGTTTGAGACGGCGATCGAAGC
GGCTAGAGCTTACGACAAAGAAGCGTTTAGAC→TACGAGGATCAAAGGCGATTCTGAATTTCCCGCTTGAAG
TTGACAAGTGGAATCCACGCGCTGAAGATGGTCGTGGCCTGTACAACAAACGGAAGAGAGACGGCGAGGAGGA
GGAAGTGACGGTGGTTGAGAAAGTGCTAAAGACGGAGGAGAGTTACGACGTTAGCGGCGGCGAGAATGTTGAG
TCAGGTTTGACGGCGATAGATGACTGGGATTTGACGGAGTTTCTGAGCATGCCGCTTT(SALK087356)LB
→TATCGCCGTTATCTCCACACCCACCGTTTGGTTATCCACAATTGACCGTTGTTTGAattctatttttctgt
ttgggtcagcttgttgtatgctgacgtggaattatgctatatcttttgcttattttatgttcacattcgagca
aaatttattcattcgtttatttgtttttttaatacaactcttgatatttctaaattctaaccaatcaccttatt
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aagctccgatcaaatgacttttatcatagaacgtattatttttcaatggcatgtaatatgctcggatttatt
accgtaacatctttagcagtctcgattatatcctatttgtgacttaatatttgaaagctccatgactcttttt
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atttctcatttgtgacttcatttttgaaatatctataaatttgtaatca

Uppercase and lowercase represent exons and untranslated regions respectively. Underlined regions indicate the gene specific probe used in northern blot analyses. Blue and green sections represent primers used in PCR screening of T-DNA lines, where LB stands for left border primer. Yellow areas denote primers used for amplification of northern blot probes. Arrows indicate the primer orientation.

E.3 At4g18950: Ankyrin protein kinase (APK)

Figure E.3
GABI-Kat_626D02 (APK)

tccttttcctctccgatgactttcttaccgaccagcttagtcttctccattacggcatcattcacctataaat
ttcgccgctgggtttaattctctattcattcagaaccaaaaaagaagaaaccttttgattctctttgcgatt
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caaatcagggttttagggtttttgttggaATGGAAGAGGATTATCAACAGCCGAGGTTTACGATTGG→TAGG
CAATCATCAATGGCGCCGGAGAAGATTCCGGAGCCGTCGGTTCACTCAGAAGAAGAGGTGTTTGAGGATGGAG
AAGAGATCGATGGTGGTGTGAGACTAATGTATTTGGCTAATGAAGGTGACATTGAAGGGATTAAGGAGCTTAT
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Tgtaagaagaaactaaaccaattgcttttttttttttggtgattgtctctctggttatgttgagtaatgatg
atgtttgttagtgatctaatacgttttagaaaacagCCATTTGCAGATGCGATATTTTACAAGAACATTGATG
TTATCAAGATTCTTGAGATACATGGAGCTAAACATCCGgtaagaatctggctagtaatgttggttttggtgtt
tgattggaagcttatttgtttttctcatgattgttgacacatttgtatgttttcagATGGCTCCAATGCACGT
TAAGACTGCTCGTGAAGTCCCTGAGTATGAAATAAATCCTAGTGAGCTTGATTTCACTCAAAGCAAAGAGATA
ACAAAGgtaaaaaacatgtttcatctgtaatggatcttgtatctataagtttgagtctttttgtcttaacag
tgtgaagattcttgtttcagGGAACTTACTGTATGGCAATGTGGCGTGGTATTCAAGTTGCGGTGAAAAAGCT
GGATGATGAAGTTTTGAGCGATGACGATCAAGTgtaagtaaatatcctgtagacggttctaacttctaaggtt
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CTCCAAAGGCTTAGGCATCCAAACATTGTGCAGTTTCTTGGTGCTGTAACCCAAACTAACCCAATGATGATTC
TGACTC→AATATTTGCCCAGGgtactgactttctctgataaatttgaaattatttttgcatttagtttcacg
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CCAGCTTGATATTGCTAGgtatgcaagttgaagctatatatgtctcttcttcgttgtttatatatgttctata
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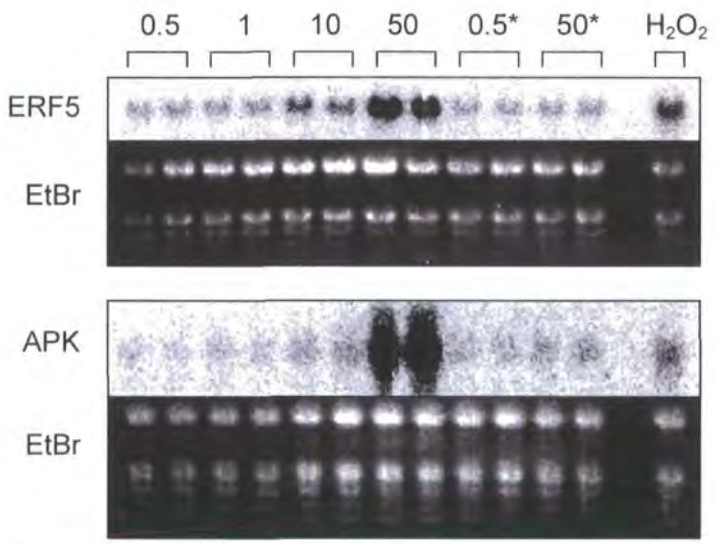
CCTATAATCCACCGCGATCTTGAACCTTCgtgagtcacactgtccatatcagtggtttctgtctcttatatgt
gcagtta←LB(GABIKat626D02)ctgacagcaaactctttctgttttcgttctgacattggtaaaactat
atttgcagAAACATTCTGCGGGATGATTCAGGGCATCTGAAAGTTGCAGACTTTGGAGTAAGCAAGCTTGTTA
CTGTTAAAGAAGACAAGCCTTTTACATGTCAAGACATTTCTTgtaagtacttttcgcacccaactagttact
gtaaattttgaatgctttaattcttgatctgcttggttaaagagtcttcattactggatgtgttataataaaa
atttatcggtgttatccacattatagGTCGATATATAGCTCC←CGAGGTTTTCACTAGTGAAGAATACGATA
CAAAAGCTGATGTTTTCTCATTTGCATTGATCGTTCAGGAGgtaagtaaaaactaaaaacttgtgactttctc
aagtatttttttcatgtcattgattccaatgtcggattctctagATGATCGAAGGCCGAATGCCGTTTGCTG
AAAAGGAAGACAGTGAAGCTTCTGAAGCTTATGCTGGCAAACATCGGCCATTATTCAAAGCTCCATCAAAGAA
TTACCCACATGGTCTTAAAACgtgagtctttcttctctctttcaccaagaaactaaatacttatgaacagttt
tgcatcattgattggtttggttcatgtcattaatccgatttttgaatcccatagGTTGATAGAAGAATGCTGG
CATGAGAAACCTGCAAAGCGACCAACTTTCAGAGAGATCATTAAACGACTTGAGTCCATTCTTCACCACATGG
GTCACAAGCGACAATGGAGGgtctgtttctctatatctctatcaaacacacattgagttttgtttgtttgtct
ctctgatgtgtttctacttctacatgttgtctttttctttctcagATGAGGCCATTGACATGCTTTCAGAAT
TTCGAGCACAAGAAGAAACATAATTGGGATTTGAGCAGCCATGACGGCTCATCATCCGGTTCACATTTGTGAt
tctttaaacggttaaagaaaccattcttttgtatccattccttccggtttagtttgggtttccccaaagcaac
cgaatttgactttgtatatttgttgtacactgaagaactcaagaaagtcttcgtgtatacaaaaactgcattt
ttttattctagtatacaaaattgttgtaagatctatgattttaaccgcaatatatatttgctgttgtaatct
aaaaaattggtttaagaatatttatgatttttcctcaatc

Uppercase and lowercase represent exons and untranslated regions respectively. Underlined regions indicate the gene specific probe used in northern blot analyses. Blue and green sections represent primers used in PCR screening of T-DNA lines, where LB stands for left border primer. Yellow areas denote primers used for amplification of northern blot probes. Arrows indicate the primer orientation.

Appendix F: Supplemental northern blot analysis data

F.1 Response to menadione

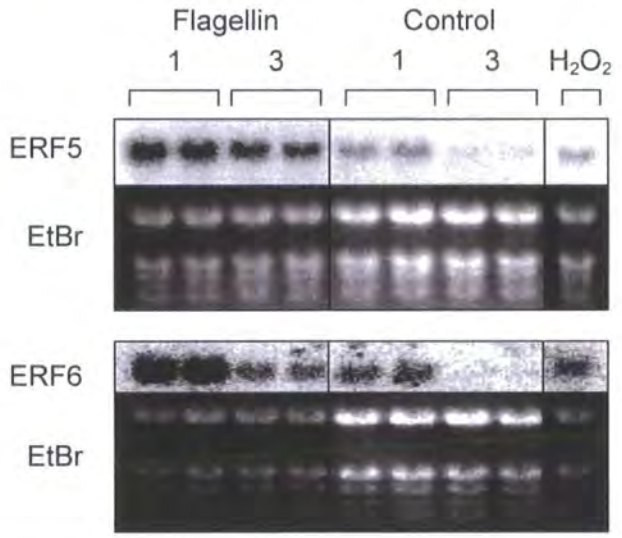
Figure F.1
Northern blot analysis of *ERF5* and *APK* in response to various different menadione concentrations.



Wild-type seedlings (10 days old) were incubated for 3 h with water prior to a 3 h menadione treatment with a final concentration of either 0.5, 1, 10 or 50 µM in DMSO. DMSO controls for are indicated by asterisks. A 10 mM H_2O_2 treatment was also included for comparison. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate (except H_2O_2).

F.2 Response to flagellin

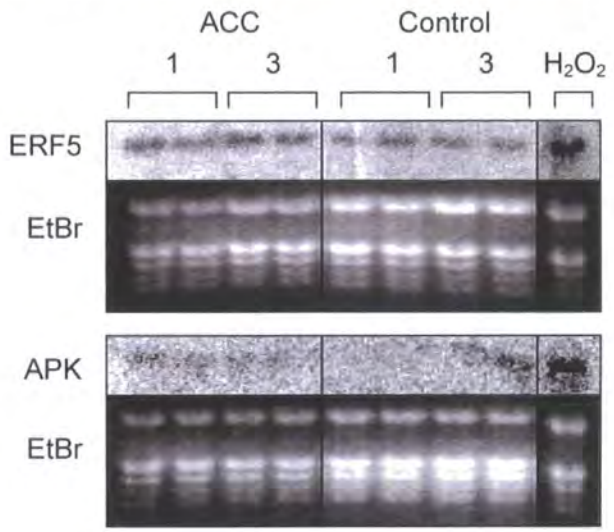
Figure F.2
Northern blot analysis of *ERF5* and *ERF6* in response to flagellin treatment.



Wild-type seedlings (10 days old) were incubated for 3 h with water prior to treatment with a final concentration of 1 µM flagellin-22 in 0.1 % DMSO or 0.1 % DMSO only (control) for 1 or 3 h. A 3 h 10 mM H_2O_2 treatment was also included for comparison. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate (except H_2O_2).

F.3 Response to ACC

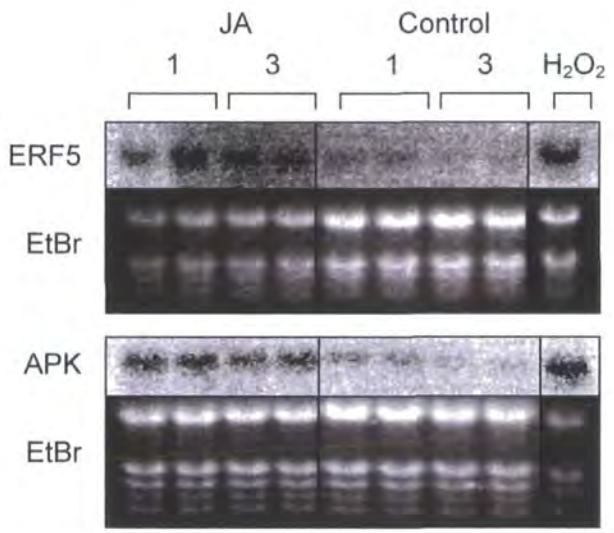
Figure F.3
Northern blot analysis of *ERF5* and *APK* in response to ethylene treatment.



Wild-type seedlings (10 days old) were incubated for 3 h with water prior to treatment with a final concentration of 100 µM ACC or water (control) for 1 or 3 h. A 3 h 10 mM H_2O_2 treatment was also included for comparison. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate (except H_2O_2).

F.4 Response to methyl jasmonate

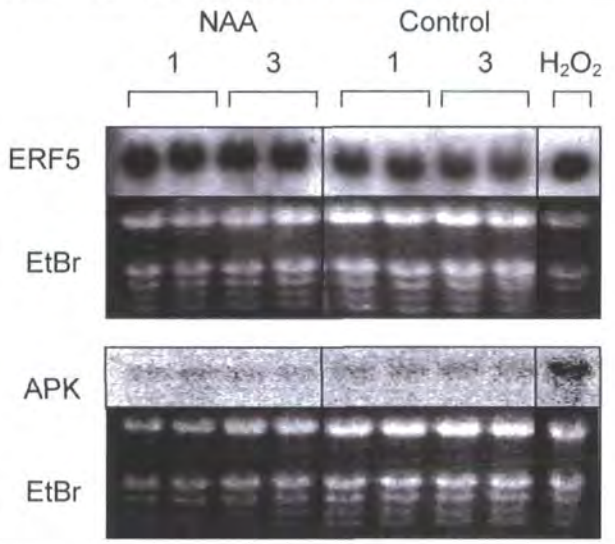
Figure F.4
Northern blot analysis of *ERF5* and *APK* in response to meJA treatment.



10 day old wild-type seedlings were incubated for 3 h with water prior to treatment with a final concentration of 100 µM meJA in 0.1 % ethanol or 0.1 % ethanol only (control) for 1 or 3 h. A 3 h 10 mM H_2O_2 treatment was also included for comparison. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate (except H_2O_2).

F.5 Response to auxin

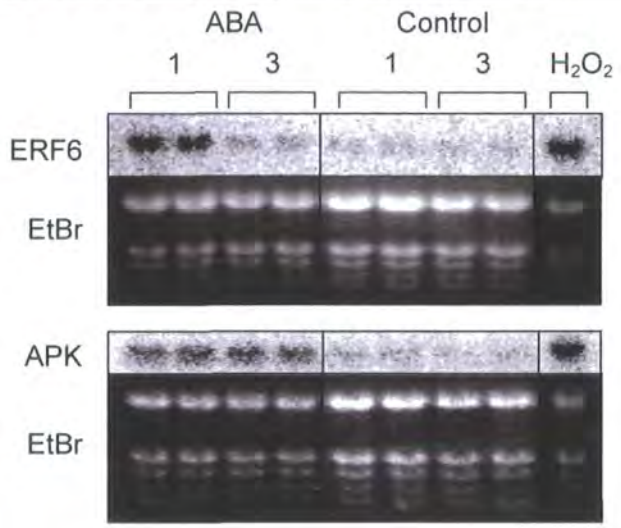
Figure F.6
Northern blot analysis of *ERF5* and *APK* in response to auxin treatment.



Wild-type seedlings (10 days old) were incubated for 3 h in water prior to treatment with a final concentration of 1 µg/ml NAA or water (control) for 1 or 3 h. A 3 h 10 mM H_2O_2 treatment was also included for comparison. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate (except H_2O_2).

F.6 Response to abscisic acid

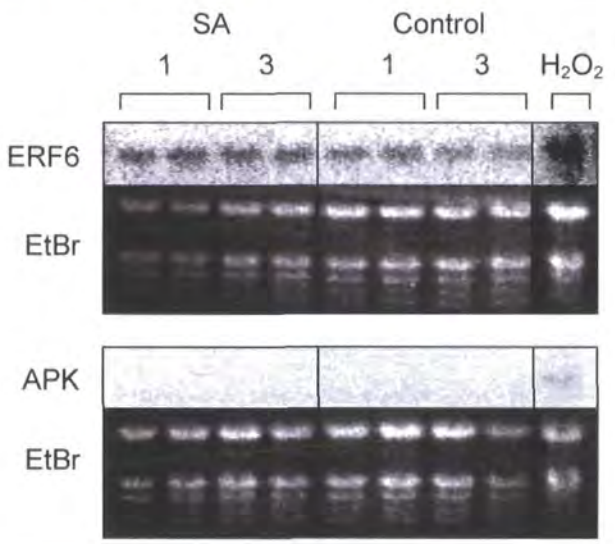
Figure F.7
Northern blot analysis of *ERF6* and *APK* in response to ABA treatment.



Wild-type seedlings (10 days old) were incubated for 3 h in water prior to treatment with a final concentration of 100 µM ABA in 0.1 % ethanol or 0.1 % ethanol only (control) for 1 or 3 h. A 3 h 10 mM H_2O_2 treatment was also included for comparison. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate (except H_2O_2).

F.7 Response to salicylic acid

Figure F.5
Northern blot analysis of *ERF6* and *APK* in response to SA treatment.



Wild-type seedlings (10 days old) were incubated for 3 h with water prior to treatment with a final concentration of 100 µM sodium salicylate or water (control) for 1 or 3 h. A 3 h 10 mM H_2O_2 treatment was also included for comparison. Ethidium bromide (EtBr) staining of the corresponding RNA gel is shown (10 µg total RNA per lane). Samples are in duplicate (except H_2O_2).

Appendix G: Transcriptomic Changes in Response to *APK* over-expression

G.1 Transcript induction by APK over-expression

Only transcripts with at least a 1.5-fold induction and present only calls in all 3 slides are shown. Annotations are according to TAIR version 6 genome release (2007). For full details of the microarray experiment please refer to Results Chapter 6.

AGI code	Gene annotation	Fold induction			
		Slide 1	Slide 2	Slide 3	Average
At4g18950	**ankyrin protein kinase (over-expressed gene)**	**3.83**	**7.31**	**12.95**	**8.03**
At3g14890	phosphoesterase	5.24	3.94	1.53	3.57
At2g43950	expressed protein	1.68	4.02	4.18	3.29
At1g12630	AP2 domain-containing protein similar to DREB1B	3.37	2.68	3.12	3.06
At5g28270	hypothetical protein	1.67	1.64	5.10	2.80
At2g45940	hypothetical protein	1.71	1.74	4.74	2.73
At2g20465	expressed protein	2.52	2.30	3.14	2.65
ath-MIR398c	miRNA gene Arabidopsis thaliana miR398c stem-loop	2.13	2.39	3.32	2.61
At4g02810	expressed protein	2.51	2.03	3.07	2.53
At1g23270	hypothetical protein	1.56	1.82	3.72	2.36
At5g09510	40S ribosomal protein S15 (RPS15D)	1.52	3.66	1.51	2.23
At3g16120	dynein light chain, putative	1.53	2.24	2.80	2.19
At1g52700	phospholipase/carboxylesterase family protein	1.74	2.94	1.85	2.18
At2g35612	expressed protein	1.86	2.65	1.86	2.12
At2g25630	glycosyl hydrolase family 1 protein	2.26	2.22	1.86	2.11
At1g64100	pentatricopeptide (PPR) repeat-containing protein	2.68	1.54	1.98	2.07
At2g27402	expressed protein	2.87	1.58	1.66	2.04
At5g50930	hypothetical protein	1.53	1.60	2.79	1.97
At5g39000	protein kinase family protein	2.10	2.23	1.57	1.97
At3g44920	cation/hydrogen exchanger, putative (CHX11)	2.64	1.69	1.54	1.96
At2g39650	expressed protein	2.67	1.62	1.51	1.94
At1g60640	expressed protein	2.46	1.67	1.60	1.91
At3g45150	TCP family transcription factor, putative (TCP16)	2.32	1.74	1.60	1.89
At2g20815	expressed protein	1.91	1.77	1.91	1.86
At3g43750	zinc finger (C3HC4-type RING finger) family protein	1.86	1.68	1.96	1.83
At1g13750	calcineurin-like phosphoesterase family protein	1.62	2.15	1.70	1.82
At1g11120	expressed protein	1.64	1.52	2.24	1.80
At3g61660	hypothetical protein	2.13	1.75	1.52	1.80
At3g14370	protein serine/treonine kinase protein (WAG2)	1.56	1.66	2.14	1.79
At1g49570	peroxidase, putative identical to peroxidase	2.25	1.50	1.52	1.76

	ATP5a				
At5g36925	expressed protein	1.91	1.68	1.59	1.73
At5g57240	oxysterol-binding family protein	1.81	1.55	1.80	1.72
At3g51950	zinc finger (CCCH-type) family protein / RNA recognition motif (RRM)-containing protein	1.54	2.04	1.53	1.70
At3g48080	lipase class 3 family protein / disease resistance protein-related	1.65	1.67	1.77	1.70
At2g40910	F-box protein-related similar to F-box protein family, AtFBX9	1.83	1.55	1.70	1.69
At5g24105	expressed protein	1.74	1.53	1.54	1.60

Appendix H: Transcriptomic Changes in Response to *ERF5* over-expression

H.1 Transcript induction by ERF5 over-expression

Only transcripts with at least a 1.5-fold induction and present only calls in all 3 slides are shown. Grey highlighting denotes genes common to the *ER6* up-regulated gene list (Appendix I2). Annotations are according to TAIR version 6 genome release (2007). For full details of the microarray experiment please refer to Results Chapter 6.

AGI code	Gene annotation	Fold induction			
		Slide 1	Slide 2	Slide 3	Average
At5g44420	plant defensin protein, putative (PDF1.2a)	24.74	28.72	11.94	21.80
At5g47230	**ethylene-responsive element-binding factor 5 (ERF5) (over-expressed gene)**	**40.90**	**2.32**	**12.36**	**18.53**
At4g33720	pathogenesis-related protein, putative similar to Pathogenesis-related protein 1 precursor (PR-1)	20.21	20.33	8.62	16.39
At1g55010	plant defensin-fusion protein, putative (PDF1.5)	12.29	19.92	15.49	15.90
At2g26020	plant defensin-fusion protein, putative (PDF1.2b)	13.18	23.72	5.29	14.06
At1g75830	plant defensin-fusion protein, putative (PDF1.1)	15.84	19.16	4.20	13.07
At2g26010	plant defensin-fusion protein, putative (PDF1.3)	11.74	20.83	3.06	11.88
At5g44430	plant defensin-fusion protein, putative (PDF1.2c)	7.47	24.35	2.81	11.54
At1g02930	glutathione S-transferase, putative	15.42	4.00	3.60	7.67
At1g02920	glutathione S-transferase, putative	13.59	4.41	2.90	6.97
At3g53260	phenylalanine ammonia-lyase 2 (PAL2)	7.33	5.75	4.07	5.72
At3g15356	legume lectin family protein	3.05	9.00	2.07	4.70
At1g78860	curculin-like (mannose-binding) lectin family protein	3.80	6.60	1.99	4.13
At3g49110	peroxidase 33 (PER33) (P33) (PRXCA) / neutral peroxidase C (PERC)	3.45	6.50	1.68	3.88
At4g16260	glycosyl hydrolase family 17 protein	4.24	4.47	2.53	3.75
At3g04720	pathogenesis-related protein 4 (PR-4)	5.86	1.92	3.13	3.63
At1g02030	zinc finger (C2H2 type) family protein identical to C2H2 zinc finger protein ZAT1	3.56	2.21	4.97	3.58
At1g78850	curculin-like (mannose-binding) lectin family protein	4.11	2.44	3.77	3.44
At4g06746	AP2 domain-containing transcription factor family protein	2.17	5.54	1.74	3.15
At4g38390	expressed protein	2.43	3.62	3.26	3.10
At3g49620	2-oxoacid-dependent oxidase, putative (DIN11)	2.78	2.78	3.62	3.06
At5g18980	expressed protein	3.77	1.68	3.55	3.00
At3g32130	hypothetical protein	2.89	3.32	2.22	2.81
At5g48430	expressed protein	3.62	3.01	1.75	2.80
ath-MIR157c	miRNA gene Arabidopsis thaliana miR157c	1.58	5.03	1.54	2.72

	stem-loop				
At2g25735	expressed protein	3.54	2.55	2.05	2.71
At5g18130	expressed protein	1.53	3.30	3.21	2.68
At2g02930	glutathione S-transferase, putative	3.30	2.61	2.10	2.67
At2g41640	expressed protein	2.11	3.80	1.93	2.61
At2g26560	patatin, putative	3.39	2.69	1.67	2.58
At5g31752	hypothetical protein	1.62	2.68	3.36	2.55
At1g45545	hypothetical protein	3.02	2.85	1.73	2.53
At3g55230	disease resistance-responsive family protein	1.97	3.82	1.67	2.49
At5g42370	expressed protein	2.51	1.67	3.27	2.49
At5g57785	expressed protein	2.12	2.57	2.25	2.31
At1g31000	F-box family protein	2.29	2.85	1.79	2.31
At2g44900	armadillo/beta-catenin repeat family protein / F-box family protein	2.85	1.89	2.19	2.31
At2g28190	superoxide dismutase [Cu-Zn], chloroplast (SODCP) / copper/zinc superoxide dismutase (CSD2)	2.31	1.56	3.01	2.29
At1g21110	O-methyltransferase, putative	2.61	2.31	1.92	2.28
At1g55450	embryo-abundant protein-related	2.75	1.98	2.06	2.26
At1g73580	C2 domain-containing protein	1.78	2.25	2.66	2.23
At2g30810	gibberellin-regulated family protein	2.09	2.41	2.18	2.23
At2g32275	Expressed protein	2.31	2.28	2.01	2.20
At1g48070	expressed protein	2.13	1.69	2.74	2.18
At4g33495	expressed protein	1.57	3.18	1.80	2.18
At1g03905	ABC transporter family protein	1.58	2.67	2.20	2.15
At5g48770	disease resistance protein (TIR-NBS-LRR class)	2.89	1.86	1.64	2.13
At3g55710	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1.53	3.09	1.73	2.12
At4g35750	Rho-GTPase-activating protein-related	1.54	2.66	2.11	2.10
At2g45220	pectinesterase family protein	2.65	1.71	1.93	2.10
At5g57910	expressed protein	1.51	2.42	2.34	2.09
At1g53990	GDSL-motif lipase/hydrolase family protein	2.60	1.68	1.97	2.08
At2g18010	auxin-responsive family protein	2.54	1.94	1.75	2.08
At2g28755	UDP-D-glucuronate carboxy-lyase-related	2.59	1.56	2.00	2.05
At5g40610	glycerol-3-phosphate dehydrogenase [NAD+] / GPDH	2.24	1.51	2.38	2.04
At3g57710	protein kinase family protein	1.63	2.05	2.45	2.04
At4g35890	La domain-containing protein	1.91	1.73	2.48	2.04
At3g54880	expressed protein	1.76	2.28	1.98	2.01
At5g28630	glycine-rich protein	2.44	1.54	1.95	1.98
At5g33220	hypothetical protein	1.94	2.20	1.76	1.97
At2g22080	expressed protein	1.58	1.97	2.35	1.97
At3g22640	cupin family protein	1.52	2.23	2.14	1.97
At3g50210	2-oxoacid-dependent oxidase, putative	1.58	2.62	1.69	1.96
At4g21350	U-box domain-containing protein similar to immediate-early fungal elicitor protein CMPG1	1.85	2.47	1.56	1.96
At5g47640	CCAAT-box binding transcription factor subunit B (NF-YB) (HAP3) (AHAP3) family (Hap3b)	1.74	1.70	2.43	1.96
At2g35030	pentatricopeptide (PPR) repeat-containing protein	1.86	1.89	2.12	1.96
At5g26670	pectinacetylesterase, putative	1.71	1.61	2.49	1.94
At3g04290	GDSL-motif lipase/hydrolase family protein	2.25	1.79	1.76	1.93
At5g10760	aspartyl protease family protein	1.82	1.58	2.36	1.92
At5g46680	pentatricopeptide (PPR) repeat-containing	1.97	2.03	1.75	1.92

	protein				
At1g78820	curculin-like (mannose-binding) lectin family protein / PAN domain-containing protein	2.25	1.89	1.57	1.90
At1g30250	expressed protein	1.55	1.68	2.42	1.88
At1g49340	phosphatidylinositol 3- and 4-kinase family protein	1.62	1.62	2.41	1.88
At4g22210	hypothetical protein	1.88	1.72	1.96	1.85
At1g53890	expressed protein	1.64	1.62	2.29	1.85
At1g55870	CAF1 family ribonuclease	1.66	1.64	2.24	1.85
At5g61010	exocyst subunit EXO70 family protein leucine zipper-containing protein	1.66	1.85	1.98	1.83
At5g49530	SIN-like family protein	1.85	1.74	1.87	1.82
At3g62760	glutathione S-transferase, putative	1.64	1.90	1.87	1.80
At1g14610	valyl-tRNA synthetase / valine--tRNA ligase (VALRS)	1.57	1.51	2.27	1.78
At5g45360	F-box family protein similar to SKP1 interacting partner 2 (SKIP2)	1.83	1.85	1.66	1.78
At4g11290	peroxidase, putative identical to peroxidase ATP19a	1.83	1.58	1.88	1.76
At5g11670	malate oxidoreductase, putative similar to NADP-dependent malic enzyme	2.00	1.77	1.51	1.76
At1g33680	KH domain-containing protein similar to FUSE binding protein 2	1.89	1.61	1.73	1.74
At5g47840	adenylate kinase, chloroplast, putative / ATP-AMP transphosphorylase, putative	1.57	1.66	1.90	1.71
At5g45070	disease resistance protein (TIR class)	1.95	1.57	1.59	1.70
At5g57970	methyladenine glycosylase family protein	1.80	1.67	1.61	1.69
At1g47870	E2F transcription factor-2 (E2F2)	1.84	1.61	1.59	1.68
At1g79400	cation/proton exchanger, putative (CHX2)	1.98	1.52	1.52	1.68
At4g04780	expressed protein	1.55	1.52	1.84	1.64
At2g22010	zinc finger (C3HC4-type RING finger)	1.54	1.56	1.74	1.61

H.2 Transcript repression by ERF5 over-expression

Only transcripts with at least a 1.5-fold repression and present only calls in all 3 slides are shown. Grey highlighting denotes genes common to the *ER6* down-regulated gene list (Appendix I2). Annotations are according to TAIR version 6 genome release (2007). For full details of the microarray experiment please refer to Results Chapter 6.

AGI code	Gene annotation	Fold repression			
		Slide 1	Slide 2	Slide 3	Average
At1g08800	expressed protein	12.87	1.61	9.40	3.73
At4g21820	calmodulin-binding family protein	1.70	3.39	6.22	2.88
At3g30720	expressed protein	3.06	2.60	2.19	2.57
At1g77120	alcohol dehydrogenase 1 (ADH1)	2.53	2.80	2.12	2.45
At4g25210	expressed protein	1.52	3.90	2.75	2.35
At1g10585	expressed protein	3.41	2.34	1.68	2.28
At2g42540	cold-responsive protein / cold-regulated protein (cor15a)	2.57	2.24	1.78	2.15
At1g52310	protein kinase family protein / C-type lectin domain-containing protein	1.72	1.90	3.02	2.08
At3g29610	hypothetical protein	1.64	1.52	5.08	2.05
At5g67350	expressed protein	1.54	1.66	3.99	2.00
At2g28900	mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	4.53	1.53	1.56	1.98
At2g17850	senescence-associated family protein	2.49	1.65	1.91	1.96
At5g66890	disease resistance protein (CC-NBS-LRR class), putative	2.07	1.80	2.01	1.95
At3g58930	F-box family protein	1.82	1.77	2.20	1.91
At3g56450	alpha-soluble NSF attachment protein 2 / alpha-SNAP1 (ASNAP2)	1.89	1.74	2.16	1.91
At1g01225	NC domain-containing protein	1.64	2.42	1.74	1.88
At3g17150	invertase/pectin methylesterase inhibitor family protein	1.67	1.77	2.26	1.87
At3g01830	calmodulin-related protein, putative	1.69	1.98	1.84	1.83
At2g37770	aldo/keto reductase family protein	2.34	1.51	1.77	1.81
At4g09360	disease resistance protein (NBS-LRR class), putative	2.18	1.69	1.57	1.78
At3g56660	bZIP transcription factor family protein	1.92	1.71	1.66	1.76
At1g02800	endo-1,4-beta-glucanase / cellulase (CEL2)	1.63	1.70	1.61	1.65
At3g14170	expressed protein	1.68	1.56	1.60	1.61

Appendix I: Transcriptomic Changes in Response to *ERF6* over-expression

I.1 Transcript induction by ERF6 over-expression

Only transcripts with at least a 1.5-fold induction and present only calls in all 3 slides are shown. Grey highlighting denotes genes common to the *ER5* up-regulated gene list (Appendix H1). Annotations are according to TAIR version 6 genome release (2007). For full details of the microarray experiment please refer to Results Chapter 6.

AGI code	Gene annotation	Fold induction			
		Slide 1	Slide 2	Slide 3	Average
At5g44420	plant defensin protein, putative (PDF1.2a)	11.79	16.07	26.24	18.03
At1g75830	plant defensin-fusion protein, putative (PDF1.1)	9.49	13.47	24.09	15.68
At2g26020	plant defensin-fusion protein, putative (PDF1.2b)	6.49	17.78	19.87	14.71
At4g33720	pathogenesis-related protein, putative similar to Pathogenesis-related protein 1 precursor (PR-1)	11.24	16.02	14.80	14.02
At5g44430	plant defensin-fusion protein, putative (PDF1.2c)	4.32	11.63	16.07	10.68
At2g26010	plant defensin-fusion protein, putative (PDF1.3)	3.48	11.70	16.66	10.61
At1g55010	plant defensin-fusion protein, putative (PDF1.5)	6.44	4.58	16.38	9.14
At1g02920	glutathione S-transferase, putative	7.35	3.48	6.82	5.88
At1g02930	glutathione S-transferase, putative	10.12	2.02	4.32	5.49
At2g18980	peroxidase, putative	3.53	2.52	4.63	3.56
At1g06100	fatty acid desaturase family protein	2.62	6.26	1.76	3.55
At3g04720	pathogenesis-related protein 4 (PR-4)	5.00	1.96	3.45	3.47
At3g15356	legume lectin family protein	1.76	4.94	3.56	3.42
At1g03905	ABC transporter family protein	2.26	3.77	4.17	3.40
At4g17615	calcineurin B-like protein 1 (CBL1)	5.66	2.40	1.88	3.32
At4g16260	glycosyl hydrolase family 17 protein	3.28	3.32	3.14	3.25
At3g49110	peroxidase 33 (PER33)	2.68	3.43	3.27	3.13
At4g36030	armadillo/beta-catenin repeat family protein	2.00	4.07	2.65	2.91
At3g45500	hypothetical protein	2.44	2.71	3.54	2.90
At4g06746	AP2 domain-containing transcription factor family protein	1.92	2.93	3.63	2.83
At1g27020	expressed protein	2.75	3.00	2.70	2.82
At3g20840	plethora 1 (plt1)	2.21	4.58	1.61	2.80
At2g26560	patatin, putative	3.03	1.94	3.35	2.77
At1g74420	fucosyltransferasen 3 (FUT3)	1.66	1.54	4.47	2.56
At1g78860	curculin-like (man0se-binding) lectin family protein	2.28	2.96	2.40	2.55
At4g11650	osmotin-like protein (OSM34)	2.16	2.73	2.70	2.53
At5g09970	cytochrome P450 family protein	2.02	3.57	2.01	2.53
At2g33050	leucine-rich repeat family protein	1.91	1.59	3.81	2.44
At1g78850	curculin-like (man0se-binding) lectin family protein	2.90	1.59	2.70	2.40

At5g06390	beta-lg-H3 domain-containing protein / fasciclin domain-containing protein	2.75	1.62	2.27	2.21
At1g21120	O-methyltransferase, putative	1.57	3.53	1.50	2.20
ath-MIR169b	miRNA gene	2.19	1.80	2.51	2.17
At5g61070	histone deacetylase 18 (HDA18)	2.25	1.83	2.37	2.15
At5g47450	arabidopsis thaliana intrinsic protein 2;3 (ATTIP2;3)	1.53	3.16	1.62	2.10
At1g77550	tubulin-tyrosine ligase family protein	2.95	1.56	1.61	2.04
At3g52010	serine carboxypeptidase S10 family protein	1.77	1.96	2.37	2.03
At4g17490	**ethylene-responsive element-binding protein 6 (ERF6) (over-expressed gene)**	**2.11**	**1.82**	**2.15**	**2.03**
At2g25735	expressed protein	1.82	2.25	1.99	2.02
At5g26670	pectinacetylesterase, putative	2.37	1.96	1.56	1.97
At1g49960	xanthine/uracil permease family protein	1.71	2.20	1.83	1.91
At1g33670	leucine-rich repeat family protein	2.22	1.98	1.51	1.90
At5g23850	expressed protein	1.72	1.89	1.95	1.85
At3g24260	hypothetical protein	1.81	1.87	1.80	1.83
At4g37520	peroxidase 50 (PER50)	2.04	1.50	1.83	1.79
At1g59870	ATP binding cassette transporter (PEN3)	1.77	1.80	1.79	1.79
At5g07590	WD-40 repeat protein family contains 3 WD-40 repeats	2.07	1.61	1.66	1.78
At2g18550	homeobox-leucine zipper family protein	1.89	1.87	1.53	1.76
At4g11290	peroxidase, putative identical to peroxidase ATP19a	1.92	1.73	1.64	1.76
At5g43030	DC1 domain-containing protein	1.99	1.53	1.68	1.73
At1g79630	protein phosphatase 2C family protein / PP2C family protein	1.85	1.79	1.52	1.72
At1g64710	alcohol dehydrogenase, putative	1.53	1.76	1.51	1.60
At5g40380	protein kinase family protein	1.55	1.54	1.58	1.56

I.2 Transcript repression by ERF6 over-expression

Only transcripts with at least a 1.5-fold repression and present only calls in all 3 slides are shown. Grey highlighting denotes genes common to the *ER5* down-regulated gene list (Appendix H2). Annotations are according to TAIR version 6 genome release (2007). For full details of the microarray experiment please refer to Results Chapter 6.

AGI code	Gene annotation	Fold repression			
		Slide 1	Slide 2	Slide 3	Average
At4g30040	aspartyl protease family	8.70	2.97	2.73	3.67
At3g21230	4-coumarate--CoA ligase 5 (4CL5)	3.41	3.45	2.48	3.04
At1g02965	hypothetical protein	3.54	3.17	2.23	2.87
At1g22130	MADS-box family protein	2.00	4.54	3.01	2.85
At1g06260	cysteine proteinase, putative	2.34	1.59	3.87	2.28
At2g32610	cellulose synthase family protein	2.10	3.51	1.74	2.25
At2g05370	expressed protein	2.48	1.76	2.53	2.20
At2g34440	MADS-box family protein	2.11	3.96	1.54	2.18
At1g10585	expressed protein	2.61	2.12	1.70	2.08
At4g32370	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	2.56	2.40	1.50	2.03
At3g52940	C-14 sterol reductase / delta(14)-sterol reductase / FACKEL (FK) / HYDRA 2	2.06	2.73	1.59	2.03
At1g50360	myosin family protein contains	1.76	1.80	2.69	2.01
At5g28190	hypothetical protein	1.51	1.69	3.54	1.95
At3g55630	dihydrofolate synthetase/folylpolyglutamate synthetase (DHFS/FPGS4)	2.10	1.52	2.37	1.93
At1g02850	glycosyl hydrolase family 1 protein	1.97	1.68	2.14	1.91
At2g29470	glutathione S-transferase, putative	1.56	2.26	1.91	1.87
At1g16850	expressed protein	1.50	2.21	1.98	1.85
At1g54700	hypothetical protein	1.60	1.62	2.58	1.84
At5g24750	expressed protein	2.16	1.86	1.56	1.83
At4g39810	exonuclease family protein	1.96	1.55	1.61	1.69
At2g24950	hypothetical protein	1.65	1.73	1.52	1.63