The role of SENSITIVE TO FREEZING6 in plant tolerance to stress

WATHUGALA-GAMAGE, DEEPTHI,LAKMINI

The role of SENSITIVE TO FREEZING6 (SFR6) in plant tolerance to stress

Wathugala GD Lakmini

Submitted for the Degree of Doctor of Philosophy by Research

School of Biological and Biomedical Sciences

April 2010

Statement of Authorship

I certify that all work described in this thesis is my own original research unless acknowledged in the text or by references, and has not been subtitled for a degree in this or any other university.

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Dedication

This thesis is dedicated to my daughter **IG Jayadhi Nuwandana** and my husband **IG Janaka Manohara** for their love and endless support during this study.

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Abstract

The current rate of climate change predicts that plants will become subject to increasing extremes of environmental stress. Rapid population increases in developing countries also demand higher yield from crop production, often from sub-optimal agricultural areas. Genetic engineering can help meet these needs through the development of crops with greater stress tolerance. Identification of *Arabidopsis* mutants unable to tolerate stress is a powerful approach to investigate the molecular basis of stress tolerance in plants and so identify targets for modification. The *Arabidopsis* mutant s*fr6* (***s****ensitive to* ***fr****eezing****6***) is unable to tolerate freezing stress, due to failure of CBF transcription factors to activate downstream cold responsive (*COR*) gene expression.

In this study, I assessed the suitability of using SFR6 as a molecular tool to improve low temperature tolerance of crop plants. These results show that complementation of *sfr6* with the wild type *SFR6* gene restores *COR* gene expression and freezing tolerance; however over-expression of the gene in wild type has no effect. This suggests that SFR6 is essential but not sufficient to initiate *COR* gene expression controlled by CBF transcription factors. *At4g04920,* the gene encoding SFR6, has been identified as a component of the multi-subunit transcriptional co-activator, known as mediator, which controls eukaryotic inducible gene expression, which explains these findings.

A homologue of SFR6 was cloned from rice (OsSFR6) and over-expression of *OsSFR6* in the *sfr6-1* mutant led to complementation, demonstrating orthology. Rice is a freezing-intolerant species, and therefore the presence of a functional SFR6 orthologue in rice capable of restoring *Arabidopsis* CBF function suggests that OsSFR6 may play a role in chilling tolerance in rice analogous to that of CBF/DREB1 in other chilling sensitive species.

The responses of *sfr6* mutants to a range of biotic and abiotic stress stimuli revealed a hypersensitivity to both UV radiation and infection by both virulent and avirulent *Psuedomonas syringae* DC3000. This reduced tolerance correlates in *sfr6* with

reduced mRNA levels of UV irradiance and pathogen infection response genes, including *META CASPASE8* (*MC8*), *PATHOGENESIS RELATED1* (*PR1*) and *ENHANCED DISEASE SUSCEPTIBILITY5* (*EDS5*).

Keywords: SFR6, cold acclimation, *Arabidopsis*, freezing tolerance, *COR* genes, rice, orthologue, defence gene expression, *Psuedomonas*, UV radiation

Abbreviations

Standard abbreviations for length (mm, cm), weight (ng, µg, mg, g), volume (µl, ml), temperature (^{o}C) amount (µmol), molarity (µM, mM, M), time (s, min, h) energy (KJ) and pressure (pa, psi) are used.

Standard chemical element symbols, amino acid and protein codes are also used.

Standard convention for gene and protein naming is used: genes are italicised and proteins are not, wild type genes and proteins are capitalised and mutant genes and proteins are not.

All other abbreviations used in this thesis are defined below.

ABA- Abscisic acid
ABA1- Abscisic acid deficient
ABF- Abscisic acid binding factor
ABI1- Abscisic acid insensitive
ABRE- Abscisic acid responsive element
ADH- Alcohol dehydrogenase
AFP- Anti freeze protein
AP2/EREBP- Apetala2/ethylene-responsive element binding proteins
bHLH- basic helix–loop–helix
bZIP- basic-domain leucine zipper
CAS- Cold accumulation specific
CBF- C-repeat binding factor
CDK- Cyclin dependent Kinase
COR- Cold regulated
COS- Cold standard set
CRT- C-repeat
DEAR- DREB and EAR motif protein
DRE- Drought responsive element
DREB- Drought responsive element binding factor

EAR- ERF-associated amphilic repressor

EE- Evening element

EEL- EE-Like

EIL- Expansion induced lysis

EM- Electron microscope

EMS- ethane methyl sulfonate

ERF- Ethylene responsive factor

ERF- Ethylene responsive factor

ESK- Eskimo

GFP- Green florescence protein

GTF- General transcription factor

HOS- High expression of osmotically sensitive

ICE- Inducer of CBF expression

KB- King's broth

KIN- Cold induced

LB- Luria bertani

LEA- Late embryogenesis abundance

LOR- Los of osmotic responsiveness

LOS- Low temperature sensitive

LTI- Low temperature induced

MED- mediator

MS- Murashing and Skoog

NPR- Non-expressor of PR1

OBF- *ocs*- Element binding factor

OD- Optical displacement

ORF- Open reading frame

P5CS- Δ^1-pyrolline-5-carboxilase synthetase

PFT- Phytochrome and flowering time

PR- Pathogenesis responsive

RD- responsive to desiccation

RNAPII- RNA polymerase II enzyme

ROS- Reactive oxygen species

SA- Salicylic acid

SDS- Sodium dodecyl sulphate

SFR- Sensitive for freezing

STET- Sucrose-Tris-EDTA-Triton

STZ- Salt tolerance zinc finger

SWP- Struwwelpeter

TAF- TBA associated factor

TBA- TATA binding protein

T-DNA- transfer DNA

USA- Upstream stimulatory activity

UTR- Un-translated region

UV- Ultra violet

WT- Wild type

ZAT- C2H2-EAR zinc finger protein

Chapter 1

Introduction

Environmental stress is the major limitation to plant growth and productivity. The sessile nature of plants means that they are unable to avoid the constraints imposed by their immediate environment. Therefore, plants have evolved complex and sophisticated protective mechanisms to withstand conditions such as drought, freezing and pathogen attack. However, the degree of tolerance varies between species.

The current rate of environmental degradation and anthropogenic climate change predicts that plants will become subject to increasing extremes of environmental stress. Previous efforts to improve plant stress tolerance have typically used classical breeding methods. However, these methods are slow in practice. In addition, the need for greater food production, to sustain the expanding global population, means that development of better crop strains using classical breeding methods are no longer sufficiently rapid. Whilst genetic engineering offers a viable alternative, using this technology to manipulate plant defense systems is only as powerful as our comprehension of the systems themselves. A better understanding of plant stress signal perception, transduction and activation of defense responses at the cellular and molecular level is therefore imperative. Recent studies have made progress in the definition of various genetic protective responses to stress stimuli, using the model plant species *Arabidopsis thaliana*.

This study aims to investigate the regulation of gene expression in response to environmental stresses in the *sfr6* (***s****ensitive to* ***fr****eezing****6***) mutant of *Arabidopsis*, which fails to cold acclimate (Warren *et al*., 1996) and shows loss of cold-inducible *COR* (***CO****LD-****R****EGULATED*) gene expression (Knight *et al*., 1999, Knight *et al*., 2009). This chapter reviews topics relevant to the present study as follows. First, cellular injuries of low temperature stress and plant cold acclimation are described. Next follows a general description of the regulation of gene expression in response to stress, with emphasis on the responses induced by low temperature. Crosstalk

between low temperature and other biotic and abiotic stresses is then discussed. The chapter concludes with an introduction to the *sfr6* mutant, and summarizes our current understanding of the involvement of SFR6 in the regulation of stress-associated gene expression.

1.1Low temperature stress

Low temperature is one of the most important abiotic stress factors that influence plant growth, development and geographical distribution. Low temperature reduces growth and development of plants by influencing the normal function of cellular processes. An analysis of maize growth at low temperatures showed that the cell cycle was prolonged by 64 % (Rymen *et al*., 2007). Natural tolerance to low temperatures varies widely between plant species. Those which are native to cold climatic regions can survive in freezing (<0^{o}C) temperatures. 'Extremophiles' from Arctic and Alpine environments, such as *Silene acaulis* and *Carex firma,* can survive at temperatures of less than -50^{o}C, and some cultivated crops such as winter wheat can tolerate temperatures below -25^{o}C (reviewed in Atici and Nalbantoglu, 2003). However, degrees of low temperature tolerance vary between these freezing resistant species, whilst plants growing in tropical countries are generally unable to withstand even mild chilling (0-15^{o}C; Zhu *et al*., 2007).

1.1.1 Freezing injuries of plants

Plants are most vulnerable to freezing injury at their membranes, primarily through frost-induced cellular dehydration (Steponkus, 1984.). When environmental temperatures fall below 0^{o}C, ice crystals form first in intercellular spaces, due to the lower solute concentration of intercellular fluid relative to the intracellular cytoplasm (Thomashow, 1999). In addition, intercellular fluids contain a mixture of ice nucleating agents, such as dust and bacterial proteins, which enhance ice crystal formation (reviewed in Xin and Browse, 2000). Ice particles cause mechanical

damage to membranes and the cell wall, resulting in cell rupture (Thomashow, 1999).

Intercellular ice formation also causes cellular dehydration. Because the chemical potential of ice is less than that of liquid water at a given temperature, the water potential of extracellular fluid lowers relative to the intracellular fluid (Thomashow, 1999). Water always moves from a higher to a lower water potential. Under freezing conditions this movement of water, from the cytoplasm to the intercellular space, continues until an osmotic equilibrium is reached between intracellular water and extracellular ice. The result is cellular dehydration, cell shrinkage and an increased endogenous solute concentration. Upon thawing, water moves rapidly back through the plasma membrane, causing the protoplast to burst before it regains its original size. This is known as expansion-induced lysis (reviewed in Xin and Browse, 2000). As the cells burst, ions and solutes leak into the surrounding interstitial spaces, giving water soaked appearance to the plant tissues.

At the molecular level freezing affects membrane lipids, proteins and nucleic acids (Wolkers *et al*., 2007). A membrane's fluidity is determined by the structure and composition of its lipids and proteins. Cooling-induced cellular dehydration alters the organization of the lipid bilayer, by destabilising hydrophobic lipid-lipid and protein-lipid interactions within the membrane (Pearce, 1999). Therefore, during freezing, cell membranes undergo a phase transition from a flexible crystalline to solid gel state; this can result in macroscopical damage, and local aggregations of proteins and lipids (known as lateral phase separation; Wolkers *et al*., 2007). These structural changes, and proximity of endo-membranes to each other and to the plasma membrane due to cell shrinkage, can produce ultra-structural changes such as lamella to hexagonal II phase transition (lipid molecules aggregate into cylindrical structure) and fracture jump lesions (unusual alterations in membrane structure) (Fujikawa *et al*., 1999).

Structural and functional changes can also occur through oxidative damage to membranes during freezing and thawing (Thomashow, 1999 and Wolkers *et al*., 2007). Reactive oxygen species (ROS) are present in cells at low levels during

normal conditions, but can accumulate under freezing stress through deactivation of enzymatic scavenging system during freeze induced dehydration (Wolkers *et al*., 2007).

The rate of temperature reduction determines which of these types of cell damage are most significant (Wolkers *et al*., 2007). Fast cooling promotes mechanical damage through intracellular ice formation, whereas slow cooling causes cell dehydration, results in osmotic injuries associated with high solute concentrations (Wolkers *et al*., 2007). These factors accumulate to exert a complexity of freezing-induced stresses. In an increasing order of severity, these effects result in a reduced growth rate, chlorosis, wilting, water soaked lesions on foliage and tender tissues, and ultimately death of the plant.

1.1.2 Cold acclimation

Plants growing in temperate regions have the ability to increase their level of freezing tolerance when exposed to low non-freezing temperatures; known as cold acclimation (Thomashow, 1999). Uemura *et al*. (1995) showed that the maximum freezing tolerance of *Arabidopsis* is attained after 1 week of cold acclimation at 2^{o}C. In contrast, winter oat (*Avena sativa* L. *cv* kanota), spring oat (Ogle) and rye (*Secale cereal* L *cv*. Puma) required 4 weeks of cold acclimation at 2^{o}C to attain maximum freezing tolerance (Webb *et al*., 1994).

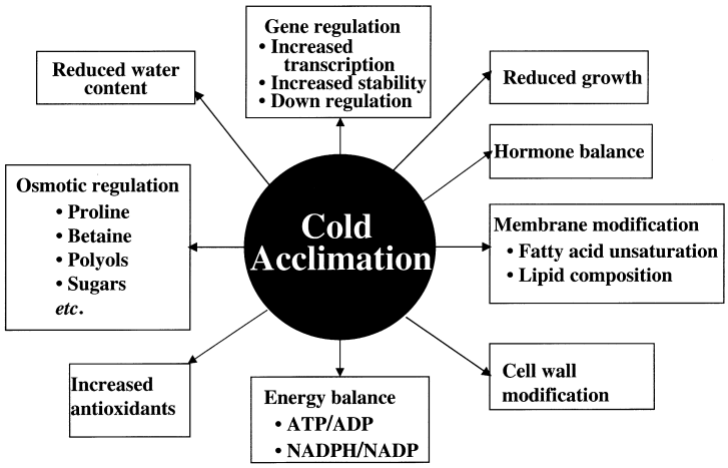
Cold acclimation initiates numerous biochemical and physiological changes as well as global alterations in gene expression (Xin and Browse, 2000). Some of the most commonly observed changes are summarized in Figure 1.1. The consequences of almost all of these for freezing tolerance are not yet well understood. However, they combine to decrease the incidence of freezing injuries in acclimated plants.

The cellular changes taking place during cold acclimation include shifts in membrane lipid composition (Steponkus *et al*. 1990), cell wall composition (Fujikawa *et al*., 1999), accumulation of soluble carbohydrates such as proline

(Tantau and Dorffling, 1991) sugar (Yoshida *et al*., 1998), antifreeze proteins (Griffith *et al*., 2004) and production of protective COR proteins (Thomashow *et al*., 1999).

Figure 1.1

Some of the cellular processes commonly occurring when plants are subjected to low non freezing temperatures. Adapted from Xin and Browse (2000).



When a plant is exposed to low non-freezing temperatures different cellular processes are altered to improve the plant's ability to tolerate subsequent freezing. This figure shows some common changes occurring during the cold acclimation process.

Uemura *et al*. (1995) anlysed changes in the lipid composisition of *Arabidopsis* membranes during cold acclimation, and found increases in the proportion of membrane pospholipid from 46.8 % to 57.1 % during cold acclimation. Steponkus *et al*. (1990) also reported differences in lipid ratios between cold acclimated and non acclimated rye plasma membranes. These analyses suggest that changes in lipid composition may reduce membrane ultra-structural changes occurring due to membrane fusion (Fujikawa *et al*., 1999).

Cell wall components also alter during cold acclimation. Acclimated cells show an elevation in wall phenolic compounds, wall-associated lipids, and increases in cell wall thickness and rigidity (Fujikawa *et al*., 1999). The antioxidant properties of

phenolic compounds may act to reduce lipid peroxidation actions of reactive oxygen species (Blokhina *et al*., 2003). In addition, accumulation of osmolytes (such as sugar, proline and glucose) in acclimated cells promotes an elevation of endogenous solutes, which can counter cellular dehydration (reviewed in Xin and Browse, 2000). The altered protein components of apoplastic sap after cold acclimation also confer antifreeze-like effects (Griffith *et al*., 2004). Fujikawa *et al*., (1999) suggest that these proteins reduce the initiation, growth and re-crystallization of extracellular ice.

The changes in gene expression during cold acclimation were first demonstrated by Guy *et al.* (1985). Since then research has focussed on identifying these genes, and seeking to understand their function and regulation. These genes are discussed further in section 1.2.7 of this chapter.

1.2 Regulation of stress response gene expression

1.2.1 Changes in gene expression in response to environmental stresses

Stress responses are initiated when plants recognize stress at the cellular level, followed by generation of second messengers such as calcium and ROS. These then trigger activation of various signal transduction pathways (Fowler and Thomashow, 2002; Knight and Knight, 2001) which transmit information and activate stress inducible gene expression. Microarrays in different plant species show a significant overlap in the types of genes whose expression is altered upon exposure to different stresses (Schenk *et al*., 2000; Chen *et al*., 2002; Seki *et al*., 2002). For example, Seki *et al.* (2002) report that 22 genes (out of 7000 from *Arabidopsis)* form part of a common transcriptional response to cold, drought and salt induced stresses. Another microarray analysis, by Chen *et al*. (2002) using 402 *Arabidopsis* transcription factors, showed extensive overlaps in expression of similar genes under different stresses. Nawrath *et al*. (2002) also showed the induction of a number of genes in common between pathogen attack and UV irradiance. The encoded proteins function either to protect cells from stress damage directly, or are involved in the generation of regulatory molecules like abscicic acid (ABA) ethylene and salicylic acid (SA) which themselves further regulate gene expression (Shinozaki *et al*., 2003). The

signalling pathways of stress induced gene expression are not yet completely understood, though the transcriptional activation of some stress induced genes has been well studied (Xiong *et al*., 2002).

1.2.2 Transcription factors regulate stress responses

Transcription factors are genetic switches which fine tune the stress responses of plants by activating promoters of stress inducible genes. Many transcription factor genes are found among the list of genes up and down regulated in response to environmental stress (Seki *et al*., 2001; Kreps *et al*., 2002; Seki *et al*., 2002; Chen *et al*., 2002; Shinozaki *et al*., 2003).

Stress-inducible expression of transcription factors include members of the AP2/EREBP (apetala2/ethylene-responsive element binding proteins) family, the zinc-finger family, the WRKY family, the MYB family, the basic helix–loop–helix (bHLH) family, the basic-domain leucine zipper (bZIP) family, the NAC family, and the homeodomain transcription factor family (Shinozaki *et al*., 2003). The AP2/EREBP transcription factor family, bZIP family and WRKY family proteins are reported as major players in the regulation of gene expression in response to environmental stresses (Singh *et al*., 2002). Furthermore, significant improvement of stress tolerance was observed upon over-expression of individual transcription factors from these families. As examples, over-expression of *CBF1/DREB1B* and *DREB1A* in *Arabidopsis* greatly increases the plant's resistance to cold, drought and salt (Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999).

Both induction and repression of specific transcription factors are required to fine tune the plant to various environmental stresses. For example, MYB transcription factors are associated with responses to UV light and pathogen infection (Rushton and Somssich, 1998; Singh *et al*., 2002), though Jin and colleagues (2000) report that AtMYB4 represses target genes under UV-B irradiance, and the over-expression of *AtMYB4* reduces the UV-B tolerance of *Arabidopsis*.

AP2/EREBP comprises the biggest transcription factor family in *Arabidopsis*, with 45 members (Riechmann *et al*., 2000). These transcription factors are linked with a wide range of stress responses. One subfamily of AP2/EREBP, known as CBF/DREB1 (C-REPEAT BINDING FACTOR/DROUGHT RESPONSIVE ELEMENT BINDING FACTOR1), bind a *cis*-element known as the CRT/DRE (C-repeat/drought responsive element), common to the promoters of *COR* genes, induced by cold, drought and salt-stress (Stockinger *et al*., 1997; Liu *et al*., 1998). In *Arabidopsis* there are four CBF/DREB1 proteins. Another DRE binding protein, DREB2 (DROUGHT RESPONSIVE ELEMENT BINDING FACTOR2); is from a subfamily of AP2/EREBP transcription factors that regulate several drought and salt stress induced genes (Liu *et al*., 1998). A further transcription factor subfamily, known as ERFs (ethylene responsive element binding factors), bind to GCC box *cis*-element in the promoters of several *PATHOGENESIS RELATED* (*PR*) genes (Singh *et al*., 2002). Therefore, members of the AP2/EREBP family regulate both biotic and abiotic stress responsive gene expression.

The bZIP family of transcription factors also function to control stress responsive gene expression. One type of bZIP family transcription factors, the ABFs (ABA responsive factor), bind ABREs (Abscisic acid responsive elements). These cis-elements regulate expression of cold, drought and salt induced genes via ABA-dependent signal transduction (Choi *et al*., 2000). Over-expression of ABF3 and ABF4 transcription factors in *Arabidopsis* alters the expression of stress responsive genes and increases drought tolerance in transgenic plants (Kang *et al*., 2002). Another bZIP subfamily, the TGA/OBF, bind to the *as-1*/*ocs* element of *PR1* genes (Zhang *et al*., 1999; Despres *et al*., 2000). NPR1 (NON EXPRESSOR OF PR1), a key component of SA (Salicylic Acid) mediated signalling, interacts with TGA/OBF family proteins upon pathogen infection. This physical interaction is essential to enhance the DNA-binding properties of TGA/OBFs which lead to the induction of *PR* gene transcripts (reviewed in Eulgem, 2005).

WRKY transcription factors are unique to plants, and are involved in stress responsive gene expression. Members of the WRKY family show enhanced expression following pathogen infection (reviewed in Singh *et al*., 2002). As an

example, the expression of WRKYs is essential to activate *NPR1* activity upon pathogen infection (Yu *et al*., 2001). WRKY factors interact with two W boxes in the NPR1 5' UTR upon pathogen infection, activating *NPR1* transcription. Over-expression of several *WRKY* genes in *Arabidopsis* has produced an enhanced resistance to pathogens and altered expression of several stress responsive genes (reviewed in Singh *et al*., 2002).

The MYB and bHLH families of transcription factors are also reported to be involved in stress-responsive gene expression. These transcription factors specifically bind to MYC and MYB recognition sites in promoters of ABA responsive genes (Abe *et al*., 2003). Transgenic *Arabidopsis* over-expressing *AtMYC2* and *AtMYB2* transcription factors genes showed a higher sensitivity to ABA and altered expression of ABA induced genes (Abe *et al*., 2003).

As mentioned above, many transcription factors are involved in regulation of stress responsive gene expression. Some stress genes are induced by several transcription factors, whilst the same transcription factor may behave differently in response to different stress stimuli. Therefore, it is important to understand the mechanism by which transcription factors are recruited to the promoters of stress induced genes, in order to manipulate the stress resistance of crop plants.

1.2.3 General RNA polymerase II transcription cycle

Synthesis of RNA from DNA is called transcription, and this process takes place in the nucleus. The cell initiates transcription by directing the RNA polymerase II complex to motifs in the DNA in proximity to the coding region of the target gene, known as the promoter. The most common promoter motif in eukaryotic DNA is the TATA box. Transcription initiates through the formation of a pre-initiation complex (PIC) near the transcriptional start (Figure 1.2).

The PIC includes 6 general transcription factors (GTFs) and the RNA polymerase II enzyme (RNAPII). These GTFs are known as TFIIA, TFIIB, TFIID, TFIIE, TFLLF,

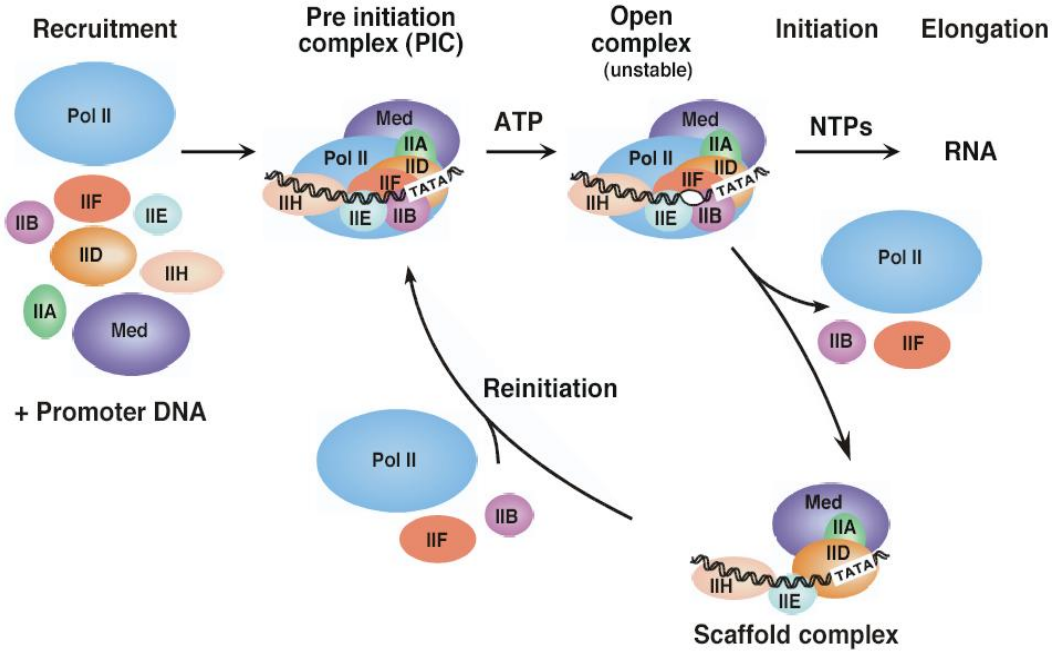
TFIIH (Hahn, 2004; Thomas and Chiang, 2006); they work together with RNAPII to specify the transcription initiation site (reviewed in Thomas and Chiang, 2006). The PIC usually begins with TFIID binding to the TATA box, followed by the entry of the other GTFs and RNAPII either in a sequential assembly or a preassembled RNAPII holoenzyme (reviewed in Thomas and Chiang, 2006). Formation of a PIC is sufficient for basal level transcription (Thomas and Chiang, 2006). However, for activator-dependent (or regulated) transcription, general co-factors are also required. These include mediator, TAFs (TATA Binding Protein associated factors) and upstream stimulatory activity (USA) derived positive and negative co-factors, and are needed to transmit regulatory signals between gene specific activators and the general transcription machinery (Thomas and Chiang, 2006).

With the completion of formation of PIC, dramatic conformational changes occur in the 11-15 base pairs of DNA surrounding the transcription start (Hahn, 2004). Then transcription begins with the synthesis of the first phosphodiester bond of RNA (Hahn, 2004). In several transcription systems, multiple short RNAs, called abortive products, are synthesized before initiating synthesis of full length transcripts (reviewed in Hahn, 2004). After synthesis of about 30 bases of RNA, RNAPII, TFIIB, and TFIIF release their contact with the core promoter and the rest of the transcription machinery enter the transcript elongation phase (Hahn, 2004). Many of the GTFs of PIC remain behind at the promoter in the scaffold complex after release of RNAPII from the PIC complex, and this enables re-initiation of transcription (Hahn, 2004). The scaffold complex accelerates subsequent transcription by bypassing the very slow recruitment of GTFs and formation of PIC at the transcriptional start site (Hahn, 2004).

Immuno-precipitation studies on recruitment of mediator to yeast *GAL* genes *in vivo* found that the recruitment of mediator to upstream activation sequences is independent of RNAPII, general transcription factors and core promoters (Kuras *et al*., 2003).

Figure 1. 2

Model of RNA polymerase II transcription initiation and re-initiation machinery. Adapted from Hahn (2004).



Schematic representation of general RNA polymerase II transcription cycle. This is initiates through the formation of a pre-initiation complex (PIC) near the transcriptional start. PIC includes 6 general transcription factors (IIA, IIB, IID, IIE, IIF and IIH), RNA polymerase II (Pol II) and mediater (MED). Then transcription begins after large conformational changes occur in the surrounding the transcription start. After the abortive transcription phase Pol II, IIB and IIF would escape the promoter leaving other general transcription factors as a scaffold complex to facilitate rapid assembly of a new PIC.

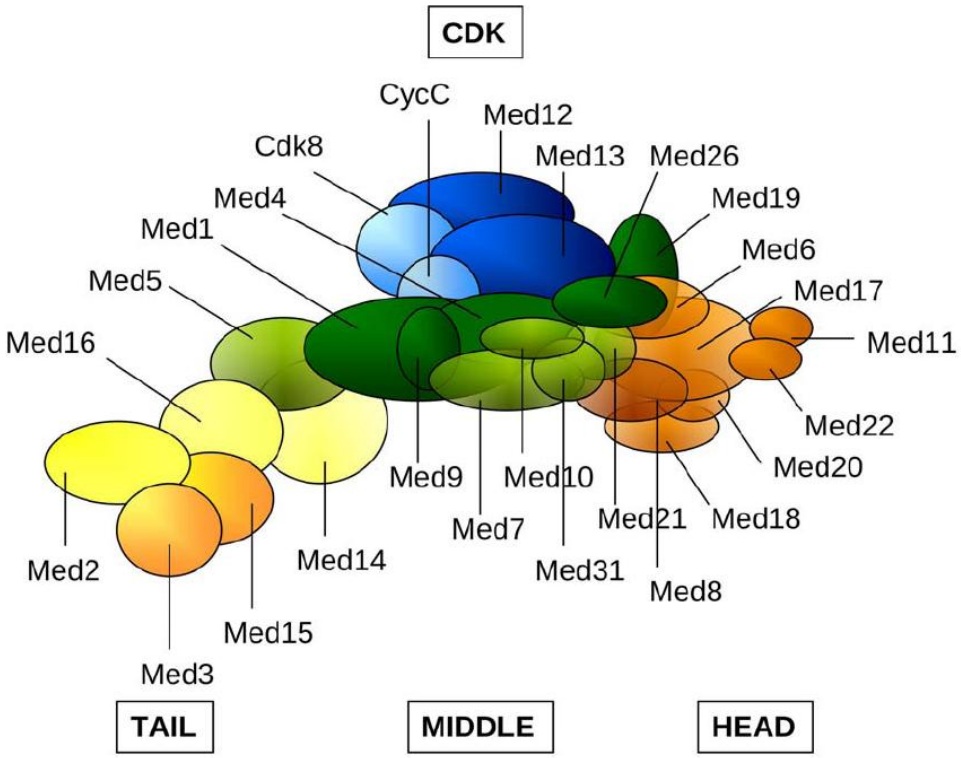
1.2.4 Mediator complex

Mediator is a transcriptional co-activator complex which acts as a bridge to convey DNA-bound transcriptional regulators and enhancers to the general RNA polymerase II transcription machinery (Chadick and Asturias, 2005; Bourbon, 2008). Components of mediator were first identified thought biochemical and genetic studies in baker's yeast (*Saccharomyces cerevisiae*), as subunits linking to the RNA

Polymerase II holoenzyme (Flanagan *et al*, 1991). Mediator complexes were subsequently found in other organisms including higher eukaryotes (Bourbon *et al*., 2004; Bjorklund and Gustafsson, 2005; Bourbon, 2008). Of these, the structure and composition of yeast mediator has been studied in most detail. The yeast complex comprises 26 subunits (Guglielmi *et al*., 2004), and has been grouped through interactor studies into 4 sub-modules known as the head, middle, tail and kinase (CDK) (Asturias *et al*., 1999) (Figure 1.3). In mammalian mediator, 30 subunits have been identified (reviewed in Bourbon *et al*., 2004). However, prior to 2007 there were no reports of a plant mediator complex, probably due to the low sequence homology between *Arabidopsis* and other non-plant eukaryotic mediators (Backstrom *et al*., 2007).

Figure 1.3

Topological organization of yeast mediator. Adapted from Guglielmi *et al*. (2004).



This model has been made by considering the direct links taking place in between different mediator subunits and the relative size of the mediator subunits found by several researchers and the work of Guglielmi *et al*. (2004).

Physical interactions between mediator subunits were revealed in 1992 by Jiang and Stillman, through research on yeast SIN4 (MED16) subunit mutant strain. A mutation in the SIN4 (MED16) subunit of yeast showed loss of function of other tail subunits and therefore, it was suggested that mediator subunits physically interact with each other (Jiang and Stillman, 1992). Consistent with these results, electron microscopic (EM) studies of holoenzyme complexes purified from mutant yeast lacking MED16, showed that the tail mediator modules (MED15, MED3 and MED2) were absent (Chadick and Asturias, 2005). Therefore they also suggested that the tail portion of mediator formed a subset of physically interacting units. Moreover, the physical association of yeast MED16 and MED14 (Rgr1) proteins were also apparent with the same spectrum of phenotypes observed in *sin4* and *rgr1* mutant strains (Jiang *et al*., 1995).

1.2.5 Mediator in transcriptional regulation

During transcriptional activation and repression, the mediator complex is not directly in contact with gene-specific regulatory sequences (enhancers or silencers). Rather, mediator subunits bind to specific transcriptional activators or repressors, and with other components of PIC (Figure 1.4) (Bjorklund and Gustafsson, 2005; Bourbon, 2008).

Studies have shown that yeast mediator is present both free and in a complex with RNAPII (mediator+RNAPII) (Kim *et al*., 1994). However, whether the mediator recruit either as free complex or mediator+RNAPII complex to PIC to initiate transcription is not well understood yet.

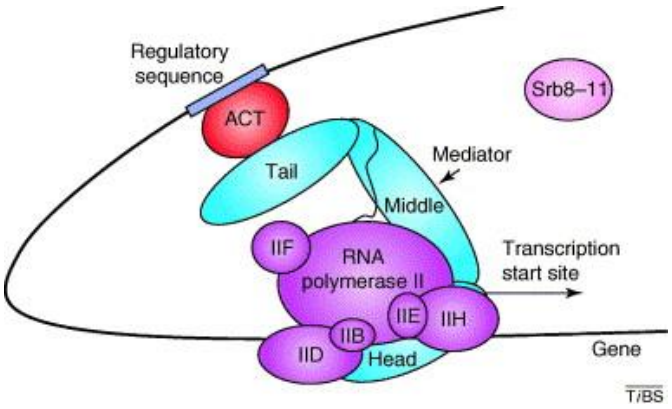
The CDK module (Sbr8-11 or according to the new nomenclature MED12, MED13, Cdk8 and CycC respectively) of mediator has been identified as a negative regulator of transcription. Therefore, mediator complexes with the CDK module present, prevent interaction with RNAPII. Degradation of the CDK module is necessary for mediator to interact with RNAPII and subsequently initiate transcription (Samuelsen *et al*., 2003) (Figure 1.4). These same authors proposed a three step model for the

degradation of CDK before transcription can commence. First, mediator complexed with CDK binds the transcriptional activation domain, and then to the promoter of the gene; finally at the promoter, the CDK module degrades to facilitate interaction with RNAPII. This means that the mediator attached to PIC comprises only the head, middle and tail modules. Consistent with these results, EM visualization of yeast mediator attached to PIC did not revealed the CDK module (Chadick and Asturias, 2005).

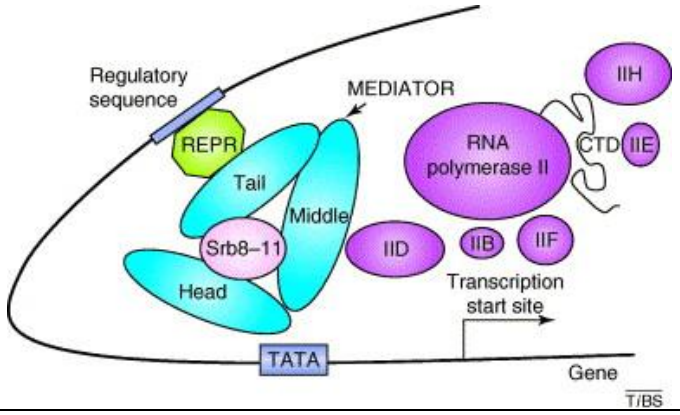
Figure 1.4

Function of mediator in transcriptional activation and repression. Adapted from Bjorklund and Gustafsson (2005).

A. Transcription activation



B. Transcription repression



A. Mediator functions as a bridge between gene-specific activators and the general polymerase II transcription machinery at the promoter. Activator interactions mainly take place within the tail region of mediator, whereas contacts with polymerase II are localized to the head and middle region.

B. Gene specific repressors (REPR) interact with specific mediator subunits and recruit the complex to upstream regulatory DNA sequences. Mediator that is recruited by repressors contains the Srb8-11 (Med12-Med13-Cdk8-CycC) module, which prevents interactions with polymerase II and the basal transcription machinery.

EM images of yeast PIC revealed that the tail domain extends away from RNAPII, though there are multiple physical contacts between RNAPII and the middle and

head mediator domains (Chadick and Asturias, 2005). However, large areas of the RNAPII surface (~ 75%) remain available for interaction with other components of the PIC (Chadick and Asturias, 2005). When considering the structural changes of mediator complex during RNAPII interaction, it appears that the structure of the head domain remains same but the tail and middle domains unfold (Chadick and Asturias, 2005).

The carboxyl terminal domain (CTD) of Rpb1 (the largest RNAPII subunit) was identified as necessary for regulation of transcription through mediator (Myers *et al*., 1998; Chadick and Asturias, 2005). However, more than CTD is required to facilitate the conformational changes necessary for the interaction of mediator with RNAPII (Chadick and Asturias, 2005). In addition, the mediator-RNAPII interaction appears to be at least kingdom-specific, since no mediator+RNAPII complex was formed when yeast mediator was incubated with mammalian RNAPII. This is surprising since the sequences of yeast and mammalian CTDs are highly homologous (Chadick and Asturias, 2005).

1.2.6 Plant mediator

In 2007 Backstrom *et al*. were able to identify plant mediator complexes from *Arabidopsis thaliana*, using a combination of bioinformatics and biochemical analytical techniques. They found that most of the yeast and metazoan subunits are present in plant mediator, but with a very low sequence homology. Since the *Arabidopsis* genome encodes ~700 plant specific transcription factors, to regulate these via mediator, the plant protein recognition motifs would have to differ from those of yeast and other eukaryotes subunits (Backstrom *et al*., 2007). Their study found homologues for most head and middle domain subunits known from yeast, but very low homology for the candidates of tail domain. This section of the mediator complex also showed greatest variability when comparing proteins from yeast and metazoans (Backstrom *et al*., 2007). As discussed earlier, tail domains make contact with gene-specific enhancers and suppressors of transcriptional regulation. It is likely that the *Arabidopsis* mediator includes plant specific tail domains (Backstrom

et al., 2007). Apart from that they identified potential plant specific mediator subunits named as MED32-37. Backstrom *et al*. (2007) also identified *PFT1* (*PHYTOCHROME AND FLOWERING TIME*; Cerdan and Chory, 2003) as *MED25* and *SWP* (*STRUWWELPETER*; Autran *et al*., 2002) as *MED14*. These genes were already known as being involved in regulation of flowering time via phyB-dependent pathway (*PTF1*), and defining the duration of cell proliferation (*SWP*).

1.2.7 Regulation of gene expression in response to low temperature

1.2.7.1 Cold acclimation utilises signalling networks also common to drought, salt and ABA responses

Several research groups have shown that cold acclimation activates multiple low temperature responsive pathways (Seki *et al*., 2001; Thomashow, 2001; Fowler and Thomashow, 2002). Many of these are also induced by other stresses such as drought, salt and ABA, suggesting common signal transduction pathways are activated by these different stress stimuli (Thomashow, 1999; Seki *et al*., 2001, 2002, Shinozaki *et al*., 2003). Several research teams are now seeking to group and assign functions to these cold induced genes.

Members of one such gene set were already known by a range of different names, including *COR* (cold-regulated), *LTI* (low-temperature induced), *CAS* (cold accumulation specific), *KIN* (cold induced) and *RD* (responsive to desiccation). Those genes (COR) show strong induction (typically 50-100 fold) after cold treatment have been the subject of particular interest (Xin and Browse, 2000). Most of the promoter sequences for this group of genes contain C-repeat (CRT) elements (Baker *et al*., 1994). A similar *cis*-acting element was reported concurrently by Yamaguchi-Shinozaki and Shinozaki (1994), and designated as the 'Dehydration Responsive Element' (DRE). CRT/DRE elements contain a conserved CCGAC core sequence which is sufficient to induce *COR* gene accumulation under cold stress (Baker *et al*., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). Yamaguchi-Shinozaki and Shinozaki (1994) also found another *cis*-acting element responsive to ABA in the promoter of *rd29A* genes, now designated as the ABA responsive

element (ABRE). Therefore, the genes which contain both CRT/DRE and ABRE elements in their promoters can response to both cold/drought/salt and ABA.

Consistent with these results Seki *et al*. (2002) found a common set of genes responding to both ABA and cold in their microarray analysis. They also found more overlap between 'drought v ABA' than 'cold v ABA'. However, several genes that responded to dehydration and low temperature did not respond to exogenous ABA (Shinozaki and Yamaguchi Shinozaki, 1997; Thomashow, 1999). This implies that *COR* genes are regulated by both ABA independent and ABA dependent pathways (explored further in section 1.2.7.3) (Shinozaki and Yamaguchi Shinozaki, 1997; Thomashow, 1999). The CBF cold response is the most well-known ABA independent low temperature signal transduction pathway (Vogel *et al*., 2005). As a compliment to these studies, *Arabidopsis* mutants altered in low temperature responsiveness have helped identify several other negative and positive regulators of low temperature signal transduction. Some of these mutants will be discussed in later sections of this chapter.

1.2.7.2 The CBF cold response pathway

A group of transcription factors called C-repeat binding factors (CBF) (Stockinger *et al*., 1997), and also known as dehydration responsive element binding factors (DREB1) (Liu *et al*., 1998), control cold induced *COR* gene expression in an ABA-independent manner. CBF/DREB1 transcription factors belong to the EREBP/AP2 family of DNA binding proteins (Stockinger *et al*., 1997; Liu *et al*., 1998); AP2 is an evolutionary conserved plant-specific domain (Weigel, 1995). There are 3 closely related cold inducible CBF/DREB1 genes, named CBF1/DREB1B, CBF2/DREB1C and CBF3/DREB1A all located on chromosome 4 of *Arabidopsis* with approximately 88 % identical and 91 % similar amino acid sequences (Gilmour *et al*., 1998; Liu *et al*., 1998). CBF/DREB1 transcripts increase markedly within 15 mins of transfer to low temperature, and continue to increase over next 1-2 h. These 3 genes encode transcriptional activators which bind CRT/DRE elements (Gilmour *et al*., 1998; Liu *et al*., 1998). After about 2 h, the transcripts of COR genes containing

CRT/DRE in the promoters accumulate (Gilmour *et al*., 1998). Although *CBF/DREB1* genes strongly and transiently express under cold stress, these genes do not themselves carry the CCGAC sequence in their promoters (Gilmour *et al*., 1998).

Another CBF protein named CBF4 has also been identified, though this is induced under dehydration stress, rather than low temperatures unlike the other 3 *CBF/DREB1* genes (Haake *et al*., 2002). However over-expression of *CBF4* in *Arabidopsis* increases downstream *COR* gene transcription, and therefore increases tolerance to both drought and low temperature stresses (Haake *et al*., 2002). These authors propose that the transcription factors controlling both drought and cold responses in plants are all derived from a common CBF-like ancestral protein.

DREB2A was also identified as a DRE-binding protein with an EREBP/AP2 DNA binding domain (Liu *et al*., 1998). Apart from the EREBP/AP2 DNA binding domain, DREB1A and DREB2A proteins do not have strong sequence similarities (Liu *et al*., 1998). Furthermore, DREB2A proteins express in response to dehydration and salt stresses but not to low temperatures (Liu *et al*., 1998). Therefore, it has been suggested DREB1A and DREB2A proteins function in separate signalling pathways under cold and dehydration stress conditions (Liu *et al*., 1998). Later Nakashima *et al*. (2000) was able to identify and clone DREB2B protein with same function as DREB2A.

Transgenic *Arabidopsis* plants over-expressing *CBF1*/*DREB1B* showed increased tolerance to freezing stress without a low temperature stimulus (Jaglo-Ottosen *et al*., 1998). Over-expression of *CBF3*/*DREB1A* genes in *Arabidopsis* also induced expression of *COR* genes, resulting in an increased freezing, drought and salt tolerance (Liu *et al*., 1998; Kasuga *et al*., 1999; Gilmour *et al*., 2000). However, Novillo *et al*. (2004) report that the *Arabidopsis* mutant *cbf2* has a higher capacity for freezing tolerance than WT plants before and after cold acclimation, and also greater tolerance to dehydration and salt stresses. This is due to the increased expression of *CBF1*/*DREB1B* and *CBF3*/*DREB1A* in *cbf2* mutants, compared with WT. These authors suggest that CBF2/DREB1C negatively regulates CBF1/DREB1B and CBF3/DREB1A.

CBF-like proteins have been identified in both freezing sensitive and freezing tolerant species such as Canola (*Brassica napus*), wheat (*Triticum aestivum*), rye (Secale cereal), tomato (*Lycopersicon esculentum*) (Jaglo *et al*., 2001) and rice (*Oriza sativa*) (Dubouzet *et al*., 2003). Over-expression of these proteins in their host species all enhances tolerance to low temperature and other abiotic stresses. As an example, over-expression of rice *DREB1*/*CBF*-type genes in rice increased tolerance to drought, salt and low temperature stresses (Ito *et al*., 2006). These genes also convey cross-species function; e.g. over-expression of *OsDREB* genes in *Arabidopsis* increased freezing tolerance in a manner similar to that produced by over-expression of *AtDREB* (Dubouzet *et al*., 2003). Similarly, over-expression of *AtCBF*/*DREB1* genes in other species such as canola (Jaglo *et al*., 2001), tobacco (Kasuga *et al*., 2004) and rice (Oh *et al*., 2005) also increased freezing/low temperature tolerance. These observations imply that *CBF*/*DREB1* type genes could be useful molecular tools for improving stress tolerance in various agriculturally important plants.

1.2.7.3 Regulation of the CBF pathway

Due to the rapid and transient expression of *CBF* genes under low temperature conditions, and the evidence of CBF regulation occurring at the transcriptional level, Gilmour *et al*. (1998) suggested that a transcription factor recognizing the CBF promoters becomes activated in response to low temperature. This unknown transcriptional activator was named ICE (inducer of CBF expression) and it was suggested that ICE is present though in an inactive state at ambient (non-stressed) temperatures (Gilmour *et al*., 1998).

Later, 2 ICE proteins; ICE1 and ICE2, were identified (Chinnusamy *et al*., 2003; Fursova *et al*., 2009). Both ICE1 and ICE2 are members of the basic helix-loop-helix (bHLH) family of transcriptional activators; these bind to MYC binding sites of CBF promoters (Chinnusamy *et al*., 2003; Fursova *et al*., 2009). However, mutation of the *Arabidopsis ICE1* gene blocks only expression of *CBF3* (Chinnusamy *et al*., 2003), and *ICE2* mainly affects *CBF1* expression (Fursova *et al*., 2009).

Interestingly, enhanced expression of *CBF2* was observed in *ice1* mutant suggesting possible negative regulation of *CBF2* by CBF3 (Chinnusamy *et al*., 2003). The over-expression of either *ICE1* or *ICE2* increases freezing tolerance of *Arabidopsis* upon cold acclimation (Chinnusamy *et al*., 2003; Fursova *et al*., 2009). The freezing tolerance of non-acclimated *ICE1/2* over-expressing lines was not mentioned. However, two *ICE*-like genes (*TaICE41* and *TaICE87*) have been cloned from wheat; their over-expression in *Arabidopsis* enhanced freezing tolerance only upon cold acclimation, suggesting that other factors induced by low temperature are required for their activity (Badawi *et al*., 2008).

It has been also reported that the expression of CBF genes are repressed by their own gene product, or by the products of their downstream genes (Guo *et al*., 2002). Other known negative regulators of low temperature induced signal transduction are discussed below in section 1.2.7.6

1.2.7.4 The ABA-dependent cold response

Two lines of evidence suggest that ABA plays an important role in increasing freezing tolerance in cold acclimated plants; (1) ABA levels increase in cold acclimating plants after low temperature stress, but not in non-acclimated plants, and (2) exogenous application of ABA at non-stressing temperatures increases freezing tolerance of treated plants (reviewed in Thomashow, 1999). Supporting this evidence, two *Arabidopsis* mutants, *aba1* (ABA deficient) and *abi1* (ABA insensitive), showed less freezing tolerance than WT plants (Gilmour and Thomashow, 1991). The freezing sensitivity of *aba1* is complemented by exogenous application of ABA (Heino *et al*., 1990). However, since *abi1* and *aba1* have a wilted phenotype and reduced vigour, their freezing sensitivity might not be a direct effect of ABA on the cold acclimation process (Gilmour and Thomashow, 1991; Thomashow, 1999). Moreover, the cold induced expression of *COR78*, *COR47* and *COR6.6* is normal in both of these mutants (Gilmour and Thomashow, 1991; Thomashow, 1999). These results and several other observations of low temperature response gene expression showed both ABA dependent and independent pathways

are involved in low temperature response gene expression (reviewed in Thomashow, 1999 and Xin and Browse, 2000). These two pathways have the potential for cross-talk.

Knight *et al*. (2004) showed that CBFs themselves are also induced by exogenous ABA. Many microarray analyses also revealed group of genes express to both ABA application and low temperature stimuli (Kreps *et al*., 2002; Seki *et al*., 2002).

1.2.7.5 Cold acclimation independent of the CBF pathway

Although most reports discuss regulation of cold induced genes by CBF transcription factors, there is also evidence that cold acclimation involves activation of cold response pathways other than the CBF system. Microarrays of cold induced gene expression show that not all genes up-regulated by cold contain CRT/DRE motif in their promoters (Fowler and Thomashow, 2002; Oono *et al*., 2003). The microarray analysis performed by Fowler and Thomashow (2002) using 8000 *Arabidopsis* genes, found 4 % of genes either up- or down-regulated by low temperature stress. Of these genes, only 12 % were identified as part of the CBF regulon. In addition, 15 known or putative transcription factors were identified in this analysis, suggesting low temperature response gene products participate in several low temperature regulons operating in tandem.

One CBF-independent system is highlighted by certain of the *hos* (*high expression of osmotically sensitive*) mutants of *Arabidopsis.* The *hos9-1* and *hos10-1* mutants which display hypersensitivity to freezing display normal accumulation of the gene transcripts regulated by CBF transcription factors (Zhu *et al*., 2004; Zhu *et al*., 2005). The induction of *COR15A*, *ADH* and *KIN1* in these mutants is also not altered after cold treatment, indicating that CBF regulated genes are not sufficient for full cold acclimation in *Arabidopsis* (Zhu *et al*., 2004; Zhu *et al*., 2005). *HOS9* encodes a putative homeodomain transcription factor (Zhu *et al*., 2004) whereas, *HOS10* gene encodes a putative R2R3-type MYB transcription factor (Zhu *et al*., 2005). Both

HOS proteins localize to the nucleus. Interestingly *hos9-1* is late flowering and *hos10-1* mutant exhibits an early flowering phenotype.

A second example of CBF-independent signalling is provided by the *eskimo1* mutant of *Arabidopsis* (Xin and Browse, 1998). Loss of function at this locus correlates with an increased resistance to freezing, but does not result in increased transcripts of CBF-regulated *COR* genes (Xin and Browse, 1998). However, this mutant does accumulate high levels of the compatible osmolyte proline, suggesting that the *ESK1* gene product regulates proline synthesis and degradation (Xin and Browse, 1998). P5CS (Δ^1-pyrolline-5-carboxilase synthetase) is the first enzyme in proline synthesis. The expression of *P5CS* in the *esk1* mutant is 8-fold higher than in non-acclimated wild type plants (Xin and Browse, 1998).

The non-acclimated *ada2b-1 Arabidopsis* mutant highlights a third novel pathway leading to freezing tolerance without over-expression of *COR* or *P5CS* genes (Vlachonasios *et al*., 2003). Complementation studies have shown that the ADA2b-associated system affects freezing tolerance by a completely different mechanism from CBF or ESK1 signal transduction (Vlachonasios *et al*., 2003). Transcripts of several transcription factors such as ERF5 and ERF6, WRKY33 and WRKY53, ZAT10 and ZAT12, along with a number of putative zinc finger proteins, are up-regulated in non-acclimated *ada2b-1* plants (Vlachonasios *et al*., 2003). The up-regulation of these genes was also reported by Fowler and Thomashow (2002) in cold acclimated wild type *Arabidopsis.* Therefore, freezing tolerance of the non-acclimated *aba2b-1* mutant may result in activation of downstream genes from these signalling pathways (Vlachonasios *et al*., 2003).

Finally, the *los1* (*low temperature sensitive1*) mutant, which is defective in expressing *COR* gene transcripts, also has increased freezing tolerance after cold acclimation, although this increase is less than that seen in wild type plants (Guo *et al*., 2002).

Together, these results suggest that cold stress activates a number of independent signalling pathways. Therefore the coordination of these pathways is important for full freezing tolerance in plants.

1.2.7.6 Negative regulators of low temperature signal transduction

Negative transcriptional regulation is required to reduce levels of certain genes after cold acclimation and so facilitate subsequent freezing tolerance. For instance, the *Arabidopsis* gene *HOS1* (*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE1*) has been identified as encoding a negative regulator of low temperature gene induction (Ishitani *et al*., 1998; Lee *et al*., 2001; Dong *et al*., 2006). Ishitani *et al*. (1998) reported that *hos1* shows altered expression of cold induced genes. The genes *RD29A*, *KIN1*, *COR15A* and *COR47* are hyper-induced in this mutant after cold; also this mutant can induce these cold responsive genes in temperatures as high as 19^{o}C. Most of the altered genes in *hos1-1*, e.g. *ADH* (*ALCOHOL DEHYDROGENASE*), are not controlled by the CBF/DREB1. Ishitani *et al*. (1998) also showed that the *hos1-1* mutant is less cold tolerant than wild type plants, but after 2 days of cold acclimation mutant can acquire freezing tolerance equivalent to wild type. Interestingly, this mutant did not show significantly altered expression of genes induced by osmotic stress and ABA. These authors therefore suggest that HOS1 may affect a step prior to convergence of the cold and ABA/osmotic responses. In addition *hos1-1* is an early flowering mutant, leading Ishitani *et al*. (1998) to suggest that HOS1 has multiple roles in cold acclimation, freezing tolerance and flowering in *Arabidopsis*.

HOS1 encodes a novel protein with a modified ring finger domain (Lee *et al*., 2002); this was the first RING finger protein reported to function in low temperature signal transduction. HOS1 localizes to the cytoplasm at warm temperatures but upon cold exposure translocates to the nucleus (Lee *et al*., 2002). Since some ring finger proteins act as E3 ubiquitin ligases, degrading specific proteins, Lee *et al*. suggest that HOS1 may target CBFs. Later it was shown that the HOS1 is a functional E3 ubiquitin ligase, which targets the ICE1 protein for degradation (Dong *et al*., 2006).

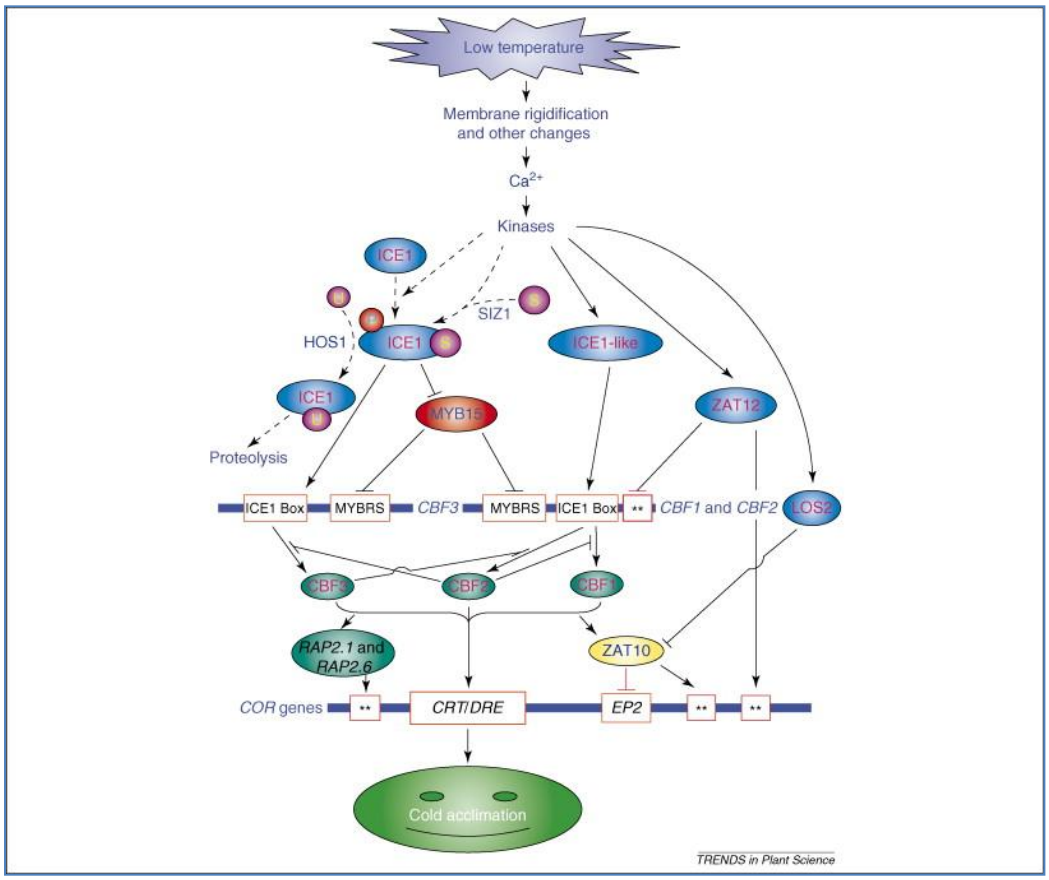
In *hos1-1* mutants, ICE1 does not degrade, resulting in higher levels of CBF transcription factors and downstream effecter proteins. In HOS1 over-expression lines, ICE1 protein levels are low even at warm temperatures compare to the wild type level (Dong *et al*., 2006). These data suggest that HOS1 negatively regulates downstream cold gene expression by degrading ICE1.

ZAT12 (a C_2H_2 zinc finger transcription factor) is another protein with a role in the negative regulation of cold induced genes. Induction of *ZAT12* was observed in parallel with *CBF1-3* after cold exposure in *Arabidopsis;* it regulates 24 *COS* (*COLD STANDARD SET*) genes, of which 9 are cold induced and 15 are cold repressed (Vogel *et al*., 2005). The over-expression of *ZAT12* did not cause any alterations in freezing tolerance (Vogel *et al*., 2005). However, transgenic plants over-expressing *ZAT12* accumulate much lower levels of *CBF1-3*, suggesting a negative regulation of the CBF cold response pathway (Vogel *et al*., 2005). The ZAT12 protein contains an EAR-like (ERF-associated amphiphilic repression-like) repressor domain, and therefore Vogel *et al*. (2005) suggest that ZAT12 might directly repress expression of *CBF1-3*. ZAT10/SIZ, another C_2H_2 zinc finger transcription factor, has also been identified as a negative regulator of cold induced gene expression (Lee *et al*., 2002). Transient expression assays showed that ZAT10 can negatively regulate *RD29A* expression under cold treatment, whilst LOS2 acts as a negative regulator of ZAT10 (Lee *et al*., 2002).

The transcription factor MYB15 also appears to function as an upstream negative regulator of CBFs. *MYB15* is up-regulated by cold treatment, and binds to MYB recognition sequences in the promoters of CBF genes (Agarwel *et al*., 2006). Over-expression of *MYB15* leads to reduced *CBF* transcripts along with a reduction in freezing tolerance (Agarwel *et al*., 2006). These authors also found that *myb15* mutant plants showed enhanced expression of CBFs during cold acclimation, and had enhanced freezing tolerance (Agarwel *et al*., 2006). Therefore, MYB15 is thought to be an upstream negative regulator of CBFs expression. An interaction of MYB15 with ICE1 has also been proposed as over-expression of *MYB15* is observed in *ice1* mutants under cold stress conditions (Chinnusamy *et al*., 2003; Agarwel *et al*., 2006).

Figure 1.5

Cold responsive transcriptional network in *Arabidopsis*. Adapted from Chinnusamy *et al*. (2007).



According to the review of Chinnusamy *et al*. (2007) plants probably sense low temperatures through membrane rigidification and/or other cellular changes, which induce a calcium signature and activate protein kinases. This leads to the activation of transcription factor proteins, as indicated. Broken arrows imply post-translational regulation; solid arrows indicate activation; lines ending with a bar suggest negative regulation, and two asterisks (**) indicate unknown *cis*-elements. Abbreviations: CBF, C-repeat binding factor; CRT, C-repeat elements; DRE, dehydration-responsive elements; HOS1, high expression of osmotically responsive genes 1 ; ICE1, inducer of CBF expression 1 ; LOS2, low expression of osmotically responsive genes 2 ; MYB, myeloblastosis; MYBRS is a MYB transcription factor recognition sequence; SIZ1 (a SUMO E3 ligase); P, phosphorylation; S, SUMO (small ubiquitin-related modifier); U, ubiquitin.

Most of the currently known components of the cold response transcriptional network, as described in the above sections, are summarised in Figure 1.5.

1.2.7.7 Function of cold regulated genes leading to freezing tolerance

As discussed above, large numbers of genes are activated during cold acclimation. These genes can be categorized into groups according to their function, e.g. enzymes required for biosynthesis of osmo-protectants, late embryogenesis abundant (LEA) proteins, antifreeze proteins, chaperones, detoxification enzymes, transcription factors, kinases and enzymes involved in phosphoinositide metabolism (reviewed in Puhakainen *et al*., 2004). Over-expression of some low-temperature-induced transcription factor genes produces an increased freezing tolerance in non acclimated plants (Jaglo-Ottosen *et al*., 1998 and Liu *et al*., 1998). Whilst over-expression of certain other cold induced genes does not increase freezing tolerance to significant levels, their increased expression during low temperature exposure indicates that these genes have a role in freezing tolerance. Possible actions for these gene products may include stabilization of cell membranes against frost damage, reversion of the freezing-induced denaturation of proteins, prevention of molecules from precipitating, and reduction of the direct physical damage caused by intracellular ice (Thomashow, 1998). However, the mechanism of action for most of these other cold induced genes is unknown.

The first attempts at understanding the function of *COR* genes in freezing tolerance were made by Artus *et al*. (1996). These authors produced transgenic plants over-producing the COR15a polypeptide in *Arabidopsis*, and compared the freezing tolerance of chloroplasts and protoplasts. *COR15a* is expressed in response to cold, drought and ABA (Thomashow, 1994), and encodes a protein which is targeted to the chloroplast. The COR15 polypeptide is processed by the chloroplast (and thence designated as COR15am); later work showed that COR15am was not retained exclusively in plastids (Thomashow *et al*., 1997). The chloroplasts and protoplasts of transgenic plants over-expressing COR15am had increased freezing tolerance. (Thomashow *et al*., 1997). Further investigations, on the cryostability of liposomes *in*

vitro, showed that COR15am interacts with lipid bilayers, although precisely how this relates to cryoprotection is not known (Thomashow *et al*., 1997).

Apart from *COR15a*, over-expression of cold-induced genes such as tomato *Le25* gene in yeast (Imai *et al*, 1996) also increased low temperature tolerance.

A number of cold-responsive genes such as *KIN1*, *KIN2*, *COR15*, *COR24*, *COR78 etc*. are classified in to LEA or LEA-like proteins (reviewed in Pearce, 1999) because of their similarities in protein sequence (Thomashow *et al*., 1997). LEA proteins are synthesized in embryos just prior to seed desiccation, and in seedlings in response to water deficit. These proteins are highly hydrophilic and boiling-stable. Thomashow *et al*., (1997) therefore suggest that LEA proteins may have a role in stabilising membranes under dehydration conditions. The sequence similarities of COR proteins with LEAs likewise imply a role for *COR* gene products in tolerance to the dehydration induced by freezing (Thomashow *et al*., 1997).

Dehydrins are also LEA type proteins, produced in response to drought, low temperature, high salt and ABA application (Park *et al.*, 2006). A common feature of most dehydrins is a repeated lysine-rich block called the K segment (consensus sequence: EKKGIMDKIKEKLPG). It is postulated that this ubiquitous K segment probably form an amphipathic α-helix, which may help stabilise macromolecules and sub-cellular structures (reviewed in Pearce, 1999). Campbell and Close (1997) also suggested that the highly polar segment of dehydrins might form hydrogen bonds with polar regions of other macromolecules, forming a 'coat' which would act as a surfactant to decrease dehydration induced by drought or freezing. Apart from this, an immuno-electron microscope analysis by Danyluk *et al*. (1998) revealed that dehydrin-like proteins of wheat accumulate in the plasma membrane upon low temperature exposure, specifically in the areas where freeze-induced dehydration is likely to be more severe. They suggested that these proteins may be involved in the cryoprotection of the plasma membrane against freezing. However, an exact biological function for dehydrins has not yet been proved.

Certain antifreeze proteins (AFPs) were found to accumulate in the apoplast of cold acclimated plants, implying a role for these molecules in prevention of frost- induced damage (Griffith and Yaish, 2004). AFPs accumulate in the apoplast only upon low temperature stimuli; they have multiple hydrophilic ice-binding domains (Griffith and Yaish, 2004). AFPs are proposed to inhibit ice re-crystallization and ice nucleation during prolonged low temperature exposure (Griffith and Yaish, 2004). Microscopic studies on the shape of the ice-crystals formed with and without AFPs showed that AFP proteins bind to the face of prism like ice crystals to form hexagonal shaped crystal; this inhibits the binding of subsequent water molecules (Griffith and Yaish, 2004).

It is interesting to note that some AFP protein sequences have high homologies to PR (pathogenesis-related) proteins (Hon *et al*., 1995). Accumulation of *PR* genes during low temperature stress has been observed in several plant species (Gaudet *et al.*, 2000). The proposed function of these proteins is plant protection from attack by pathogens, e.g. snow mould, to which frost-damaged plant tissues are particularly susceptible (Pearce, 1999; Gaudet *et al.*, 2000). In addition to these specific low temperature induced genes, the enhanced expression of genes encoding general metabolic enzymes, such as sucrose phosphate synthase, galactinol synthase and Δ(1) pyroline-5-carboxylase, is assumed to promote an enhanced accumulation of metabolic solutes, and so help offset cold-induced dehydration (reviewed in Pearce, 1999).

1.3 Cross-talk between low temperature stress and other abiotic and biotic stresses

Several research groups have identified points of convergence between low temperature and other biotic and abiotic stress response pathways (reviewed by Fujita *et al*., 2006). These convergences are achieved mainly via the actions of phytohormones, ROS (Reactive Oxygen Species) and transcription factors. Overlap of gene expression between low temperature, drought and salt stress has been

observed in large scale microarray studies; e.g. Seki *et al*. (2001 and 2002), Chen *et al*. (2002), Shinozaki *et al*. (2003). Some stresses result in specific cellular effects, such as ion toxicity during salt stress, reduction of nutrient availability during drought stress and direct effects on metabolism caused by freezing such as reduced metabolic rates. In addition to their specific actions, these stresses also cause general cellular injury through dehydration. (Chinnusamy *et al*., 2004). Therefore, expression of common sets of genes for these three stresses can be expected when the plant activates injury prevention mechanisms in response to cellular dehydration.

The overlapping gene expression profiles in response to UV, oxidative stress and pathogen attack has also been discussed (Nawrath *et al*., 2002). In plants, ROS are continuously produced in chloroplasts, mitochondria and peroxisomes; scavenging of ROS by various mechanisms maintains an equilibrium in the cell between their production and removal (reviewed in Apel, 2004). Most abiotic stresses, such as high light, drought and extremes of temperature, affect these steady state levels of ROS, and can cause cellular damage (reviewed in Apel, 2004). Although high accumulation of ROS are toxic to plants, these chemical species also act as signalling molecules to activate stress response pathways such as ABA signalling (reviewed in Fujita *et al*., 2006). Similarly, with pathogen invasion, a rapid defence mechanism observed in plants is the 'oxidative burst'; a biphasic production of cytosolic ROS which kills the infected cell (and its resident pathogen), so limiting spread of the invading microorganism within the plant body (reviewed in Apel, 2004). Therefore, ROS production is a point of convergence for both biotic and abiotic stress signal relays. Consistent with this, expression of large set of genes that encode ROS scavenging enzymes has been observed under both biotic and abiotic stress conditions (reviewed in Fujita *et al*., 2006).

ABA (Abscisic Acid), SA (Salicylic Acid), JA (Jasmonic Acid) and ET (Ethylene) are the main phytohomones which regulate plant biotic and abiotic signalling pathways. Of these, SA, JA and ET participate in biotic stress responses by activating different signalling mechanisms (Kunkel and Brooks, 2002). ABA's main role is in response to abiotic stresses such as drought, salt and to some extent in low temperature tolerance (Xiong *et al*., 2002; Shinozaki *et al*., 2003). However, several

reports have indicated a antagonistic action of plant disease resistance by ABA (reviewed in Fujita *et al*., 2006). Exogenous applications of ABA to various plant species including rice, tobacco and tomato reduced their resistance to various pathogens, as well as reducing the accumulation of transcripts of pathogen responsive genes such as β-1-3-glucanase, an antifungal protein (reviewed in Yasuda *et al*., 2008 and Fujita *et al*., 2006). Therefore, low temperature stress response relays converge with other biotic and abiotic stresses via ABA signalling.

Many similarities in the expression profiles of transcription factor genes under cold, drought and salt treatments, and their known targets, have been mentioned in earlier sections. In contrast, there is scant evidence of cross talk in transcriptional regulation between low temperature stress and biotic stress treatments. A rare example is *DEAR1* (*DREB and EAR motif protein 1*), a transcriptional repressor of DREB proteins, identified as a transcription factor protein which mediates both plant defence and freezing tolerance (Tsutsui *et al*., 2009).

1.4 The SFR6 locus of *Arabidopsis*, and the *sfr6* mutant phenotype

1.4.1 Identification of *sfr* mutations

Arabidopsis mutants impaired in freezing tolerance are important resources through which to identify components of low temperature induced signalling. In the last 2 decades several *Arabidopsis* mutants defective in freezing tolerance have been isolated. The molecular analysis of these mutants has accelerated our understanding of cold acclimation and freezing tolerance of *Arabidopsis* as well as other crop species. The *Arabidopsis* mutant ***s****ensitive to* ***fr****eezing****6*** (*sfr6*), used for this study, is one such mutant, impaired in cold acclimation and freezing tolerance (Warren *et al*., 1996).

Screening of mutant populations of plants is one way to identify new functional genes relevant to specific biological functions. Using this approach, Warren *et al*., (1996) targeted genes whose proteins performed a role in freezing tolerance. They identified 8 *Arabidopsis* mutants with a low-temperature sensitive phenotype from an

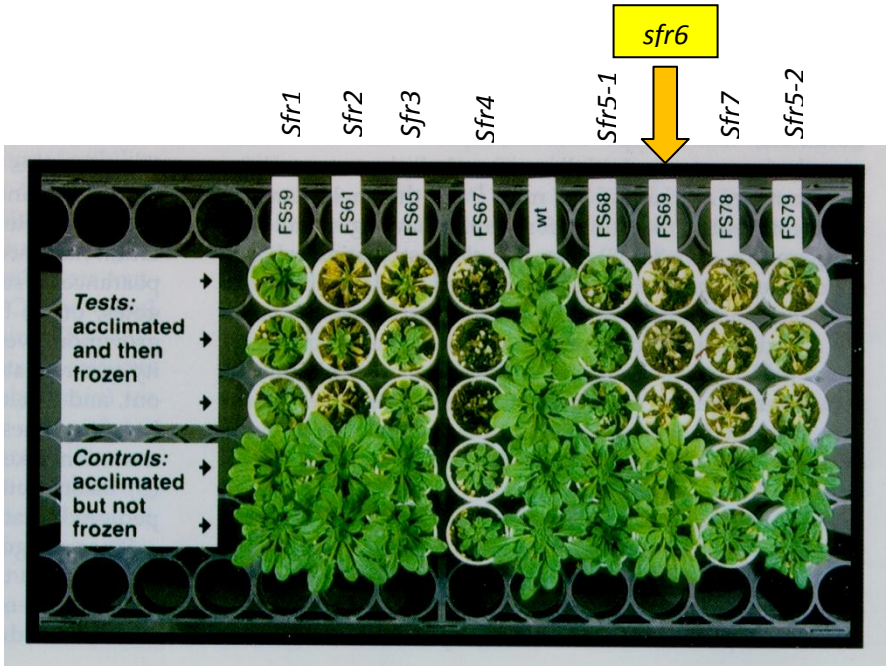
EMS-mutagenized population. The screening treatment involved exposure to chilling (4^{o}C) to trigger the cold acclimation response, followed by 24 hours at freezing temperatures (-6°C). Only those mutants showing hypersensitivity to freezing, but not chilling, were selected in their study.

These authors isolated 8 lines for further study; their response to freezing is illustrated in Figure 1.6 below. The mutants were tested for dominance; 6 of these mutations were recessive and the remaining 2 were co-dominant. Genetic complementation tests were performed to test the allelism of these 8 mutations. With the exception of 2 lines all other lines complimented each other for sensitivity to freezing. It was thus suggested that the 8 lines isolated defined 7 genes affecting the induction of freezing tolerance after cold acclimation. These loci were defined as *sfr* mutants (*sensivity to freezing) 1-7*.

Warren *et al.* (1996) pointed out that the degree of sensitivity to freezing temperatures varied between the mutants. They suggested that the 7 *SFR* genes have a role specific to freezing tolerance, since their phenotype was normal during cold acclimation; significant damage was seen in these plants only after expose to freezing (Figure 1.6). Mutants varied in the extent and type of leaf damage incurred during freezing. In *sfr1* only young leaves are affected, whilst in other mutants all leaves showed signs of damage (Warren *et al*., 1996). Electrolyte leakage (a well known assay for membrane stability) was performed on mutant tissues with and without cold acclimation, and compared to leaves from wild type plants subjected to similar treatment. The inferred LT50 value of cold acclimated mutant tissue was significantly higher than that of wild type control, demonstrating that the increased freezing sensitivity of 6 of these mutants is associated with a modulation of membrane properties. The exception is *sfr2* which showed no significant difference in electrolyte leakage compared to wild type plants (Warren *et al*., 1996).

Figure 1.6

Freezing sensitivity of 7 *sfr* mutants after cold acclimation. Adapted from Warren *et al*. (1996).



Phenotypes of *sfr* mutant lines in comparison to wild type. Three plants of each line are shown 9 days (16-20^{o}C) after freezing under 24 h at -6.0^{o}C. Two plants cold acclimated but not frozen from each line are shown as controls for any injuries due to the effect of the cold acclimation treatment itself.

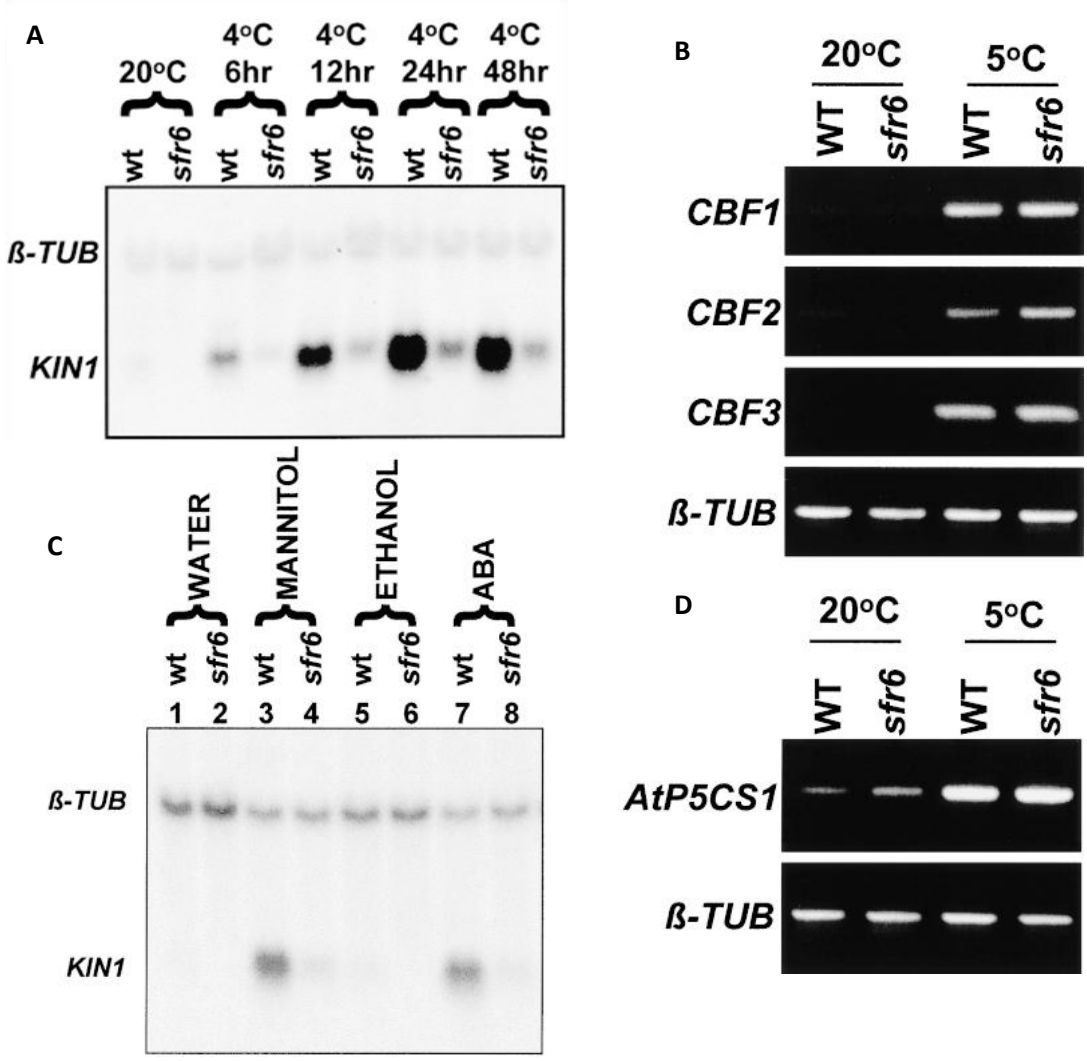
An analysis of protein, fatty acid, sugar, glucose and anthocyanin levels in the *sfr* mutants after cold acclimation showed wide variations (McKnow *et al*., 1996). A comparison of *sfr1*, *sfr2* and *sfr5* showed no significant differences from wild type for these parameters, *sfr4* prevented the normal accumulation of sucrose and glucose, and both *sfr4* and *sfr7* affected fatty acid composition after cold acclimation (McKnow *et al*., 1996). *sfr3*, *sfr4*, *sfr6* and *sfr7* showed reduced accumulation of anthocyanin during cold acclimation (MaKnow *et al*., 1996). The accumulation of three cold induced boiling soluble proteins in *sfr* mutants were as wild type, indicating that the detection and transduction of cold signals might not altered by these mutations (McKnow *et al*., 1996).

1.4.2 Evidence for the effect of SFR6 on the CBF cold response pathway

With the exception of *sfr6, COR* gene expression in the *sfr* mutants was normal under both low temperature and drought stress (Knight *et al*., 1999). The promoters of the *COR* genes (*COR78, KIN1 and COR15a*) tested in this experiment all contain CRT/DRE *cis*-acting elements (Baker *et al*., 1994), targeted by transcription factors; CBF1-3 upon low temperature exposure (Stockinger *et al*., 1997) and DREB2 upon drought treatment (Liu *et al*., 1998). In additon, CBF4 (Haake *et al*., 2002) activates this element (see section 1.2.7.2 for more information). However, *CBF1-3* expression under low temperature conditions (Figure 1.7), and CBF protein levels were found to be normal in the *sfr6* mutant (Knight *et al*., 1999; Knight *et al*., 2009). This indicates that SFR6 acts downstream of CBF activity. Over-expression of CBF1/2 did not restore *COR* gene expression in *sfr6-1*, confirming that SFR6 acts downstream of CBF transcription (Knight *et al*., 2009).

Figure 1.7

Stress induced gene expression in *sfr6-1* mutant. Adapted from Knight *et al*. (1999).



(A) *KIN1* expression of 7-day-old seedlings of *sfr6-1* and wild type plants grown on MS agar and placed in a growth chamber at 4^{o}C for the length of time indicated.

(B) Cold induced expression of *CBF1*, *CBF2* and *CBF3* in *sfr6-1* mutant and wild type Col-0 treated either 5 or 20^{o}C for 3 h.

(C) Osmotic stress-induced and ABA-induced *KIN1* expression in *sfr6-1* and Col-0 treated with water, 0.44 M mannitol, 0.1% (v/v) ethanol or 100 µM ABA for 3 h.

(D) Cold induced expression of *AtP5CS1* in *sfr6-1* mutant and wild type Col-0 treated either 5 or 20^{o}C for 3 h. B-TUB (β-Tubulin).

1.4.3 Cloning the *SFR6* gene

SFR6 transcript levels during low temperatures remain unchanged, suggesting that SFR6 is activated at the post transcriptional level to exert its effect on cold gene regulation (Knight *et al.*, 2009). The SFR6 predicted protein is 1268 amino acids long (Figure 1.8) with a molecular mass of 137 kDa (Knight *et al*., 2009). It was found that the SFR6 is a unique gene in *Arabidopsis*; close homologues are present in higher plants including rice, wheat, potato and other *Brassicaceae* (Knight *et al*., 2009). However, there are no similar protein sequence matches outside the plant kingdom (Knight *et al*., 2009).

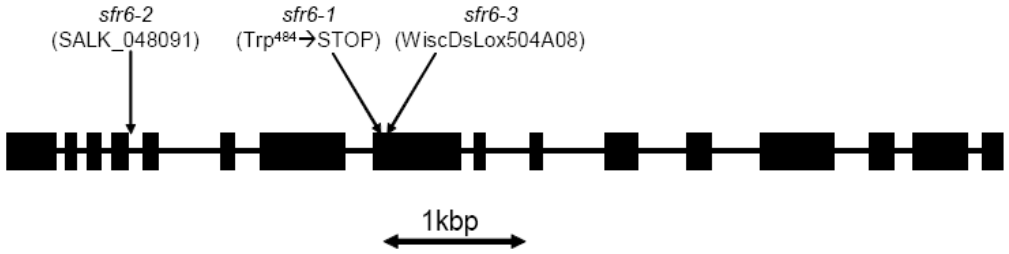
Attempts to map the chromosomal location of *SFR6* indicated that it lies close to the centromere of chromosome 4 (Knight *et al.*, 2009). The locus was identified as *At4g04920* by screening T-DNA insertion lines within the interval identified by mapping; this method identified 2 T-DNA lines (*sfr6-2:*- SALK-048091, NASC ID N048091 and *sfr6-3:*- WiscDsLox504A08, NASC ID N859103). These lines both have insertions in the same locus, and both show a phenotype similar to *sfr6-1* mutant, *i.e.* reduced expression of a group of *COR* genes, freezing sensitivity after cold acclimation and visible phenotypes such as enlarged cotyledons and pale coloured foliage (see Figure 1.9 for the visible phenotype) (Knight *et al*., 2009). The point mutation resulting in *sfr6-1* changes a nucleotide from "G" to "A", changing a UGG tryptophan codon to UGA stop in exon 8 (Knight *et al*., 2009) (Figure 1.8).

At4g04920 is a large gene with 16 exons and a predicted coding region of 3807 bp (without introns). *sfr6-2* and *sfr6-3* have an T-DNA insertion into the 4th and 8th exons respectively (Knight *et al*., 2009). All these mutations occur in the first third of the SFR6 protein; the EMS point mutation of *sfr6*-1 is at bp 1452 (amino acid 484) and the *sfr6-3* insertion is very close to this original mutation (Knight *et al*., 2009) (Figure 1.8).

Figure 1.8

Map and predicted protein sequnce of *At4g04920* showing the T-DNA insertion sites of *sfr6* alleles and the point mutation of original *sfr6* EMS mutant. These pictures were adapted from Knight *et al*. (2009).

(a)



(b)

```
   1  MNQQNPEEEVSLVNNSGGGGIIEAPAIVEEKEEEGLQQKQEETIESTDPILVVVEEKLLEKSVDGEKEDDNSSSSNMEIDPVSPATVFCV
  91  KLKQPNSNLLHKMSVPELCRNFSAVAWCGKLNAIACASETCARIPSSKANTPFWIPIHILIPERPTECAVFNVVADSPRDSVQFIEWSPT
 181  SCPRALLIANFHGRITIWTQPTQGSANLVHDATSWQCEHEWRQDIAVVTKWLTGASPYRWLSSKPSSGTNAKSTFEEKFLSQSSESSARW
                                 ↑ sfr6-2
 271  PNFLCVCSVFSSGSVQIHWSQWPSNQGSTAPKWFSTKKGLLGAGPSGIMAADAIITDSGAMHVAGVPIVNPSTIVVWEVTPGPGNGLQAT
 361  PKISTGSRVPPSLSSSSWTGFAPLAAYLFSWQEYLISEIKQGKKPSDQDSSDAISLSCSPVSNFSAYVSPEAAAQSAATTTWGSGVTAVA
 451  FDPTRGGSVIAVVIVEGQYMSPYDPDEGPSITG*WRVQRWESSVQPVVLHQIFGNPTSNFGGQVPTQTVWVSRVDMSIPPTKDFKNHQVAA
                                 sfr6-1 ↑                                    ↑ sfr6-3
 541  AGPSVDAPKEPDSGDEKANKVVFDPFDLPSDIRTLARIVYSAHGGEIAIAFLRGGVHIFSGPTFSPVENYQINVGSAIAAPAFSPTSCCS
 631  ASVWHDAAKDCAMLKIIRVLPPALPRNQSKVDQSTWERAIAERFWWSLLVGVDWWDAVGCTQSAAEDGIVSLNSVIAVMDADFHSLPSTQ
 721  HRQQYGPNLDRIKCRLLEGTNAQEVRAMVLDMQARLLLDMLGKGIESALVNPSALVFEPWRVDGETITGINPEAMAVDPALVSSIQAYVD
 811  AVLDLASHFITRLRRYASFCRTLASHAASAGTGSNRNNVTSPTQNASSPATPQVGQPTTTTTTTATTNSSGSSHVQAWMQGAIAKISSSN
 901  DGSNSTASPISGSPTFMPISINTGTFPGTPAVRLIGDCHFLHRLCQLLLFCFLQRSSRFPQRNADVSSQKLQTGATSKLEEVNSAKPTPA
 991  LNRIEDAQGFRGAQLGTGVKGIDENSARTTKMGSGNAGQGYTYEEVRVLFHILMDLCKRTSGLAHPLPGSQVGSGNIQVRLHYIDGNYTV
1081  LPEVVEAALGPHMQNMPRPRGADAAGLLLRELELHPPSEEWHRRNLFGGPGSEPEDMILTDDVSKLSNSLDLPDTNFSGICDGYNRVHSL
1171  WPRKRRMSERDAAFGSNTSVGLGAYLGIMGSRRDVVTATWKTGLEGVWYKCIRCLRQTSAFASPGATKQPNPNERETWWTSRWVYCCPMC
1261  GGTWVRVV*
```

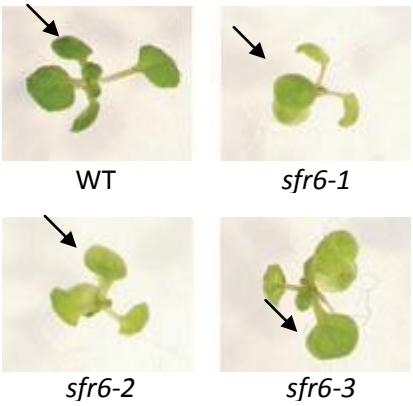
(a) Representation of the *At4g04920* genomic coding sequence, in which black blocks represent exons and thin lines represent introns. The *sfr6-1* EMS point mutation causes a premature stop codon in exon 8. The T-DNA insertion sites of *sfr6-2* and *sfr6-3* are shown by the arrows.

(b) Predicted protein sequence with sites of T-DNA insertion and the premature stop codon (*sfr6-1*) marked. Again the T-DNA insertion sites of *sfr6-2* and *sfr6-3* are indicated.

Protein fusion constructs tagging SFR6 with GFP, and transiently expressed in leek cells (by particle bombardment), as well as stably integrated into the *Arabidopsis* genome, both show that the SFR6 protein is localized in the nucleus (Knight *et al*., 2009) (see Figure 1.10 below).

Figure 1.9

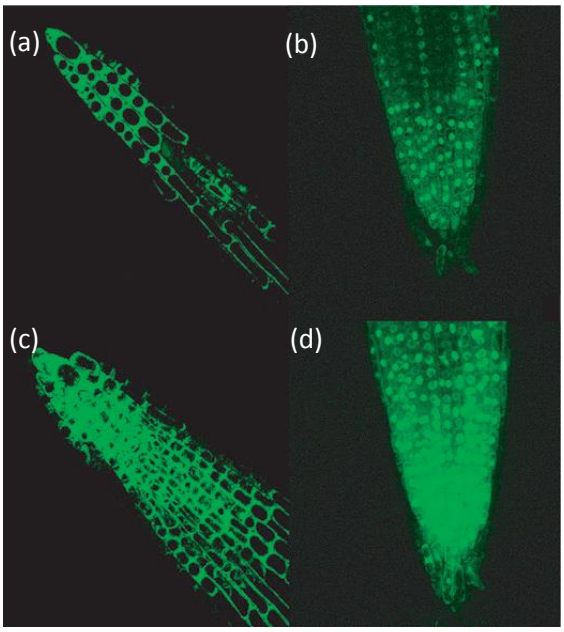
Twelve-day-old Columbia wild type and *sfr6* mutants. Adapted from Knight *et al*. (2009).



sfr6 mutants show paler colouring and larger cotyledons (indicated by arrows) than wild type Columbia plants. *sfr6-1* is the original EMS mutant, *sfr6-2* is line SALK_048091 and *sfr6-3* is line WiscDsLox504A08.

Figure 1.10

Nuclear localization of SFR6 using GFP fusions. Adapted from Knight *et al*. (2009).



Confocal microscopy images of N-terminal GFP-tagged GUS (a,c) and N-terminal GFP-tagged SFR6 (b,d) in the root tip are shown in this figure. One optical section (a,b) and the complete z-stack (c,d) are shown for each construct.

1.4.4 Other *Arabidopsis* mutations which repress the cold-induction of *COR* genes

The *los2* mutant of *Arabidopsis* is reported to reduce cold induction of *COR* genes without altering *CBF* gene expression (Lee *et al*., 2002), whilst another mutation, *los1*, also reduces expression of *COR* genes upon low temperature stress (Guo *et al*., 2002). However, unlike *los2*, the *los1* mutant shows super induction of *CBF* expression (Guo *et al*., 2002). The effects of *LOS1* and *LOS2* are specific to cold because both these mutations do not block osmotic, salt and ABA induction of *COR* genes (Guo *et al*., 2002; Lee *et al*., 2002). The *los* mutations therefore contrast with the *sfr6* phenotype, which blocks osmotic and ABA induced *COR* gene expression (Knight *et al*., 1999). Another contrast with *sfr6* is the sensitivity of *los2* to chilling

stress in light but not dark (Lee *et al*., 2002) (their data makes no mention of chilling sensitivity assays for *los1*). Since *CBF*s are induced to normal levels in *los2* mutant and are 'super-induced' in *los1*, accompanied by significant reduction of *COR* gene expression, these authors suggest that both LOS1 and LOS2 have essential functions downstream of CBF transcription factor activity upon low temperature induction of *COR* genes.

Cloning of *LOS1* revealed LOS1 to be a translation elongation factor 2-like protein, essential for peptide chain elongation during protein synthesis. However, protein synthesis in the *los1* mutant at ambient temperatures was not altered, indicating a function for LOS1 specifically at cold temperatures (Guo *et al*., 2002). Since the *los1* mutant super-induces *CBF*/*DREB1* during cold stress, the authors also suggest that the CBF/DREB1 protein may feedback-suppress its own level within the cell, either by regulating its own transcription directly, or via some downstream components of the signalling pathway.

LOS2 encodes an enolase that converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway (Lee *et al*., 2002). According to these authors, the *LOS2* transcript was detectable at ambient temperatures, and increased substantially in response to cold, although LOS2 protein levels only increased slightly. In human cells, the α-enolase protein act as transcriptional repressors by binding to the promoter of the *c-myc* gene, so inhibiting transcription by blocking TATA binding proteins from reaching their docking site in the DNA (Lee *et al*., 2002). Therefore, these authors suspect that LOS2 might function as similar transcriptional repressor. The *Arabidopsis* zinc finger gene *STZ/ZAT10,* encoding a protein which represses the trans-activation of genes, contains a *cis* element in its promoter which is similar to the enolase recognition sequence from the c-myc promoter (Lee *et al*., 2002). *STZ*/*ZAT10* is transiently expressed in *Arabidopsis* within 1h of exposure to low temperature (Fowler and Thomashow, 2002). A stronger and more sustained transient expression of *STZ*/*ZAT10* was observed in *los2* (Lee *et al*., 2002). Therefore, these authors suggest that LOS2 increases *COR* gene expression indirectly upon low temperature exposure by repressing levels of the *STZ/ZAT10* transcript (Lee *et al*., 2002).

1.4.5 Effect of SFR6 on other cellular processes

Recent work has shown that in addition to modulating *COR* gene expression; the *SFR6* gene product also has a role in determination of flowering time and circadian clock function (Knight *et al*., 2008). *sfr6* is late flowering when grown under a long day photoperiod. Microarray analysis of gene expression showed that the *sfr6-1* mutation down-regulates circadian clock control genes with an evening element (EE-*AAAATATCT*) in their promoters (Knight *et al*., 2008); this mechanism is a key component of circadian regulated gene expression. Further, the analysis of leaf movement rhythms in *sfr6* mutant plants showed a sucrose dependent long-period phenotype, i.e. *sfr6* did not shift its periodicity to a shorter rhythm in the presence of sucrose, as seen in wild type *Arabidopsis* (Knight *et al*., 2008).

The genes involved in flowering time and circadian clock function are independent of the CBF controlled *COR* regulon. However, several lines of evidence suggest that cross-talk occurs between pathways regulating the clock and low temperature responses (Mikkelsen and Thomashow, 2009; Seo *et al*., 2009). Kreps *et al*. (2003) report that the promoters of *COR* genes are enriched with *EE*- evening element motifs as well as *CRT*/*DRE, ABRE* and *G*-Box elements. More recently Mikkelsen and Thomashow (2009) reported that *EE*-like (*EEL*) elements are required for the cold induction of *COL1* (CONSTANS-like) and *COR27* (*COLD REGULATED GENE27*). Further, these authors found that cold induction via the *EEL* element is amplified through coupling it with the *ABRE*/*ABA* response element-like (*ABREL*) motif. Both *COL1* and *COR27* are low temperature induced, and have *EE* sequences in their promoters. The promoters of the 218 CBF-independent cold induced genes are highly enriched with *EE* and *ABREL* motifs in combination (Mikkelsen and Thomashow, 2009). This means that the effect of *sfr6* on clock and flowering time gene expression may not be a direct consequence of the failure of *CBF* gene induction. Instead, it is possible that *SFR6* might also regulate other transcriptional events, and so mediate a wider range of transcriptional response in plants to environmental stimuli.

1.4.6 The *sfr2* and *sfr4* mutations

Out of 7 *sfr* mutations identified by Warren *et al*. (1996) apart from the *sfr6* mutant, the freezing sensitivity of *sfr2* and *sfr4* mutants has also been further studied. According to the electrolyte leakage assay conducted by Warren *et al.* (1996) all these *sfr* mutants gain some freezing tolerance during cold acclimation, although less than wild type. They also observed that *sfr4* has a higher degree of sensitivity to freezing than the other 6 mutants. Subsequently, they observed that *sfr4* mutant plants accumulate considerably less sucrose and glucose than other *sfr* mutants and wild type *Arabidopsis* (McKnow *et al*., 1996). Therefore, Uemura *et al*. (2003) used this characteristic of the *sfr4* phenotype to understand the role of sugars in protection against freezing.

A comparison of non-acclimated *sfr4* mutants and wild type plants showed little or no difference for electrolyte leakage, (Warren *et al*., 1996), freeze induced membrane lesions (e.g. expansion induced lysis (EIL)), loss of osmotic responsiveness (LOR) (Uemura *et al*., 2003) and soluble proteins, sugar and fatty acid content (McKnow, 1996). After cold acclimation, isolated *sfr4* protoplasts showed freezing tolerance similar to wild type; injury by EIL was completely eliminated in the mutant, and the temperature at which LOR was detected in *sfr4* was considerably higher. This indicates that the freezing tolerance of *sfr4* is due to a high incidence of LOR (Uemura *et al*., 2003). The incidence of LOR in *sfr4* was complemented by supplementing plants with sucrose. Since *COR15A* and lipid composition affects LOR, these authors suggest that the elevated sugar levels in sucrose-supplemented *sfr4* might change *COR* gene expression and/or membrane lipid composition, so restoring freezing tolerance (Uemura *et al*., 2003).

Thorlby *et al*. (2004) identified that the mutation in *sfr2-1* mutant is in gene *At3g06510*. Whilst this protein is a predicted to be a 1-glycosyl hydrolase (ß-glycosidase) it is divergent from most ß-glycosidases of *Arabidopsis*, and shows a closer homology to ß-glycosidases from thermophilic archea and bacteria. However, homologues of AtSFR2 from other plants, including both freezing-tolerant and freezing-sensitive species such as rice, could qualitatively and quantitatively

complement *SFR2* phenotype (Fourrier *et al*., 2008). The transcript levels of *SFR2* in wild type after cold acclimation or other stress treatments did not increase (Thorlby *et al*., 2004). The SFR2 protein localizes to the chloroplast envelope, suggesting a possible role in chloroplast protection under cold stress (Fourrier *et al*., 2008).

1.5 Summary

Cold acclimation is important in mediating freezing tolerance in over-wintering plants. During this process low non-freezing temperatures lead to the activation of signalling pathways that effect changes in the expression of genes protective proteins, and conferring freezing tolerance. Therefore, understanding the process of cold acclimation is necessary in order to manipulate freezing tolerance in agriculturally important crop plants. Identification of mutants which are unable to cold acclimate is a powerful approach for investigating and comprehending the signalling pathways involved.

The *sfr6* mutant of *Arabidopsis* was isolated on the basis of its reduced ability to cold acclimate (Warren *et al*., 1996). Further studies revealed that the freezing sensitivity of *sfr6* is due to defective expression of COR genes controlled by CBF/DREB1 transcription factors (Knight *et al*., 1999; Knight *et al*., 2009). However, *CBF/DREB1* gene expression is not mis-regulated at the transcriptional and translational level in *sfr6*, indicating that SFR6 operates downstream of CBF translation (Knight *et al*., 1999; Knight *et al*., 2009). SFR6 also mediates tolerance to osmotic stress induced by drought and salinity (Knight *et al*., 1999; Boyce *et al*., 2003) and gene expression affecting other important developmental processes such as flowering (Knight *et al*., 2008). Mapping and cloning of *SFR6* identified the locus as *AT4g04920* on chromosome 4 close to the centromere (Knight *et al*., 2009). Molecular study of SFR6 function in stress tolerance in *Arabidopsis* and other crop plants, as a possible route to crop improvement in the future was the central objective of this study.

1.6 Aims of this study

This study aimed to examine the role of the *SFR6* gene and its protein product in stress tolerance, in both *Arabidopsis* and important crop plants. This involved:

- Further characterising the actions of SFR6 in *Arabidopsis* by over-expressing *SFR6* in both wild-type and the *sfr6* mutant backgrounds, and assessing stress responses in these stable transformants (Chapter 3).
- Examining the role of SFR6 in tolerance to multiple environmental stresses, by assessing the response of *sfr6* mutants to a variety of stresses such as drought, salinity, oxidative stress, UV irradiance and pathogen attack (Chapter 4 and 5).
- Cloning a *SFR6* homologue from rice and testing its functional equivalence to the *Arabidopsis* gene by using it to complement the *Arabidopsis sfr6* mutant (Chapter 6).
- Looking for other components of SFR6-associated signalling by (i) screening EMS-mutagenized plants for mutations that modify the *sfr6* phenotype, (ii) out-crossing to isolate these as single mutants, and (iii) assessing their stress tolerance (Chapter 7).

Chapter 2

Materials and methods

2.1 Chemicals

All chemicals and media used were purchased from one of the following companies:

BDH Laboratory Supplies Ltd, Lutterworth, Leicestershire, UK

Melford laboratories Ltd, Bildeston road, Chelasworth, Ipswich, Suffolk

Fisher Scientific UK Limited, Bishop Meadow Road, Loughborough, Leicestershire, UK

Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK

MERCK chemicals Ltd, Padge Road, Beeston Nottingham, UK

2.2 Plant materials and growth conditions

2.2.1 Plant materials

Arabidopsis thaliana (L.) Heynh. (*A. thaliana*) ecotype Columbia (Col-0) and EMS mutagenised *sfr6-1* seeds were obtained from Lehle Seeds (Round Rock, Texas, USA). The seeds of the *sfr6* original mutant (*sfr6-1*) and two alleles (*sfr6-2* and *sfr6-3*) and *eds-1* mutant (Ler-0 background) were already available as lab stocks.

35S::*AtCBF1* and *35S*::*AtCBF1*::*GFP* seeds were a kind gift from Prof. M. Thomashow's lab (MSU-DOE plant research lab, Michigan State University).

The rice (*Oryza sativa* L.) seeds of *cv*. Japonica *var*. Lemont and *var*. Nipponbare (Herbiseed, New Farm, Mire Lane, West End, Twyford, RG10 0NJ) were used to generate callus for *Agrobacterium* transformation.

2.2.2 Seed sterilization

2.2.2.1 *Arabidopsis*

1. Ethanol surface sterilization

Seeds were sterilized with 70 % (v/v) ethanol, by shaking (Labnet votex mixture, Labnet international Inc., Woodbridge) in 1.5 ml microtubes for 5-10 min. Thereafter seeds were pipetted onto sterile filter paper (Whatman International Ltd, Maidstone, Kent, UK) and air dried in a laminar flow cabinet before being sprinkled on to solid agar medium (section 2.2.3).

2. Bleach surface sterilization

Seeds obtained from plants dipped in *A. tumefaciens* (section 2.11) were first surface sterilized with ethanol as described above. Then seeds were shaken in a solution of 10 % (v/v) sodium hypochlorite (NaOCl) and 0.25 % (w/v) sodium dodecyl sulphate (SDS) for 10 min. After that, the seeds were washed 6 times in sterile water, spread directly onto agar plates (section 2.2.3) and left to dry in a sterile laminar flow cabinet.

2.2.2.2 Rice

Rice seeds were surface sterilized according to the method described by Nishimura *et al*. (2006) with some modifications. First seeds were de-hulled manually, surface sterilized in 70 % ethanol for 15 min, followed by 2 % Sodium hypochlorite for 30 min with vigorous shaking. Seeds were then rinsed 5 times with copious amount of sterile water, and placed on a filter paper to remove excess water before plating on to N6D medium (see appendix A section A.10 for recipe).

2.2.3 Plant growth medium

Sterilized *A. thaliana* seeds were grown on MS medium agar plates (Murashing and Skoog, 1962); this comprised 0.8 % (w/v) plant tissue culture grade agar (Sigma-Aldrich) and 1 × Murashige and Skoog salts (Duchefa Biochemie BV, Haarlem, Netherlands). All growth media were sterilized by autoclaving at 121^{o}C for 20 min at 10^5 Pa. The pH of the medium was adjusted before autoclaving to 5.8. If required appropriate antibiotics were also added to the liquid medium after autoclaving and cooling to 50^{o}C.

Seeds collected from *Agrobacterium* dipped plants (section 2.11) were sown on full strength MS medium prepared as above, supplemented with 10 g/l sucrose and appropriate antibiotics.

Seeds collected from *Agrobacterium* dipped plants harbouring Basta as a selectable marker were directly sown on soil (compost:sand 1:1).

Seedlings were grown to maturity by transferring 7-day-old seedlings from agar plates onto re-hydrated peat discs (Jiffy products International, Norway). Individual plants were grown on small (38 mm diameter) peat discs, whilst large (42 mm in diameter) discs were used to grow up to 3 plants per disc for *A. tumefaciens* dipping (section 2.11).

2.2.4 Plant growth condition

A. thaliana seeds sprinkled onto germination medium as described in section 2.2.3, were cold-stratified at 4^{o}C for at least 2 days, (at least 4 days for seeds from *Agrobacterium* dipped plants) to synchronize germination. Seed plates were subsequently transferred to a Percival growth cabinet (CLF PlantClimatics, Germany, Model CU36L5D) at 20 ± 1^{o}C set for a long day (16 h light/8 h dark) photoperiod, with a light level at about 150 µmol m^{-2} s^{-1}.

For seeds sown onto soil as described in section 2.2.3, trays were covered with a clingfilme (Catering PVC clingfilme, Terinex, Clingorap®) and stratified at 4°C for 3-4 days and then transferred to growth rooms. Once seedlings emerged, a few holes were cut in the film for ventilation, and after two days it was removed completely.

Seedlings transferred onto peat plugs in trays were covered with film as above, and transferred to a growth room maintained at approximately 20 ± 1°C with a long day photoperiod (16 h light/8 h dark) and light level of 100-150 µmol m^{-2} s^{-1}. The film was ventilated and removed after two days as previously described. If seeds needed to be collected from individual plants, the Aracon system was used (BetaTech, Ghent, Belgium). Individual plants were contained by an Aracon transparent plastic tube in a cup-shaped base, which isolates each plant from its neighbours. Plants were regularly watered until seed set had occurred. When pods began to yellow, plants were allowed to dry out properly prior to seed collection.

2.3 Bacterial strains and growth conditions

2.3.1 Bacterial strains

Escherichia coli (*E. coli*) strains DH5α and DH5α (DB3.1) were obtained from Bioline (London, UK) and Invitrogen (Renfrewshire, UK) respectively. Homemade *Agrobacterium tumefaciens* (*A. tumefaciens*) strains C58C1 (Holsters *et al*., 1978) and ADH105 (Hood *et al*., 1993) were used for plant transformation. *Pseudomonas syringae* pv. Tomato (Pst) (P. *syringae*) DC3000 (virulent) (Grant *et al*., 1995) strain was generously provided by Lindsay Petersen (University of Cape Town, South Africa). *P. syringae* DC3000-lux and *P. syringae* DC3000-lux-avrRpm 1 strains were kingly provided by Chris Lamb (Department of Disease and stress biology, John Innes centre, Norwich, UK)

2.3.2 Antibiotics

All antibiotics used in this study were purchased from Melford laboratories Ltd are listed in the Table 2.1.

Table 2.1
Concentrations of antibiotics used in this study

Antibiotic	Stock concentration (mg / ml)	Working concentration (µg / ml)
Kanamycin	100	For plants: 50 For bacteria: 100
Timentin	200	200
Ampicillin	100	100
Spectromycin	50	50
Rifampicin	200	200
Hygromycin	50	50
Phosphinothricin	10	25

Antibiotic stock solutions were filter sterilized using 0.22 µm filters (Millipore Corporation, Bedford, USA) attached to syringes (VWR International Ltd, Magna Park, Lutterworth, UK), prior to addition to liquid plant media.

2.3.3 Bacterial growth medium

E. coli and *A. tumefaciens* were grown on solid agar plates consisting of 1.5 % (w/v) micro agar (Melford Laboratories Ltd, Ipswich, Suffolk, UK) and 2 % (w/v) Luria-Bertani (LB) (Melford Laboratories Ltd), or liquid media made from 2 % (w/v) LB. *P. syringae* were grown on either solid agar plates or liquid media consisting of King's Broth medium (KB; Melford Laboratories Ltd; King *et al.*, 1954; see appendix A.1 for the recipe). These media were autoclaved at 121^{o}C for 20 min at 10^5 Pa and cooled to approximately 50^{o}C before the addition of appropriate antibiotics.

2.3.4 Bacterial growth conditions

Bacteria were grown overnight in liquid LB or KB media with constant agitation (150-200 rpm) in an incubator or on solid LB or KB plates without agitation. Incubation temperatures were 37^{o}C for *E. coli* cultures, 29^{o}C for *A. tumefaciens* and 28^{o}C for *P. syringae* cultures. Under the Specific Pathogen Order (DEFRA; 1988) all *P. syringae* work was performed in a Category 2 laboratory.

2.4 Stress treatments

2.4.1 Stress treatments for plant stress tolerance assays

2.4.1.1 Drought

1. Withdrawing water

Individual plants were grown on 38 mm (diameter) peat plugs (see section 2.2.3) with constant watering up to three weeks post-germination. Water was withheld for 12 d, then re-watered; survival and recovery was then assessed after a further 6 d.

2. Addition of mannitol

Individual plants of Col-0 and *sfr6-1* were grown on peat plugs with constant watering up to three weeks post-germination. Subsequent watering applied different concentrations of mannitol (50, 100, 150, 200, 250, 300 mM). For each line tested, a total of 4 plants were screened per concentration. The same volumes of either water (as control) or mannitol were applied to each batch of plants. Phenotypes were then observed three weeks after treatment.

3. Root re-orientation assay

Seedlings were germinated and grown vertically on (non-supplemented) MS agar plates. At 5 days old, seedlings were carefully transferred (using hooked forceps) onto vertical plates of MS agar supplemented with either 100, 150, 200 and 300 mM mannitol. Seedlings were transferred so that their roots pointed upwards and shoots downwards, and placed back in the growth chamber. For each line tested, a total of 12 seedlings were screened per concentration of mannitol. Plants were also

transferred onto non-supplemented MS agar plates as a control. The amount of root reorientation (past 90^{o}) and root growth was examined as a score of osmotic tolerance. 10 g/l agar was added for MS medium for these vertical plates.

4. Root elongation assay

In addition, another set of plates were prepared as described in section 2.5.1.1.3 but seedlings were transferred such that their roots pointed downward as usual. The root length was measured once every two days for the subsequent two weeks post treatment.

2.4.1.2 Salinity

1. Addition of NaCl

Individual plants of Col-0 and *sfr6-1* were grown on peat plugs with constant watering up to three weeks post-germination. Subsequently, plants were watered with equivalent volumes per batch of either water or 200 mM NaCl. For each line tested, a total of 4 plants were screened. Plants were examined for symptoms of salt stress at three weeks post treatment.

2. Root re-orientation assay

Five-day-old seedlings germinated on vertical MS agar plates were transferred to different concentrations of NaCl supplemented plates (75, 100, 150 and 200 mM NaCl) and then transferred to a growth room turning plates 180°, as described previously for the osmotic screen (see section 2.4.1.1). The amount of root reorientation (past 90^{o}) and root length were compared to wild-type to assess their degree of salt tolerance.

3. Root elongation assay

Five-day-old seedlings germinated on vertical MS agar plates were transferred to different concentrations of NaCl supplemented plates (75, 100, 150 and 200 mM NaCl) and root length was measured once every two days for two weeks post treatments.

4. Seed germination assay

Seeds were germinated and grown on MS agar plates (see section 2.2.3) supplemented with different concentrations of NaCl (75, 100, 150, 200 and 250 mM). The overall number of germinated seeds were counted 7 d later (successful germination being taken as emergence of the radicle).

2.4.1.3 Oxidative stress

H_2O_2 and methyl viologen (MV) were used to induce oxidative stress.

1. Spraying with H_2O_2

Three-week-old plants grown on peat plugs were sprayed with 100 mM of H_2O_2 until all leaves were wet and then put back into the growth room. This was repeated after three days from first spraying and plants were monitored over 14 days for leaf bleaching as a score of oxidative stress tolerance. Control plants were sprayed with water. A total of 8 plants were screened per treatment for each line tested.

2. Growth on agar plates supplemented with MV

Seedlings were germinated on vertical MS agar plates. After 5 days seedlings were carefully transferred (using hooked forceps) onto vertical MS plates supplemented with different concentrations of MV (0.1, 0.25, 0.5 and 1 µM) and placed for the subsequent two weeks into the growth room. Plants were also transferred onto non-supplemented MS agar plates as a control. Twelve plants from each line were screened per concentration. Plants were observed at 14 days post treatment.

3. Immersion of seedlings in solutions of paraquat

Seedlings were germinated on horizontal MS agar plates. After 10 days, seedlings were submerged in sterile wells (25-well plates) containing 3 ml of 0.1, 0.5, 1 and 2.5 µM (1 seedling per well) of MV and returned to the growth chamber. Control plants were treated with 3 ml of sterile water. For each concentration a total of 5 plants were tested. Plants were monitored at 24 and 48 hours after submerging for cotyledon and leaf bleaching as an indicator of oxidative stress tolerance.

2.4.1.3.1 Chlorophyll assay

A chlorophyll assay was conducted as a numerical non-subjective measurement of greening in response to MV. The leaves of four-week-old seedlings grown on peat plugs were submerged in 6-well plates (3 replicates per treatment and 2 leaves per replicate) containing 5 ml of either sterile water or different concentrations of MV for 48 h. After this treatment, 100 % acetone was added (0.4 ml) and tubes were incubated overnight at room temperature in dark conditions to limit the break-down of chlorophyll. Samples were then homogenized in the acetone using a micro-pestle (pellet pestle motor; Kontes, NJ, USA) for 60 s and vortexed for 30 s to re-suspend the material. Subsequently, tubes were centrifuged for 5 min at 20,000 g separating the plant material from the solubilised chlorophyll in the supernatant. The supernatant was collected, the pellet re-suspended in 0.4 ml acetone for further chlorophyll extraction, vortexed for 30 s and centrifuged again. These steps were repeated until a total of 1.2 ml acetone had been used to extract the chlorophyll from each sample. This pooled supernatant was made up to a volume of 1.5 ml with distilled water (resulting in an acetone concentration of 80 % v/v).

Chlorophyll content was measured at OD 663 and 645 nm using an S-20 spectrophotometer (Boeco, Germany), with 80 % acetone as a blank. The following equation was used to calculate chlorophyll concentration (Hipkins and Barker, 1986):

$$Chlorophyl\ concentration\ (g/gFW) = \frac{\{(20 \times A645) + (6.02 \times A663)\} \times V}{\mathrm{FW}}$$

V = Volume of 80 % acetone

FW = Fresh weight of tissue

2.4.1.4 Heat stress

Seeds were sown out individually and evenly on horizontal MS agar plates (approximately 30 seedlings per plate and 3 plates per treatment per line). At 14

days post-germination, the plates were transferred to growth cabinets (with lids still on) for heat treatments of 38^{o}C for 90 min, and 45^{o}C for 120 min. Control plates were maintained in a 20^{o}C Percival chamber. For all treatments, light conditions were similar (100-150 µmol m^{-2} s^{-1}). Plants were assessed for survival (seedlings with green coloured growing meristem) at 18 days post treatment.

2.4.1.5 UV stress

Seeds were sown out individually and evenly on horizontal MS agar plates. At 7 days the seedlings were irradiated with 5, 10, 15 and 20 KJ/m^2 of UV, in a UV cross linker (Uvitec Ltd, Cambridge, UK) with the plate lids off. Although control plates were not treated with UV their lids were also removed for 10 min (the maximum time taken for 20 KJ/m^2 treatments). Immediately after treatment, all plates including control plates were resealed and returned to the growth chamber. Numbers of surviving seedlings (with green coloured growing meristem) were counted 10 d later.

2.4.1.6 Freezing stress

Seven-day-old seedlings were transferred to peat plugs and put into growth chambers (Artic plant growth chambers A3655, Weiss Gallenlcamp ltd., Epinal way, Loughborough, LE11 3GE) programmed for short day conditions (8 h light and 16 h dark) 20^{o}C temperature, 60 % humidity and 150 µmol m^{-2} s^{-1} light level. Plants were grown for 5 weeks under these conditions. Cold acclimation was achieved under same day length and light level at 4^{o}C for 11 days. The temperature was subsequently reduced to freezing temperatures (-6.5, -7.5 and -8.5^{o}C) for 24 h, then returned to ambient levels. The temperature increases and decreases were achieved by ramping for 3 h (gradual decrease and increase).

2.4.1.7 Biotic stress (Pseudomonas syringae inoculation)

2.4.1.7.1 *Pseudomonas syringae* DC3000

Plants were inoculated using the syringe injection method described by Katagiri *et al*. (2002). Five-week-old plants grown individually on 38 mm peat plugs were transferred to the Category 2 Pathogen laboratory growth chamber (24 ^{o}C; 16 h photoperiod and light intensity approximately 150-200 µmol m^{-2} s^{-1}) for 4 days prior to inoculation.

Two day prior to inoculation, the *Pst* DC3000 virulent strain was streaked out on a fresh KB agar plate containing the Rifampicin (50 µg/ml) using a loop and grown at 28^{o}C. The day before inoculation, 50 ml overnight culture was prepared from a single colony. On the day of inoculation, bacteria were harvested from this 50 ml overnight culture by centrifugation for 5 min at 15, 000 g. These pelleted cells were washed by re-suspending in 10 ml of sterile water, centrifuging again for 1 min at 13000 rpm and finally resuspending in a fresh 5 ml aliquot of sterile water. The sample was adjusted to OD_{600nm}= 0.2 (equivalent to 10^8 cfu/ml). The culture was then diluted 1:100, thus infections were carried out with OD_{600nm} = 0.002 = 10^6 cfu/ml.

About 3-5 leaves of the same age from each plant were selected prior to inoculation, and a small circle punched out using a pipette tip to make it easy to identify infected leaves later. For each time point, 3 plants were needed; for the time course of day 0-3, 12 plants per line were infected using a 1 ml syringe. For the control 3 plants were infiltrated with sterile water. The infected plants were covered with cling film in order to maintain high humidity. The first sample (day 0) was harvested 4 h after inoculation. Three leaf discs from each plant were harvested (into 500 µl of sterile water) using a cork borer (0.283 cm^2). To do this, the three leaves were placed on top of each other, the circles punched out and pooled as one sample (samples were taken from three independent plants for each time point). Discs were ground using a blue plastic micro pestle in 1ml of sterile water.

The following serial dilutions were made for these samples from each time point.

Day 0: Undiluted and 10^{-1}

Day 1: 10^{-1} to 10^{-3}

Day 2: 10^{-1} to 10^{-6}

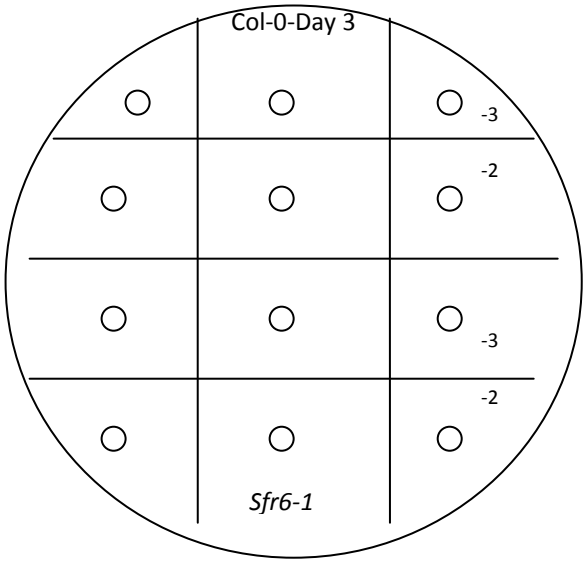
Day 3: 10^{-1} to 10^{-8}

10 µl of each dilution for each plant line was plated onto a single KB agar plate containing Rifampicin (set out as shown in Figure 2.2 below). When spots were dried enough the plates were turned upside down and incubated at 28^{o}C for 2 days.

After two days of incubation, the number of colonies were counted for each dilution. The highest dilution for which colonies were easily counted was used to calculate cfu/unit area.

Figure 2.2

Schematic diagram of the layout of plating each dilution of *P. syringae* inoculation experiment.



Small circles are the spots of 10 µl of each dilution. Three spots were plated from each dilution and one plate were used for two lines.

2.4.1.7.2 *Pseudomonas syringae* DC3000-lux

P. syringae DC3000-lux and *P. syringae* DC3000-lux-avrRpm 1 strains (Fan *et al*., 2008) were also used in this study. These strains have an insertion of the *luxCDABE* operon from *Photorhabdus luminescent* into the *P. syringae* (pv.) tomato DC3000 chromosome under the control of a constitutive promoter. Plants were inoculated by the syringe inoculation method as described in section 2.4.1.7.1.

A Photek luminometer was used to measure the bioluminescence of infected leaves. Excised leaves were placed between transparent OHP acetate sheets to flatten them, and so to ensure that the photon counting camera was a consistent distance from the sample surface. Photon count data were processed using IF32 image processing computer software (Photek Ltd).

2.4.2 Treatment of samples prior to RNA extraction

2.4.2.1 Osmotic stress

Seven-day-old seedlings grown on MS agar plates were floated in 6 ml of sterile water or 350 mM mannitol contained in transparent multi well plates. Before adding mannitol, seedlings were floated in 3 ml of sterile water for 3 h to recover from transfer to water from agar medium. Then, 3 ml of 700 mM mannitol solution was added to each well, except for control plants to which 3 ml of water was added. Fifteen to 20 seedlings were used for each treatment. Samples were collected 6 h post treatment. Immediately before harvesting, seedlings were blotted on tissue paper to remove excess solution. Samples were then quickly placed into microfuge tubes and snap-frozen in liquid nitrogen. Samples were processed so that the minimum time elapsed (less than 1 minute) between harvesting and freezing.

2.4.2.2 Cold stress

Seven-day-old seedlings grown on horizontal agar plates were transferred to 4^{o}C for the appropriate amount of time in constant light. The whole plants were collected after the indicated time of cold exposure and quickly frozen in liquid nitrogen. One biological replicate contained 15-20 seedlings.

2.4.2.3 UV stress

UV irradiations were performed as described in previously (section 2.4.1.5), then plates were resealed and returned to the growth room. Triplicate samples were collected 1, 6 and 24 h after treatment. Biological replicates comprised 15-20 seedlings; samples were collected in a microfuge tube and snap frozen in liquid nitrogen prior to RNA extraction.

2.4.2.4 Biotic stress

P. syringae inoculations were performed as described earlier (section 2.4.1.7.1). Triplicate samples (each containing 3 leaves from three different plants) from inoculated leaves were collected over different time points, placed in microfuge tubes and frozen in liquid nitrogen, prior to RNA extraction.

2.4.2.5 Salicylic acid treatments

Three-week-old plants were dipped in 1mM Sodium salicylate (SA) solution with 0.01 % Silwett L-77 surfactant (Lehle Seeds, Round Rock, Texas, USA), then returned to the growth chamber for 24 h prior to sample collection. Control plants were dipped in Silwett solution without SA. Three plants from each line were used; 1-2 leaves were collected from each plant and pooled. Samples were frozen in liquid N and kept in -80^{o}C prior to processing for RNA extraction.

2.5 DNA Techniques

2.5.1 DNA Extraction

2.5.1.1 Extraction of bacterial plasmid DNA

1. STET prep method

The STET (Sucrose-Tris-EDTA-Triton) prep is a modified alkaline lysis method for isolating crude plasmid DNA from bacterial cultures. It is a useful and cost-effective method for isolating plasmid DNA from multiple samples, e.g. when checking bacterial colonies for the presence of a plasmid vector harbouring an antibiotic resistance marker.

Individual bacterial colonies were inoculated (using sterile toothpicks) into 5 ml of liquid LB with appropriate antibiotics and bacteria were grown overnight shaking at 37^{o}C. Overnight culture (1.5 ml) was centrifuged (Eppendorf centrifuge 5415D, VWR International Ltd, Leicestershire, England) at 15,000 g for 1 min. The supernatant was removed using an aspirator and discarded. The cell pellet was re-suspended in 250 µl of pre-chilled STET buffer (see appendix A.2 for recipe). Cells were lysed by addition of 20 µl of lysozyme (10 mg/ml in STET buffer) and the tubes gently mixed by inversion, prior to incubation at 100^{o}C for 1 min in a digital dry-block (Labnet, Labnet International, Inc). Following this, 270 ml of ice-chilled 5 M LiCl was added and mixed by inversion, incubated on ice for 30 min. The tubes were then centrifuged for 15 min at 4^{o}C (at 16, 000 *g*) using the Beckman coulter centrifuge (Allegra™ X-22R, VWR international Ltd, Leicestershire, England). At this point, plasmid DNA is enriched in the supernatant, and the pellet consists mostly of proteins, polysaacharides and genomic DNA.

Pellets were removed using sterile cocktail stick. The plasmid DNA was then precipitated with ethanol. One ml of pre-chilled (-20^{o}C) 100 % (v/v) ethanol was added to each tube, mixed by inversion and incubated at -80 ^{o}C for 30 min or at -20 ^{o}C overnight. The tubes were then centrifuged (Beckman coulter centrifuge) at 16,000 *g* at for 10 min 4^{o}C to pellet the plasmid DNA, and the supernatant immediately removed. The pellets were then washed with 80 % (v/v) ethanol by inversion and centrifuged again as previously for 5 min. This supernatant was

discarded and the DNA pellets left to air dry for 10 min (to volatilise any remaining ethanol) before re-suspension in 50 µl of buffer TER (see appendix A.4 for recipe).

2. Mini prep method

For salt-sensitive applications such as DNA sequencing, the STET prep method carries through too many impurities. Hence for these applications, the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) or Wizard® plus SV minipreps (Promega) was used. These kits are useful for the extraction of high purity small scale bacterial plasmid DNA. Samples were processed according to the manufacturers' instructions. Bacterial cultures were spun down, resuspended, lysed in the presence of alkaline protease, the supernatant separated from the flocculated pellet by centrifugation, and plasmid DNA bound to the column as supplied, before washing in an ethanol-based buffer and eluting in water.

3 Maxi prep method

Quagen plasmid Maxi Kit (www.quagen.com) was used to extract high purity large scale bacterial DNA according to the manufacturers' instructions.

2.5.1.2 Extraction of DNA from an agarose gel

DNA fragments separated by electrophoresis were extracted from agarose gel slices using the GenElute Gel Extraction Kit (Sigma-Aldrich) or Qiaquick gel extraction kit (Qiagen- Qiagen Ltd, Crawley, West Sussex, UK), according to the manufacturer's instructions. Bands size range from 100-1000 bp was extracted using Ultrafree®-DA kit (MILLIPORE, Millipore corporation, Bedford, MA01730, U.S.A). An UV trans-illuminator (Ultra-Violet Products Ltd, Cambridge, Cambridgeshire, UK) was used to visualise DNA bands from agarose gels, which were excised using scalpel blades (Swann-Morton, Sheffield, Uk).

2.5.1.3 Plant genomic DNA extraction

Approximately 12 seedlings (~7-days-old) were collected in 1.5 ml micro tubes and flash frozen in liquid nitrogen. The tissue was ground with a plastic micro pestle for approximately 10 sec. Then 400 µl of Edward's extraction buffer (see appendix A.5 for recipe) was added and the tissue ground for another 10-20 sec. The micro tubes were vortexed for 5 sec and left at room temperature until all preps were ready for the next stage. Tubes were spun at 14,000 *g* for 1 min, and 300 µl of the supernatant was removed and added to 300 µl of 100 % (v/v) isopropanol in a fresh tube, and gently mixed by inversion. These tubes were left at room temperature for 2 min to allow the DNA to precipitate, and this was pelleted by centrifugation for 5 min at 14,000 *g*. The resulting supernatant was removed, and the pellet spun for a further 1min and the residual supernatant was aspirated. The pellets were dried by spinning for 5 min in a concentrator (Eppendorf concentrator 5301, VWR Internationals Ltd, Leicestershire, England), and finally re-suspended overnight at 4°C in 50 µl of cold TE buffer (see appendix A.3 for recipe).

2.5.2 Electrophoresis

DNA was size-separated by agarose gel electrophoresis. To prepare the gel, 1 % (w/v) electrophoresis grade agarose in 0.5 × TBE buffer (see appendix A.6 for recipe) was heated in a microwave oven until boiling. Ethidium bromide (10 mg/ml) was added to a final concentration 5 g/ml (1.5 µl) and allowed to cool to approximately 50^{o}C. The molten gel was poured into a gel tank and allowed to set at room temperature. 0.5 × TBE was used as the running buffer.

For small sized DNA fragments (100-1000 bp), or if bands were going to be extracted using Ultrafree®-DA kit, electrophoresis was performed using 1×TAE buffer in place of TBE (see appendix A.7 for recipe).

DNA samples were loaded into wells by mixing with 6×DNA loading buffer (see appendix A.8 and A.9 for recipes) and the gels run at 35 mA (constant current)

against a 1 Kb DNA ladder (Fermentas) molecular size standard. The DNA was visualized using a UV-trans-illuminator (UVITEC LIMITED, Avebury house, Cambridge, UK) at a wavelength of 254 nm.

2.5.3 Amplification

Polymerase chain reaction (PCR) was performed in a 96 well P×2 thermal cycler (Thermo Electron Corporation). Genomic DNA or cDNA (see section 2.6.2.1) was used as template. The PCR products were analysed by agarose gel electrophoresis.

2.5.3.1 DNA polymerase

Taq DNA or Taq Red DNA polymerases (Bioline) were used for general PCR amplification. Amplification of PCR fragments for cloning work was performed by using a proofreading polymerase with exonuclease activity. Two proofreaders were used for PCR during this project; Pyrobest DNA polymerase (Takara Bio, Shiga, Japan) or Phusion DNA polymerase (Finnzymes, Keilaranta, Finland).

2.5.3.2 PCR conditions

PCR conditions for different DNA polymerases are listed in Table 2.2. Annealing temperature was optimized for new DNA templates and primer pairs where they were used for the first time. Typically, annealing temperatures were chosen to be 5^{o}C lower than the melting temperature of the lower melting primer from the pair.

Table 2.2

PCR conditions for different DNA polymerases.

Cycle steps	Time and temperature			No. of cycles
	Taq/Taq Red	Pyrobest	Phusion	
Initial Denaturation	94^{o} C - 2 min		98^{o} C - 30 sec	1
Denaturation	94^{o} C - 10 min	94^{o} C - 30 sec	98^{o} C - 10sec	25-35
Annealing	55 - 60^{o} C 1 min/Kbp	60-70^{o} C 1 min/Kbp		
Extension	72^{o} C - 1 min	72^{o} C - 1 min	72^{o}C-30 sec/Kbp	
Final extension	72^{o} C - 10 min	72^{o} C - 10 min	72^{o} C - 10 min	1

2.5.3.3 Primers and reaction mixtures

Primers were designed to anneal to specific regions within the gene of interest using vector NTI software (Invitrogen) or by eye, if the software was unable to predict suitable primer sites. All designed primers were purchased from Fisher Scientific UK Ltd (Meadow Road, Leicestershire). The reaction mixture was prepared according to the manufacturer's instructions, using buffer as supplied with the relevant polymerase. The sequences of most of the primers used during this project are listed in appendix C.

2.5.4 Cloning

2.5.4.1 Plasmids

The details of the plasmid vectors used throughout this study are presented in appendix B, including vector maps annotated with specific features, and sequences.

2.5.4.2 Ligation

DNA fragments were ligated into a linearised vector backbone using T4 DNA ligase (Promega) in the supplied ligase buffer, according to the manufacturer's instructions. Reactions were incubated overnight at room temperature. A 1:3 molar ratio of linearised plasmid and insert was used for most experiments. The amount of insert to include in the reaction mixture was calculated using the following equation. As a control, linearised vector alone was ligated and water was added instead of DNA.

$$\frac{Amount\ of\ vector\ (ng)\ \times\ size\ of\ insert\ (kb)}{Size\ of\ vector\ (kb)} \times insert{:}\ vector\ molar\ ratio = Amount\ of\ insert\ (ng)$$

2.5.4.3 Gateway recombination

Once a DNA fragment was cloned into a Gateway entry vector, the DNA was transferred into gateway destination vectors using the LR reaction; this was performed with the Gateway LR Clonase^{TM}II Enzyme Mix (Invitrogen), using the supplied protocol. This involved performing recombination in a reaction volume of 2.5 µl, and incubating overnight at 25^{o}C. 0.5µl of proteinase K (1µg/µl) was added and incubated for 10 min at 37 ^{o}C to break down the clonase enzyme. One µl of the resultant reaction was used for *E. coli* transformation (DH5α).

2.5.5 Restriction digests

2.5.5.1 Restriction enzymes

Plasmid DNA or PCR products were digested with restriction enzymes to obtain DNA fragments for cloning or diagnosis. These reactions were performed using the manufacturer's supplied buffers at 37^{o}C for 2-4 h except where the enzymes required lower temperature for digestion. For single enzyme digestions, enzymes were added so that they comprised 1/10 of the total reaction volume. Double digestion was performed using buffer in which both enzymes could function at their optimal (or

near optimal) activity. If two enzymes required different buffers for optimal activity, sequential digestion was performed.

2.5.6 Sequencing

For sequencing of plasmids containing the cloned fragments, DNA was isolated by the mini prep method (Section 2.5.1.1). All sequencing reactions were performed by the DNA Sequencing laboratory, School of Biological and Biomedical sciences, Durham University (DBS, Durham University). DNA sequence data was analysed using BLAST 2 sequencing tool (www.ncbi.nlm.nih.gov/blast/bl2seq) and Vector NTI software (Invitrogen).

2.6 RNA techniques

2.6.1 RNA extraction

Total plant RNA extraction was performed using the RNeasy Plant Total RNA Kit (Qiagen), following the manufacturer's instructions. The isolated RNA was eluted in RNase-free water. In order to remove traces of genomic DNA from RNA, on-column DNAase digestion was performed with RNAase-free DNAase (Quiagen, Hildon; http://www.qiagen.com/), again according to the manufactures instructions.

2.6.2 RT-PCR analysis

Reverse transcription-polymerase chain reaction (RT-PCR) technique was used to amplify cDNA for gene expression studies and to amplify specific gene fragments for cloning.

2.6.2.1 cDNA synthesis for use in subsequent PCR reactions

Total plant RNA was reverse-transcribed using the H minus M-MuLV Reverse Transcriptase enzyme (Fermentas) following the protocol written for the Fermentas H Minus first strand cDNA synthesis kit. All preparations were performed on ice, using RNase free microfuge tubes and pipette tips. Five µg of plant RNA (5 µg of RNA and RNAse free water to a total volume of 10 µl) was added to 1 µl of oligo dT primer (0.5µg/µl; Fermentas). The micro tube was then incubated at 70^{o}C for 5 min in order to denature the RNA and oligo dT primer. After this period, the micro tube was immediately transferred back to ice for 2 min to allow annealing of the oligo dT with the poly-A tails of mRNA. A cocktail of 4 µl of 5× M-MuLV buffer (supplied with enzyme by Fermentase), 1 µl of "Ribo-lock" ribonuclease inhibitor (supplied with enzyme by Fermentase) and 2 µl of 10 mM dNTPs (supplied with enzyme by Fermentase) was added. Then tubes were incubated 5 min at 37^{o}C in a PCR block and 1 µl of H minus M-MuLV Reverse Transcriptase (200 U/µl) was added. The PCR machine was programmed to run at 42^{o}C for 60 min for the cDNA synthesis reaction, followed by 10 min at 70^{o}C to inactivate the enzyme at the end of the programme. The resulting cDNA was diluted 1:10 prior to use in PCR reactions.

2.6.2.2 cDNA synthesis for use in real time qPCR

Seedlings or plant tissues treated with different stresses were collected into 1.5 ml microfuge tubes, and flash frozen. Total RNA was extracted using the RNeasy kit (Qiagen) as described in section 2.6.1. Concentrations of RNA were determined using the Nanodrop, technique (see section 2.7). A high capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, USA) was used to reverse transcribe cDNA from 1.5 µg of total RNA (diluted into a total volume of 10 µl), combined with a 10 µl reverse transcriptase reaction mixture (2 µl of 10×RT buffer, 0.8 µl of 25×dNTP mix, 2 µl of RT Random primers, 1 µl of Reverse transcriptase and 4.2 µl of Nuclease-free water. All components other than water were supplied with the Reverse transcriptase enzyme (Applied Biosystems). The PCR machine was programmed to run at 25^{o}C for 10 min followed by 37^{o}C for 120 min and then

85^{o}C for 5 sec. The resultant cDNA was diluted 1:50 prior to use in quantitative real time PCR to examine gene expression.

2.7 Total DNA and RNA quantification

The concentration of DNA and RNA solutions were determined by reading the optical density of samples at a wavelength of 260 nm using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Typically, 1.5 µl of undiluted DNA or RNA applied onto the pedestal of the machine was sufficient to make a measurement. The appropriate buffer solution minus DNA/RNA was used as a zero reference in each case.

2.8 Measurement of gene expression

2.8.1 Real time PCR

The relative transcript levels of selected genes were quantified by real-time PCR using an Applied Biosystems 7300 machine. cDNA synthesised as described in section 2.6.2.2 was diluted 1:50, except for *MC8* gene probe (1:20 dilution). 10 µl of diluted cDNA was used with TaqMan Universal PCR Master mix (Applied Biosystems) in a 25 µl reaction in an optical 96 well reaction plates (MicroAmp^{TM}, Applied Biosystems) and three technical replicates were used for each sample. Three biological replicates were also carried out for each experiment. All TaqMan probes used in this research were designed using the File Builder programme available via the Applied Biosystems web site. All probes used in this study are listed in appendix C.2 with their sequences and probe identifiers. Relative gene expression was analysed using the $\Delta\Delta C_t$ method (Applied Biosystems). ΔC_t Calculations for singleolex (external control method) $\Delta\Delta C_t$ studies were performed according to the method described in User Bulletin, Applied Biosystems Real-time PCR Systems, July 2007.

2.8.2 Luciferase assay

The expression of genes fused with fire fly luciferase was measured using a luminometer. This consisted 9829A photomultiplier tube with a 1.5 KV potential from a PM28B high-voltage supply cooled to -25^{o}C by using a FACT50 air cooled thermoelectric housing and an AD2 amplifier/discriminator (Knight *et al*., 1996). An amplifier/discriminator is connected to CTI computer counter board to produce numerical output (Knight *et al*., 1996). Samples were processed according to the protocol from Promega LUC assay system (www.promega.com). First tissues were ground in a motar and pestle pre-cooled with liguid N. Then 200 µl of 1×Cell Culture Lysis reagent (Promega) for 10-15 mg of tissues was added and mixed well. Cell extracts were then transferred to a micro-centrifuge tube and supernatant was recovered by centrifugation fro 4 min at 14,000g. Eighty µl of Luciferase Assay reagent this contains luciferine was transferred into a luminometer cuvette and added 20 µl of cell extract and mixed by pipeting three times. The emitted light was measured for a period of 20 sec.

2.9 Production of artificial miRNA constructs for gene silencing

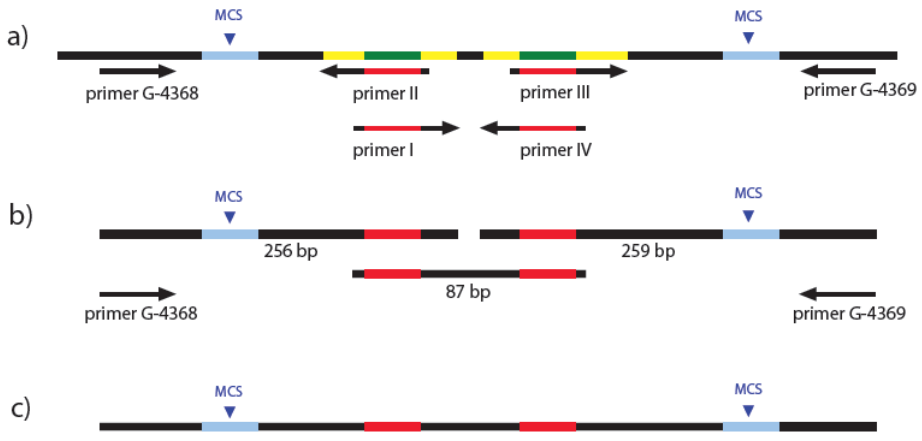
The Web MicroRNA Designer (WMD3) (www.http://wmd3.weigelworld.org) tool was used to design an artificial microRNA (amiRNA) construct for rice gene silencing. This tool is configured to optimise amiRNAs for gene silencing in >30 species. The process of PCR amplification of the RNAi cassette is summarised in Figure 2.2 below.

Entering the gene identifier (ex: Os10g35560.1) prompts the software to produce a list of amiRNA sequences according to their hybridization energy. Once the amiRNA sequence is selected, the next step is to engineer an endogenous miRNA precursor for expression. The same webpage also provides an oligonucleotide designing tool to suggest primers to produce amiRNA precursor for your selected amiRNA. Entering the gene identifier amiRNA sequence automatically designs primers for *Arabidopsis* MIR319a (ath-miR319a) and rice MIR528 (osa-miR528)

precursors. We used this tool to select amiRNA sequences suitable for silencing *OsSFR6* (the rice SFR6 homologue). The recommended strategy for PCR cloning is shown in Figure 2.2. In this strategy appends the amiRNA sequences to an osaMIR528 stemloop by sequence of PCRs with primers annealing to the vector flanking sequences. The final round of priming and extension gives the whole cassette in a single sequence for subsequent cloning. The strategy is detailed at www.http://wmd2.weigelworld.org (see appendix B.5 for the sequence of pNW55-osaMIR528 stemloop). The Pyrobest proofreading Taq polymerase was used to amplify all sequences, and the final cassette was then cloned into a binary vector, for plant transformation.

Figure 2.2

PCR scheme to produce amiRNA constructs from the pNW55 vector. Downloaded from http://wmd2.weigelworld.org.



a) Primers I-IV are designed by the WMD3 web-based programme. The original miRNA528 sequences (green) are replaced by the amiRNA sequences (red) during first round of PCRs. Sequences in pNW55 complementary to the primers are indicated in yellow and the multiple cloning sites in the blue.

b) The 3 possible primer pair combinations on the pNW55 template (G-4368+II, I+IV, III+G-4369) result in 3 PCR fragments

c) Fusion PCR on the 3 PCR products from b) with primers G-4368+G-4369 results in one DNA fragment for subsequent cloning.

2.10 Bacterial transformation and competent cell production

2.10.1 *Agrobacterium* competent cell production

An inoculated loop of a culture carrying the required *Agrobacterium* strain was streaked onto a fresh LB plate with appropriate antibiotics, and incubated for 2 days at 28°C. From this plate, a single colony of *Agrobacterium* was picked and used to inoculate a 5 ml overnight bottle of LB with appropriate antibiotics. Cultures were grown overnight at 28°C in a shaking incubator. The next day 5 ml of overnight culture were added to 100 ml of LB in a sterile 500 ml flask and shaken vigorously (250 rpm) at 28°C until the culture reached an OD_{600} of 0.5-1. Cultures were chilled on ice and centrifuged at 3000 *g* for 5 min at 4°C to pellet the cells. The supernatant was discarded and the cells resuspended in 2 ml of ice cold $CaCl_2$ solution. Finally cells were pipetted in 0.1 ml aliquots into ice cold 1.5 ml eppendof tubes, flash-frozen in liquid nitrogen, and stored at -80^{o}C until use.

2.10.2 Transformation of competent *E.coli* cells

Twenty five µl aliquots of chemically competent *E.coli* cells (Invitrogen), either DH5α (for routine cloning) or DB 3.1 (for plasmids harbouring the *ccd*B toxic cassette), were thawed on ice before transformation. Ligation mixtures were heated to 70^{o}C for 10 min to inactivate the ligase enzyme, and then transferred onto ice for 1 min. 2.5 µl of ligation reaction was added to the competent cells and incubated on ice for 30 min. The cells were then heat shocked 1 min in a 42^{o}C water bath, and rested immediately on ice for 2 min. After this time, 1 ml LB medium was added to each tube, and the tubes were shaken gently to aerate (150 rpm) at 37^{o}C for 1 h; this recovery time allows the cells to initiate replication under optimal conditions. Aliquots of each culture (over a range of concentrations) were then spread onto LB agar plates containing the appropriate antibiotics for selection of the plasmid (see table 2.1 for concentrations). Plates were incubated at 37^{o}C overnight to develop colonies, and kept at 4 ^{o}C for longer term storage.

2.10.3 Transformation of *A. tumifaciences* cells

Frozen aliquots of *A. tumefaciences* C58C1 competent cells were placed on ice and allowed to thaw. One µg of plasmid DNA was mixed with the cells, and the tube incubated on ice for 30 min. The cells were then heat shocked at 37 ^{o}C for 1 min using a 37^{o}C water bath, and the tube rested on ice for 2 min. After addition of 1 ml LB medium, tubes were agitated for 2 h at 29^{o}C to allow cells to grow. Aliquots were spread on LB agar plates containing 50 µg/ml Rifampicin (selecting for C58C1) and appropriate antibiotics for vector selection. Plates were incubated in a 29^{o}C oven for two days for colonies to develop.

2.11 Plant transformation

2.11.1 *Agrobacterium* mediated transformation of *Arabidopsis* by floral dipping

2.11.1.1 Plant preparation

Seven day old seedlings were transferred to large hydrated peat plugs (42 mm diameter) with 3 plants per plug, and grown as described in section 2.2.4. When plants progressed initially to flowering phase, bolts were clipped to encourage the formation of floral shoots from the axillary meristems. Plants were clipped several times prior to dipping, which resulted in a large number of young floral shoots; in addition the watering regime was supplemented with fertilizer to help maintain healthy growth. The final clipping was done 7 days before transformation, producing an abundance of short stems which were actively producing flowers.

2.11.1.2 Preparation of transformed *A. tumefaciens*

A single colony of *Agrobacterium* harbouring a binary vector construct was grown on LB agar plate with appropriate antibiotics (Rifampicin and the vector selective agent) and used to inoculate a 5 ml overnight culture. The following day this was used to inoculate a 200-500 ml LB flask (with the same antibiotics) and grown for a further 24 h at 29^{o}C. For transformation, cells were spun down and re-suspended in

5 % sucrose solution with Silwett L-77 surfactant (250 µl per 500 ml) added immediately prior to use.

2.11.1.3 Dipping of plants

Some of the re-suspended solution was poured into a small beaker and plants dipped into this suspension for a couple of seconds. The plants (grown in peat plugs) were then placed on their sides in a tray lined with tissue, the tray covered with cling film and returned to growth room overnight. Next day plants were uncovered and stood upright again, with watering from below to ensure that the *Agrobacterium* was not washed off.

2.11.1.4 Selection of transformants

Seeds (T_1) collected from *Agrobacterium* dipped plants transformed with a vector containing a selectable marker other than Basta resistance, were bleach sterilized as described in section 2.2.2.1 before germinating on MS agar plates with timentin (to inhibit growth of *Agrobacterium*; see table 2.1 for concentration) and appropriate antibiotics to select for the binary vector. Primary transformants (T_1) surviving selection with green cotyledons and elongating roots were transferred to peat plugs to grow to maturity, and their seeds (T_2 generation) were harvested separately.

Seeds from plants dipped with constructs carrying Basta resistance as the plant selectable marker were sown directly onto soil to germinate. After 6-7 days, Basta herbicide (250 mg/L) was sprayed onto the young seedlings; this treatment was repeated 3-4 times at two day intervals. The surviving primary transformants were transferred to peat discs to grow to maturity. Seeds (T_2) were harvested from each plant separately.

2.11.2 *Arobacterium* mediated transformation of rice

This was done using the protocol described by Nishimura *et al*., 2006 with some modifications.

2.11.2.1 Induction of callus from rice seeds

Sterilized rice seeds (section 2.2.2.2) were placed onto the surface of plates containing N6D medium (see appendix A.10 for recipe) and their edges sealed. Plates were incubated at 29.5^{o}C at about 3.5 k-Lux for 1 week, until calli formed from the scutella. If plates were contaminated, prior to callus formation uncontaminated seeds were transferred to new plates with N6D medium immediately. After 1 week, subcultures of proliferating calli were transferred to a new N6D medium after removing the seed and seedlings.

2.11.2.2 Preparation of calli for transformation

Plates of sub-cultured actively growing calli (yellowish white coloured, 1-3 mm long) on fresh N6D medium were incubated for 3 days under the same conditions as used for callus induction.

2.11.2.3 Preparation of *Agrobacterium* for transformation

Agrobacterium harbouring the gene of interest in a binary vector were streaked onto plates of AB medium (see appendix A.13 for the recipe) supplemented with appropriate antibiotics using sterile transferring loop. Plates were incubated at 28^{o}C for 3 days to allow colonies to develop.

2.11.2.4 Infection of calli with *Agrobacterium*

Agrobacterium cells were collected on a small sterile spatula and gently resuspended in 30 ml AAM medium (see appendix A.16 for recipe) containing 1000×AS (see appendix A.24 for recipe), and grown to an OD_{600} of 0.05-0.1 to use for infection. Subcultured calli were collected in a sterilized falcon tube. Calli were immersed in the *Agrobacterium* cell suspension in a falcon tube for 90 sec, decanted into a sieve, and blotted to remove excess bacteria. 1 ml AAM containing 30 µl 1,000×AS was dripped onto a disc of filter paper placed onto 2N6-AS medium (see appendix A.25 for recipe) and the calli transferred onto the paper. Plates were wrapped with aluminium foil and incubated at 28^{o}C in the dark for 48-60 h.

2.11.2.5 Washing of infected calli

Infected calli were collected in a 50 ml sterile tube and washed with sterile water by shaking the tube. Washing was repeated until the rinsing water was clear. After this, the calli were rinsed again with sterile water containing 1,000×Car (See appendix A.26 for recipe) at a concentration of 500 mg l^{-1} to kill *Agrobacterium* cells. The callus suspension was poured through stainless steel sieves held over plastic petri plates to drain, and the sieves (with calli) blotted onto paper towels to dry. Calli were transferred onto N6D-S medium (see appendix A.27 for recipe), the plates sealed, and incubate at 29.5^{o}C at about 3.5 k-Lux for 3-4 weeks. (Uncontaminated calli were transferred to new plates at this stage, and also contaminated calli were re-washed and transferred to new plates).

2.11.3 Biolastic transformation of rice

2.11.3.1 Preparation of plant materials

Rice seeds were germinated (20^{o}C and 12/12 h photoperiod) on wet filter papers for 2 weeks (two-leaf stage) and before bombarding seedlings were transferred to MS

agar plates. Seedlings were placed in the centre of the plate into a circle (about 3 cm diameter) covering about most of the area of the circle.

2.11.3.2 Preparation of the gold particles

One ml of ethanol was added to 60 mg of 1.6 μm gold micro-carriers in an eppendorf tube and vortexed for 1 min. Then spun in a micro-centrifuge for 10 sec to pellet the gold particles and removed the supernatant. Repeated washing in this way 3 times and after last wash spun for 1 min. Then added 1 ml sterilized double distilled water and resuspended the gold particles completely by vortexing for 1 min. Removed the supernatant by subsequent spinning for 1 min. Finally, gold particles were completely resuspended in 1 ml of water by vortexing and sealed the top of the eppendorf tube with parafilm and stored at 4^{o}C.

2.11.3.3 Preparation of DNA coated particles

Large macro-carrier discs (1100 and 1350 psi rupture discs) were placed into the macro-carrier rings and flatted. Gold particles were vortexed hard for 1 min. and quickly removed 50 μl into an eppendorf tube and added 5 μg plasmid DNA and vortexed hard for 30 sec. Subsequently, added 50 μl 2.5 M $CaCl_2$ and vortexed hard for 30 sec. Then 20 μl of 0.1 M spermidine free base was added and vortexed hard for 3 min. Pulse spun the tube (about 10,000 g) to pellet particles and removed supernatant as quickly as possible. Then pellet was completely resuspended in 125 μl of ethanol by vortexing. Then pipette up and down and dispensed 20 μl of suspension onto each of up to 5 macro-carrier discs and allowed to dry.

2.11.3.4 Firing at the plants

Firing DNA at the plants was performed using a Bio-Red PDS-1000/He particle delivery system. The vacuum applied to the chamber was 25 mm Hg/in. Bombarded

seedlings were incubated 48 h in the growth room for gene expression before harvest and freeze tissue in liquid N.

2.12 Enhancer/ suppressor mutant screen of *sfr6*

EMS-mutagenized seeds (Lehle seeds, Round Rock, Texas, USA, for more dovetails refer www.arabidopsis.com/main/cat/seeds) of *sfr6-1* were used to screen for mutations which enhanced or suppressed the *sfr6* mutant phenotype.

Seeds were surface sterilized (see section 2.2.2.1) and grown on MS agar plates. At 10-12 days old, mutants were screened for enhancers/suppressors.

The cotyledons of *sfr6* mutants are bigger and paler than those of wild-type plants, and the true leaves are also paler. Therefore, green coloured seedlings from the EMS population that looked more like Col-0 wild type were selected as carrying putative suppressor mutations, and seedlings with both paler leaves and larger cotyledons were selected as carrying putative enhancer mutations. Selected seedlings were transferred to soil and seeds collected separately. These seeds were grown on MS agar plates to test whether the phenotype persisted in the next generation. Lines maintaining enhancer/suppressor characteristics at this stage were selected for further experiments.

2.13 Visualization of GFP florescence

Col-0 and *sfr6* mutant plants were crossed with *35S::CBF1::GFP* over-expressers in a wild-type background. The F1 generation were selected on kanamycin (*Kan*)-supplemented agar plates, as *35S::CBF1::GFP* plants are *Kan* resistant. 12 day old F_2 plants were analysed for segregation of GFP florescence with the *sfr6* mutant phenotype. A Confocal laser scanning microscope (Zeiss LSM50) with 40× objectives was used to visualize GFP. The excitation wavelength for GFP visualization was 488 nm, with emission measured using a 505nm long pass filter.

All images were taken from seedlings grown under similar conditions, or subjected to the same treatments.

2.14 Analysis of hypocotyl elongation under different light conditions

Seeds were sown onto square petri plates (12 cm) containing 1×MS agar medium. Then plates were stratified at 4^{o}C for 3 days in darkness before transfer into constant light (50-60 µmol m^{-2} s^{-1}). All plates were subjected to 2 h white light treatment to initiate germination before being wrapped with red and blue light filters (HT019-Fire and HT363-Special medium blue). Plates were placed vertically into the growth chambers to allow germination and elongation of the seedlings. After day 7, filters were removed and the seedlings compared for rates of hypocotyl elongation under different light conditions.

2.15 Allelic discrimination assay (AD assay)

Since the *sfr6* original mutant has a single nucleotide polymorphism (SNP), an allelic discrimination assay (Applied Biosystems) was conducted to identify its genotype. This test detects single nucleotide variants of a nucleic acid sequence. The *sfr6-1* mutant plants selected after transformation were checked using the allelic discrimination assay to verify the presence of the *sfr6* mutation within those selected lines. One ng of genomic DNA (gDNA; section 2.5.1.3) and RNAse free water to a total volume of 10 µl, was used with 12.5 µl of TaqMan Universal PCR Master mix and 0.625 µl of 40× assay mix (Applied Biosystems) in a 25 µl reaction in optical 96-well reaction plates (MicroAmp^{TM}, Applied Biosystems). Two technical replicates were used for each sample. A TaqMan probe for genotyping *SFR6* (see appendix C.1 for probe details) was designed using the File Builder programme, available via the Applied Biosystems web site.

Chapter 3

Examining the effect of over-expression of *AtSFR6* in *Arabidopsis*

3.1 Introduction

Low temperature is an adverse environmental condition that affects plant growth and development and thus crop yield reduce mainly in temperate countries and higher altitudes in tropical countries. As discussed in chapter 1 the process of cold acclimation is important in mediating the freezing tolerance of plants grown in these regions. During the acclimation process numerous molecular and physiological changes take place including alterations in gene expression. The complexity of this process is implied by the number of genes altered during cold acclimation. According to some estimations 25 % of the transcriptome in *Arabidopsis* is involved in cold acclimation (Kreps *et al*., 2002). Therefore, a basic understanding of the molecular mechanisms of cold acclimation-related gene expression and the signalling pathways leading to them is important to improve the freezing tolerance of agronomic crops. A powerful approach used to understand the molecular mechanism of a specific gene is to compare *Arabidopsis* mutants that have a non-functional gene of interest with wild type *Arabidopsis.* As described in chapter 1 *sfr6-1* is such a mutant of *Arabidopsis* unable to cold acclimate (Warren *et al*., 1996) and thus provides a good resource to reveal the complex process of cold acclimation and freezing tolerance in *Arabidopsis*. It has been shown that the *sfr6-1* mutant is defective in the expression of a group of cold responsive genes (*COR* genes) such as *KIN2*, *COR15*, COR78 *etc*. which are controlled by *CBF*/*DREB1* transcription factors (Knight *et al*., 1999; Boyce *et al*., 2003). The *CBF*/*DREB1* cold response pathway has been identified as a key signalling pathway involved in freezing tolerance in *Arabidopsis* (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998; Thomashow, 2001). The over-expression of *CBF*/*DREB1* transcription factors in *Arabidopsis* result in a great increase in freezing tolerance in wild type *Arabidopsis* (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998). Taken together these results suggest the lack of freezing tolerance in *sfr6-1* is due to the defective expression of CBF/DREB1-

regulated *COR* genes. Recently, *SFR*6 has been identified as *At4g04920* (Knight *et al*., 2009). A valid hypothesis, given existing data, is that over-expression of *SFR6* will increase freezing tolerance in *Arabidopsis*.

Therefore, the specific considered hypothesis in this chapter is;

- Over-expression of *SFR6* in wild type *Arabidopsis* can increase downstream cold gene expression and freezing tolerance.

This hypothesis was tested by analysing *KIN2* expression in transgenic plants over-expressing *35S::SFR6* in Col-0 and *sfr6-1* mutant background.

3.2 Results

3.2.1 Analysis of the role of *AtSFR6* in expression of KIN2 using transgenic *Arabidopsis* plants

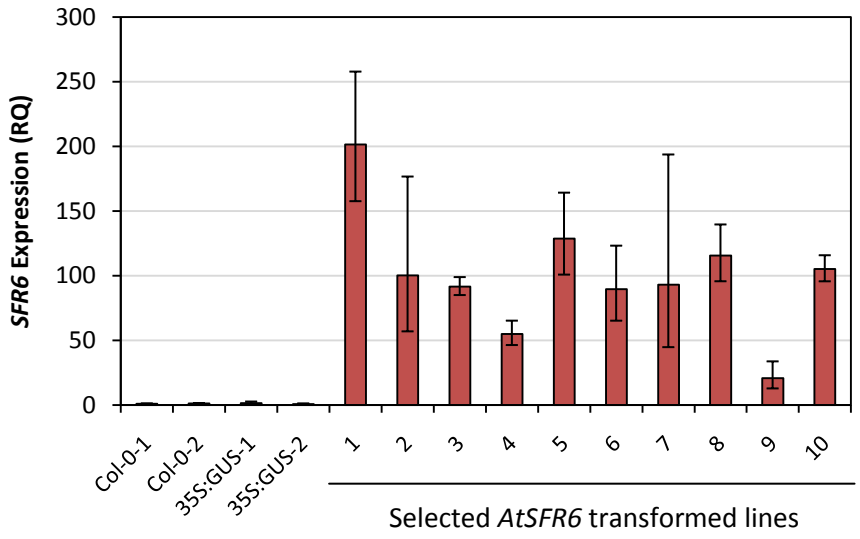
3.2.1.1 Generation of transgenic *Arabidopsis* over-expressing *AtSFR6*

Transgenic *Arabidopsis* plants over-expressing *AtSFR6* under the control of the cauliflower mosaic virus *35S* promoter were produced to investigate the function of *AtSFR6* in *COR* gene expression. Full length *AtSFR6* (7.06 kbp) was amplified from Col-0 genomic DNA using oligonucleotide primers designed to amplify the whole predicted coding sequence using Pyrobest high-fidelity DNA polymerase. It was then cloned into the *Sal*l and *EcoR*I restriction sites of *pENTR1A* (Invitrogen; http://www.invitrogen.com) gateway entry vector followed by sequencing to make sure the clone was mutation free. The *AtSFR6* ORF was sub-cloned downstream of the *CaMV 35S* promoter of the binary gateway destination vector *pB7WG2.0* (Karimi *et al*., 2002) using Gateway LR Clonase. Control plasmids were produced using same binary vector but using *pENTR-GUS* as the entry vector. Cloning of full length *AtSFR6* into *pENTR1A* was performed by Dr. Bekir Ülker (see appendix B.4 for schematic representation of cloning procedure). These constructs were transformed into *Agrobaterium tumefaciens* strain *C58*. These *Agrobacteriun* strains were used to transform *Arabidopsis* plants (both Col-0 and *sfr6-1* plants) via the floral dip method (Clough and Bent 1998; Materials and methods section 2.11.1). Transformed plants (T0) were selected on soil with herbicide "Basta - glufosinate ammonium" (250 mg/L) sprayed onto plants three times at three day intervals one week after seed germination. Following this, the surviving plants were individually transferred to peat plugs and allowed to set seed (Materials and methods section 2.2.4). Around 30 transgenic lines from each construct were obtained after Basta selection. Transgenic plants from the T2 generation were used for further analysis. Over-expression of *CBF*/*DREB1* in *Arabidopsis* with a strong constitutive *35S* cauliflower mosaic virus resulted in growth retardation compared to WT plants (Qiang Liu, 1998; Kasuga *et al*., 1999; Gilmour *et al*., 2000). Comparisons of growth between *35S*::*AtSFR6* over-expressing transgenic lines and non-transgenic Col-0 showed no obvious growth abnormality or differences under normal growth conditions (Materials and methods 2.2.4).

Over-expression of *AtSFR6* in 10 independent transgenic lines was confirmed by real-time qPCR. cDNA was synthesized (Materials and methods section 2.6.2.2) from total RNA (Materials and methods section 2.6.1) extracted from seven-day-old seedlings grown on MS agar medium (Materials and methods section 2.2.3) in a Percival chamber (Materials and methods section 2.2.4) at 20^{o}C and 150 µmol $m^{-2}s^{-1}$ light. A probe designed to detect *SFR6* (Applied Biosystems; www.appliedbiosystems .com) was used for this analysis (refer to appendix C.1 for probe descriptions). Expression values presented here are normalised to non-transgenic Col-0. As shown in Figure 3.1 all lines showed 30-200 fold over-expression of *SFR6* transcript relative to non-transgenic wild type levels.

Figure 3.1

Analysis of *SFR6* expression levels in *35S::SFR6* in Col-0 background using real-time qPCR.



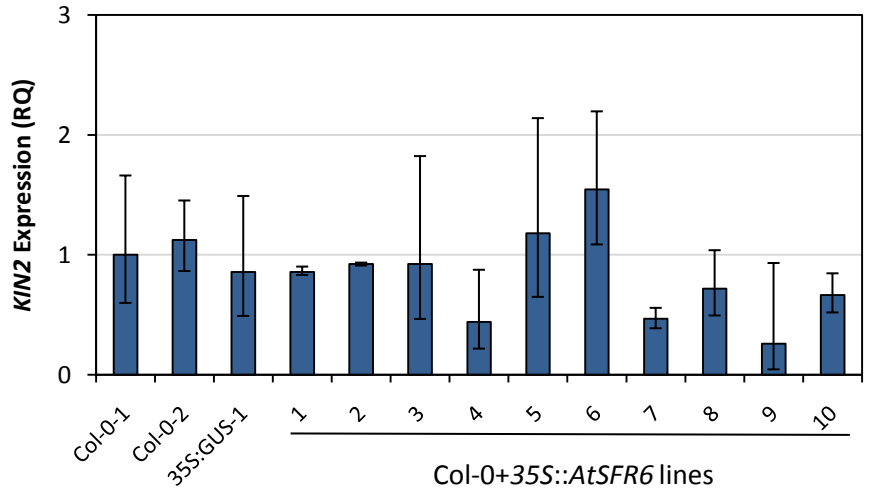
Seven-day-old seedlings were used for analysis of *SFR6* expression levels. Graph shows the relative quantification (RQ) values for *SFR6* expression relative to the *SFR6* expression in non-transgenic Col-0 plants. *ß-TUBULIN4* was used as an endogenous control. Two Col-0+*35S*::*GUS* lines were also tested as controls. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

3.2.1.2 *KIN2* expression in transgenic *Arabidopsis* over-expressing *AtSFR6*

KIN2 expression in transgenic plants was tested to evaluate the effect of *AtSFR6* over-expression upon the CBF regulated cold response pathway. The cDNA samples prepared previously for analysis of *AtSFR6* over-expression (section 3.2.1) were used. mRNA levels were measured using real time qPCR with a *KIN2* probe (Applied Biosystems; see appendix C.2 for probe descriptions). mRNA levels of *KIN2* were not increased in selected transgenic *Arabidopsis* plants (Figure 3.2). Interestingly most of the lines tested showed reduced expression of *KIN2*. There was no relationship between over-expression of *SFR6* and *KIN2* but the lowest *SFR6* over-expression line (Line #9) showed lowest average *KIN2* expression. These results suggest that *SFR6* over-expression is not sufficient to induce CBF regulated genes.

Figure 3.2

Analysis of *KIN2* expression levels in unstressed *35S*::*AtSFR6* over-expressing lines.



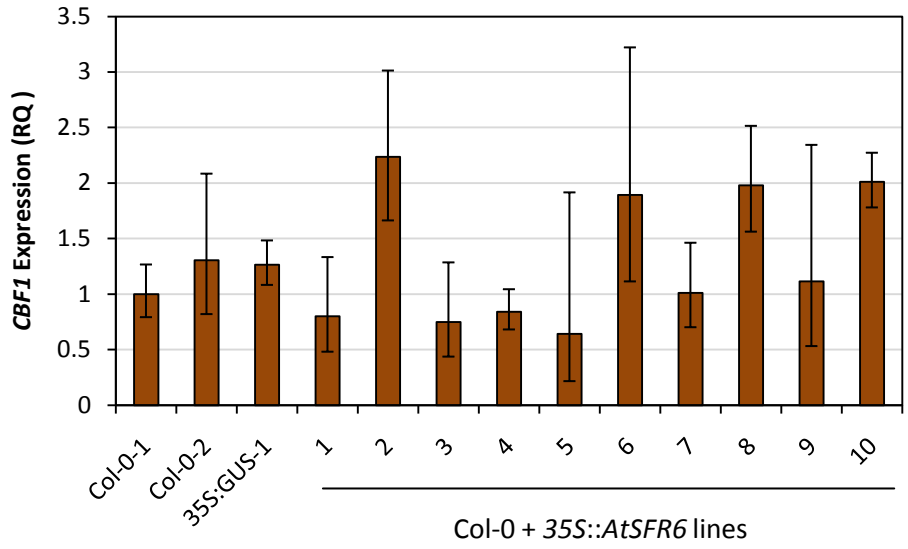
Seven-day-old seedlings grown on 1×MS agar under unstressed conditions were used to determine the effects of *SFR6* over-expression on *KIN2* expression. Graph shows the relative quantification (RQ) values for *KIN2* expression relative to the *KIN2* expression level in non-transgenic Col-0 plants. *ß-TUBULIN4* was used as endogenous control. One Col-0+*35S*::*GUS* line was tested as control. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

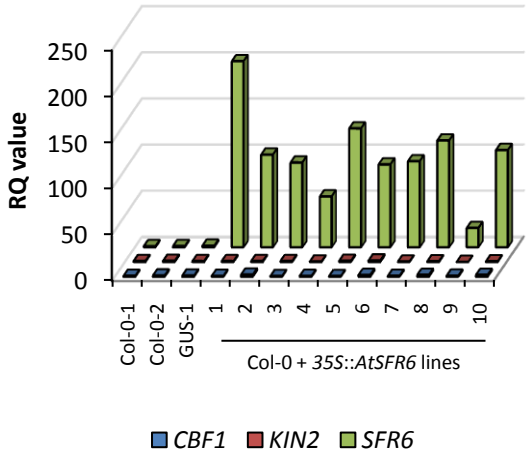
3.2.1.3 *CBF1* expression in transgenic *Arabidopsis* over-expressing *AtSFR6*

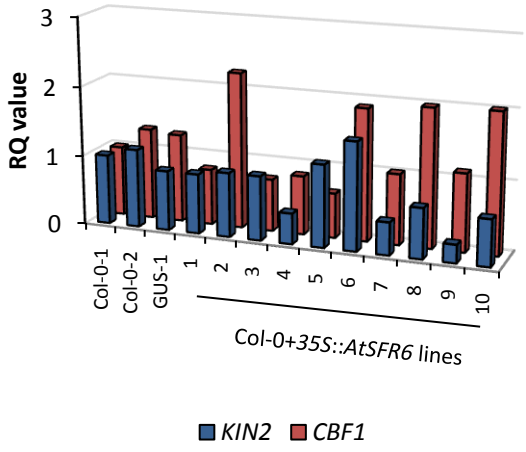
CBF transcription factors are the mediators of a group of cold expressed genes including *KIN2* (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998). As most of *SFR6* over-expressing transgenic lines showed reduced levels of *KIN2* expression compared to non-transgenic Col-0, the effect on *CBF1* expression was also tested in the same *SFR6* over-expressing lines. The same cDNA samples prepared to determine *KIN2* and *SFR6* levels were also used for this experiment. Apart from 4 lines all other lines showed reduced levels of *CBF1* expression (Figure 3.3), but there is no correlation with *SFR6* over-expression and /or *KIN2* levels and *CBF1* expression.

Figure 3.3

Expression of *CBF1* in unstressed *35S*::*SFR6* over-expressing lines.







Seven-day-old seedlings grown on 1×MS agar under normal growth conditions were used to determine gene expression levels. (a) Graph shows the relative quantification (RQ) values for *CBF1* expression relative to the level of *CBF1* expression in non-transgenic Col-0 plants. *ß-TUBULIN4* was used as endogenous control. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test. (b) Relative expression of *SFR6*, *KIN2* and *CBF1*. (c) Relative expression of *CBF1* and *KIN2*.

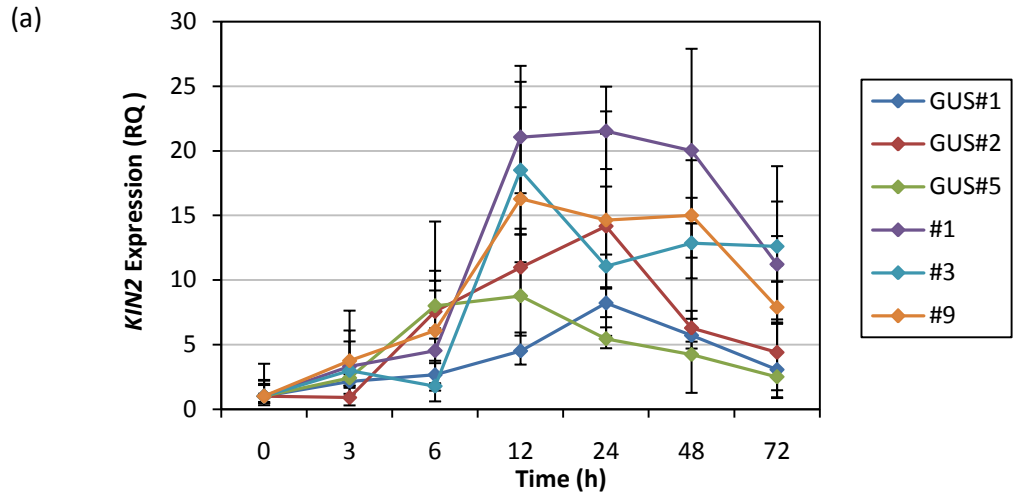
3.2.1.4 *KIN2* expression of transgenic *Arabidopsis* over-expressing *AtSFR6* during time course of cold exposure

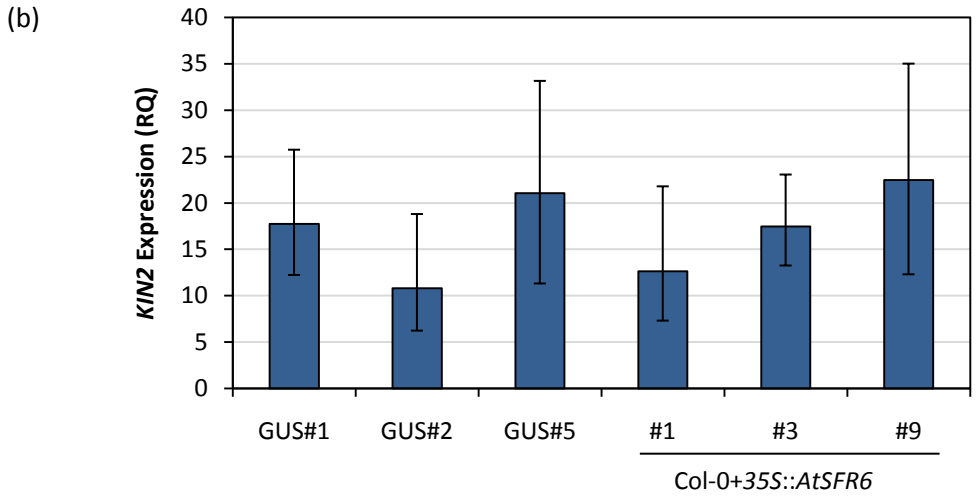
In previous experiments, *KIN2* expression in unstressed seedlings was tested to determine whether *SFR6* over-expression affects *COR* gene expression. There is, however, a possibility that SFR6 has its effect after low temperature stimuli. To test this, the expression of *KIN2* was further characterized in *SFR6* over-expressing transgenic lines during a time course of cold exposure. Seven-day-old plants grown on 1×MS agar plates were treated at 4^{o}C for different times (3, 6, 12, 24, 48 and 72 h) and tissue harvested for RNA extraction (Materials and methods section 2.6.1). Three Col-0 + *35S*::*GUS* lines were used as controls and three Col-0 + *35S*::*AtSFR6* transgenic lines (Line #1, #3 and #9) with different levels of *SFR6* expression (strong, moderate and weak respectively) were chosen.

Figure 3.4 shows the real time data of *KIN2* expression. *KIN2* transcripts increased up to a maximum in 12 h cold treatments and then gradually decreased. The three *SFR6* over-expressing transgenic lines showed higher *KIN2* transcript level than three *GUS* lines from 12 to 72 h. At the end of 3 days (72 h) of 4°C exposure transcript levels of *KIN2* in *GUS* lines had returned to ambient levels. Apart from these changes no consistent difference in expression of *KIN2* was observed in any time course between the *AtSFR6* over-expression lines and the *GUS* expression lines. These results suggest that SFR6 may have effect to keep *KIN2* expression at a higher level for a longer period of time. Therefore, to analyse this hypothesis this experiment was repeated only with unstressed samples and samples collected after 48 h cold exposure. Results displayed no significant difference in *KIN2* expression among *35S*::*GUS* and *35S*::*ATSFR6* over-expressing lines (Figure 3.4). Together, these results further suggested that over-expression of *SFR6* has no effect on KIN2 expression.

Figure 3.4

KIN2 expression during a time course of cold exposure in Col+*35S*::*SFR6* lines and Col+*35S*::*GUS*.





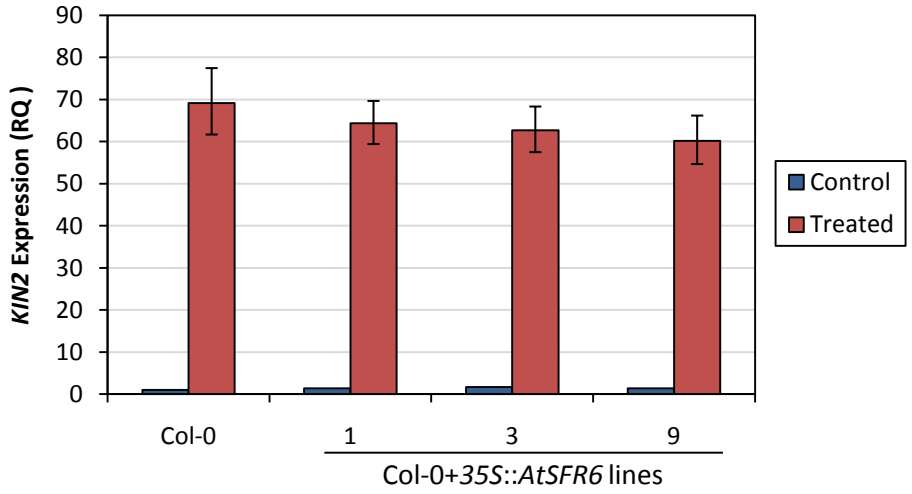
Seven-day-old seedlings grown on 1×MS agar plates were kept at 4°C. At specific times seedlings were collected for real time analysis. (a) Graph shows relative quantification (RQ) values of *KIN2* levels relative to the *KIN2* level of each line's unstressed conditions. *ß-TUBULIN4* was used as an endogenous control. (b) Graph shows the results of repeat experiment. *KIN2* levels were measured again collecting after 48 h cold exposure. Relative quantification values (RQ) of *KIN2* levels relative to *KIN2* levels of each line's unstressed conditions are presented in the graph. *PEX4* was used as endogenous control. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

3.2.1.5 *KIN2* expression of transgenic *Arabidopsis* over-expressing *AtSFR6* under osmotic stress

Finally, the expression of *KIN2* was characterized further by subjecting plants to osmotic stress, as the *sfr6-1* mutant displayed reduced expression of *COR* genes after mannitol induced osmotic stress (Boyce *et al*., 2003). Seven-day-old seedlings were immersed in 5 ml of 350 mM mannitol solution for 6 h and samples collected for RNA extraction. The three *SFR6* over-expressing lines used to analyse *KIN2* expression in response to cold (Line #1, #3 and #9) were chosen. Control seedlings were immersed in water. Mannitol treatment caused an increase in *KIN2* transcripts of 50-60 folds (Figure 3.5) but there was no significant difference between non-transgenic Col-0 and *35S*::*AtSFR6* over-expressers further suggesting that the over-expression of *SFR6* does not have an effect on *KIN2* expression.

Figure 3.5

KIN2 expression after osmotic stress in Col-0+*35S*::*ATSFR6* line.



Seven-day-old seedlings grown on 1×MS agar plates were immersed in 5 ml of 350 mM mannitol solution for 6 h. Control plants were immersed in 5 ml of water for 6 h. Graph shows the average data of three biological replicates. Relative quantification (RQ) of *KIN2* expression relative to the *KIN2* level of non-transgenic Col-0 treated with water are shown here. *ß-TUBULIN4* used as endogenous control. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

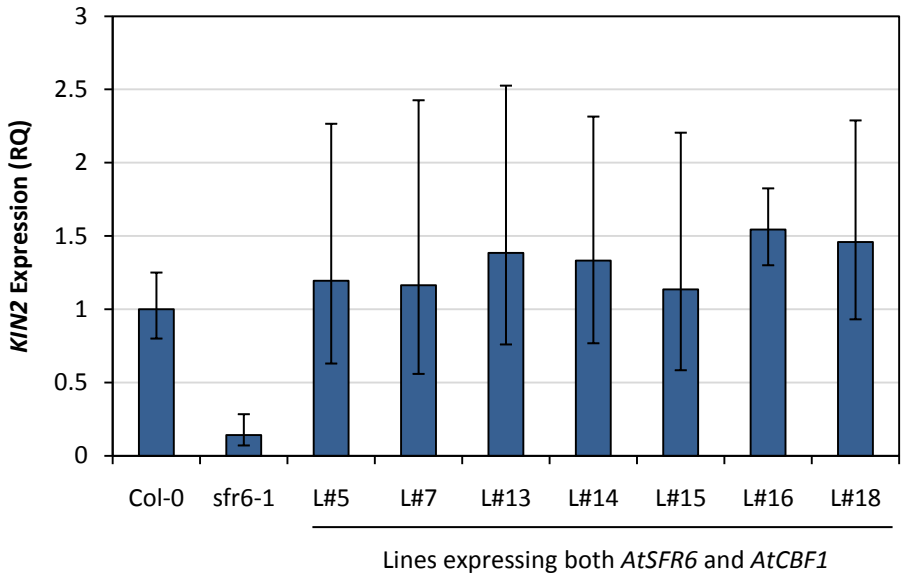
3.2.1.6 *KIN2* expression in transgenic lines expressing both *AtCBF1* and *AtSFR6*

Although we have evidence that *SFR6* is essential for *COR* gene expression of *CBF* cold response pathway, all the above analyses suggested that the over-expression of *SFR6* alone is not sufficient to induce cold gene expression. However, the over expression of *CBF1* alone significantly increases cold tolerance in *Arabidopsis* (Jaglo-Ottosen *et al*., 1998) by increasing levels of *COR* gene expression. As *SFR6* does not have effect on *CBF* gene expression, the additive effect of *CBF1* and *SFR6* over-expression on *KIN2* expression was determined.

Crosses between *35S*::*AtCBF1* plants in Col-0 background (Knight *et al*., 2009) with *35S*::*AtSFR6* in *sfr6-1* mutant plants (*sfr6-1* complemented lines, see section 3.2.2 for more details) were performed to construct *Arabidopsis* lines over-expressing both *CBF1* and *SFR6* genes. *35S*::*AtSFR6* plants were used as recipient plants when crossing. Col-0 and *sfr6-1* mutant plants were also crossed with *35S*::*AtCBF1* as controls. As *35S*::*AtCBF1* plants harbour a kanamycin (*Kan*) resistant gene F1 plants were selected on *Kan* supplemented MS agar plates. Plants were grown to maturity in normal growth conditions (Materials and methods section 2.2.4) and F2 seeds from individual plants collected. F2 seeds were germinated on MS agar plates supplemented with *Kan* and *PPT* (phosphinothricine). The seeds from Col-0 and *sfr6-1* crossed with *35S*::*AtCBF1* were selected on *Kan* supplemented agar plates. Twelve day old plants were harvested for RNA extraction. *sfr6-1* mutants have pale green coloured leaves (Knight *et al*., 2009). Therefore, seedlings with pale coloured leaves on *Kan* supplemented plates were selected as homozygous *sfr6-1* over-expressing *CBF1*. A slight increase of *KIN2* could be observed in transgenic lines over-expressing both *SFR6* and *CBF1* but these differences were not significant (95% confidence limit according to Student's *t* test) (Figure 3.6).

Figure 3.6

Transcript levels of *KIN2* in transgenic lines expressing both *35S*::*AtSFR6* and *35S*::*CBF1*.



Twelve-day-old seedlings selected on *Kan* and *PPT* supplemented MS agar plates were used for this assay. Col-0 and *sfr6-1* over-expressing *35S*::*AtCBF1* were selected on *Kan* supplemented MS plates and used as controls. Data represented here are relative quantification (s) values of *KIN2* expression relative to the *KIN2* levels of Col-0 over-expressing *CBF1*. *PEX4* was used as endogenous control. Data represented here are averages of 3 technical replicates. Error bar indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

3.2.1.7 Localization of CBF1 in the *sfr6-1* mutant

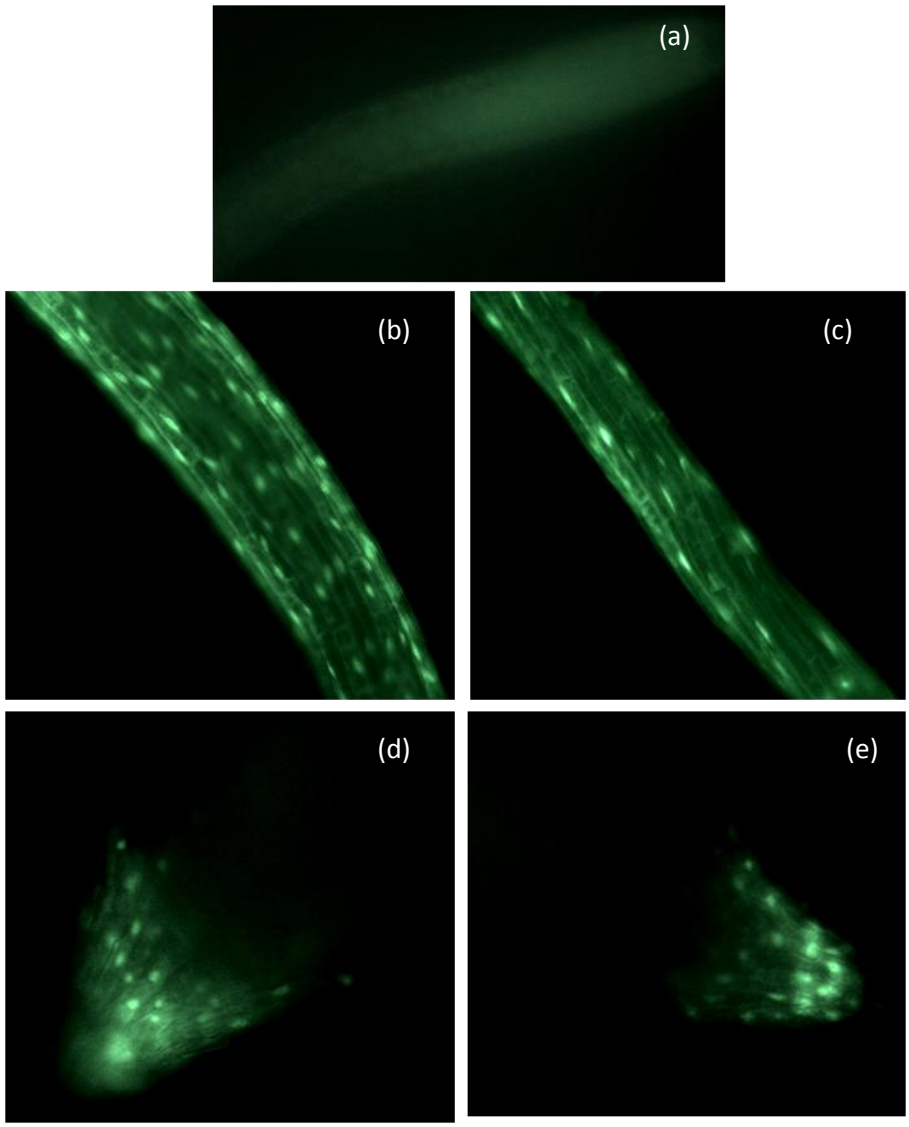
The *sfr6-1* mutant shows normal levels of *CBF* gene expression suggesting that SFR6 acts downstream of *CBF* transcription (Knight *et al*., 1999). The over-expression of *CBF1* and *CBF2* in the *sfr6-1* mutant did not increase downstream cold gene expression (Knight *et al*., 2009). Further, a CBF-aequorin fusion protein accumulated to WT levels in *sfr6-1* mutant indicating SFR6 does not affect CBF protein levels and these results suggest SFR6 acts after CBF protein translation

(Knight *et al*., 2009). Thus it is possible that SFR6 controls localization of CBF proteins in *Arabidopsis*. Therefore the localization of CBF1 protein in the *sfr6-1* mutant was examined. *sfr6-1* and Col-0 plants were crossed with wild type plants over-expressing *35S*::*CBF1*::*GFP* (Materials and methods section 2.2.1). F1 plants were obtained after *Kan* selection. F2 seeds were obtained by self pollinating F1 plants and again F3 plants were chosen by *Kan* selection. *sfr6-1* mutants have pale coloured leaves (Knight et al., 2009). Therefore, seedlings with pale coloured leaves on Kan supplemented plates were selected as homozygous sfr6-1 over-expressing *35S::CBF1:GFP*. The plants with pale green leaves were selected to visualize CBF1 localization in roots of *sfr6-1* mutant.

The localization of CBF1 protein was not altered in *sfr6-1* mutant compare to the WT (see section 2.13; Figure 3.7). These results suggest that SFR6 is not involved in localization of CBF1 in *Arabidopsis*.

Figure 3.7

Localization of CBF1 in the *sfr6-1* mutant using GFP imaging.



Localization of 35S::CBF1::GFP in root cells of *Arabidopsis* plants over-expressing *35S::CBF1:GFP*. Images were captured using a 40 × objective. (a) Root of wild type *Arabidopsis* plant over-expressing *35S*::*CBF1*. (b, c) Images of Col-0 and *sfr6-1* over-expressing *CBF1* and *GFP* respectively were taken from root elongation zone and (d, e) root cap. Images were taken with identical parameters to allow comparison.

3.2.2 Complementation of *sfr6-1* mutant by the wild type *AtSFR6* gene

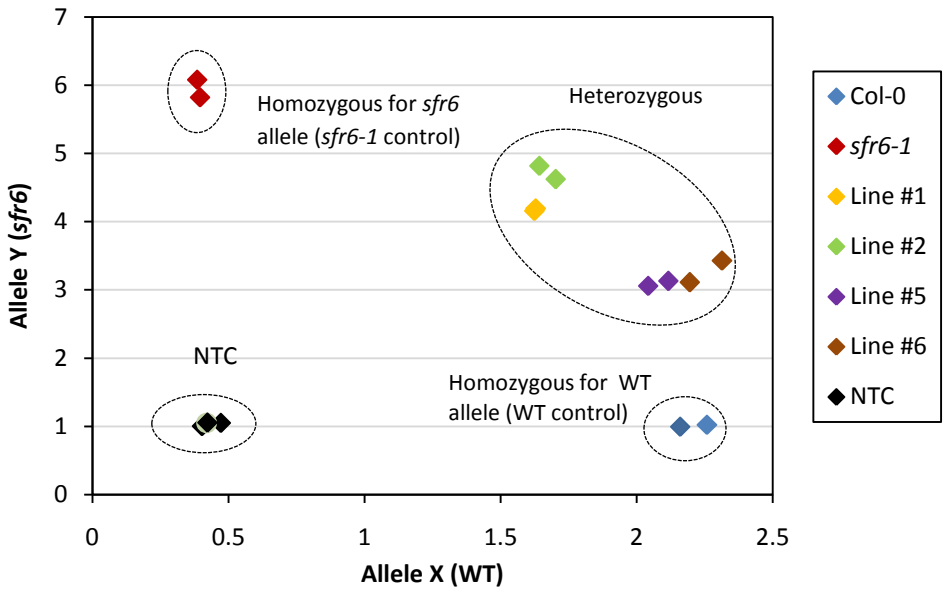
A complementation assay was conducted to confirm that the un-induced levels of *KIN2* expression in *35S::AtSFR6* over-expressing lines is not due to mutations or mis-splicing of the SFR6 construct used to generate these lines.

3.2.2.1 Selection of transgenic lines

sfr6-1 plants were transformed with the *35S::AtSFR6* construct using the floral dip method (see section 3.2.1.1 for preparation of construct). At the same time the GUS construct (as described in section 3.2.1.1) was also transformed into *sfr6*-1 as a control. Six transformants were selected and an allelic discrimination assay (Materials and methods section 2.15) was performed to ensure that the transformants were in the *sfr6-1* mutant. A mixture of two TaqMan® MGB probes which can detect wild type *SFR6* gene and mutant *sfr6* gene were used for this assay (see appendix C.1 for probe details). If transgenic lines originated from the *sfr6-1* mutant the T2 generation should heterozygous for *SFR6* (Figure 3.8). In 4 lines out of 6 both mutant and wild type alleles were detected (heterozygous) and these lines were selected for further analysis.

Figure 3.8

Results of allelic discrimination assay of *sfr6-1* complemented lines.

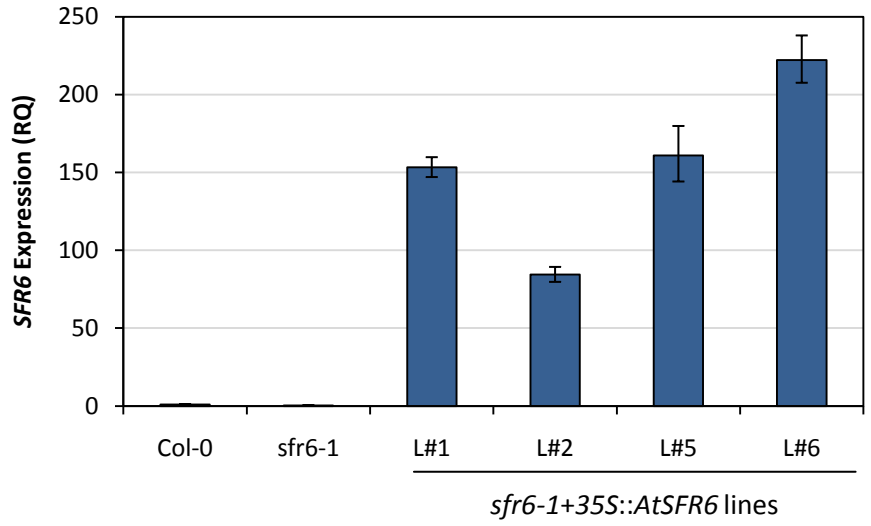


Genomic DNA was extracted from 7-day-old seedlings grown on 1×MS agar medium. Two technical replicates were performed for each sample. Water was used for non template control (NTC). Graph presents data of only four lines identified as heterozygous to *SFR6* along with *sfr6-1* and Col-0 homozygous lines.

SFR6 transcript levels of these selected lines were tested using a TaqMan probe for *SFR6* (see appendix C.2). Seventy to 230 fold inductions of *SFR6* transcripts was detected in the four lines tested relative to non transgenic Col-0 (Figure 3.9).

Figure 3.9

Analysis of *SFR6* over-expression levels due to *35S*::*AtSFR6* in *sfr6-1* mutant background.



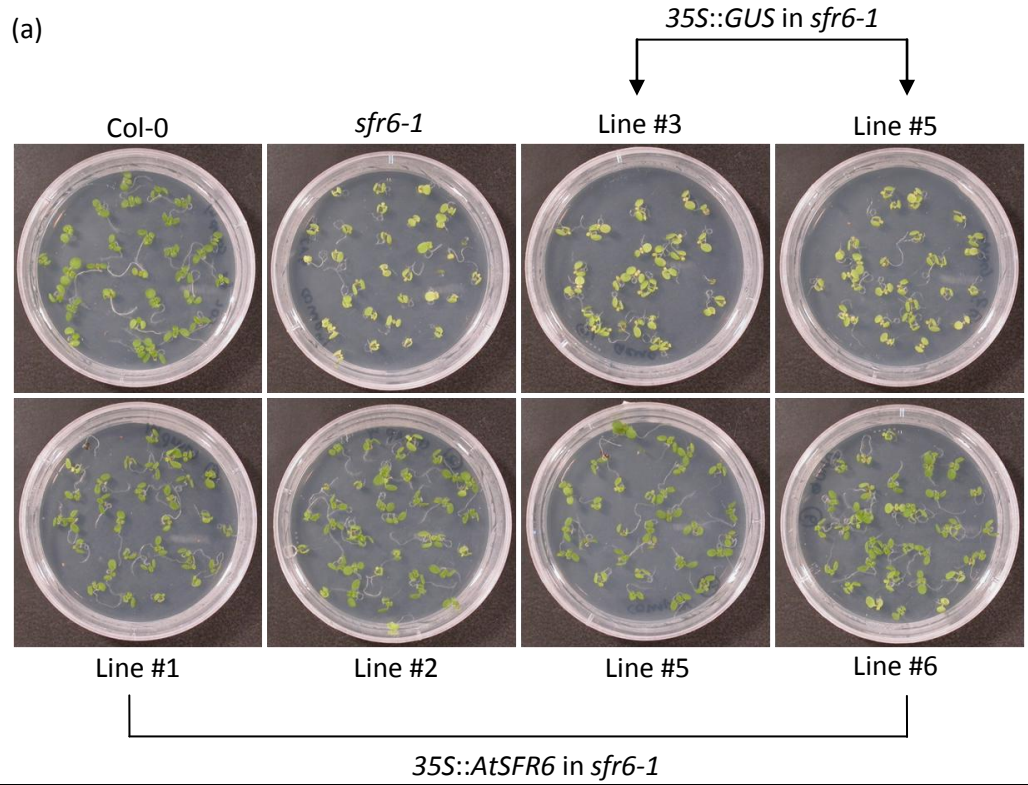
Unstressed 7-day-old seedlings grown on 1×MS agar plates were used. Graph shows relative quantification (RQ) values of *SFR6* expression relative to the *SFR6* expression level of non-transgenic Col-0 plants. *ß-TUBULIN4* was used as an endogenous control. Non-transgenic *sfr6-1* mutant was also used as a control. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

3.2.2.2 Phenotypic complementation

The original mutant of *sfr6-1* shows paler leaf colour compared to Col-0 (Knight *et al*., 2009). Therefore, we tested the colour complementation of transgenic plants. As shown in figure 3.9 *sfr6-1* mutant expressing *35S*::*AtSFR6* displayed WT phenotype and the lines with *35S*::*GUS* construct still showed the *sfr6-1* mutant phenotype.

Figure 3.10

Phenotypic complementation of *sfr6-1* by *AtSFR6* gene.



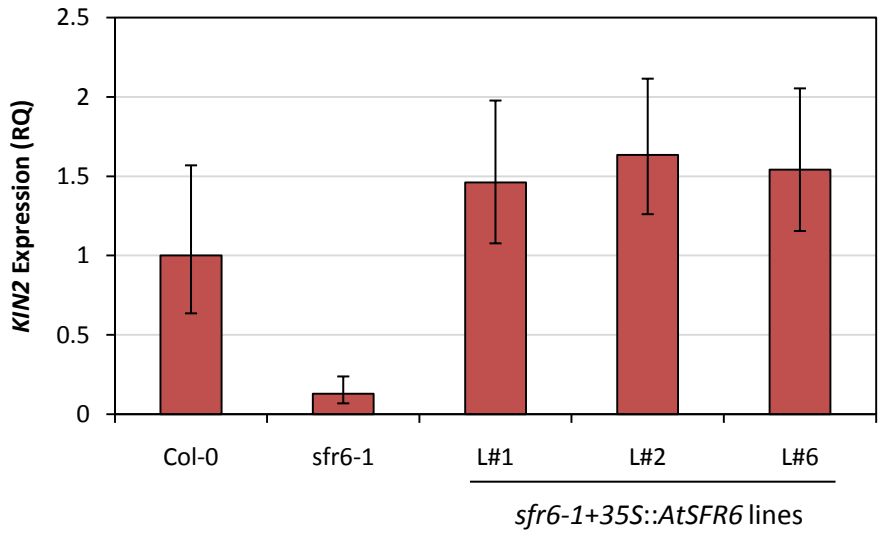
Col-0, *sfr6-1* and T2 progeny of *sfr6-1* plants containing transgene 35S::*SFR6* and 35S::*GUS* were grown on 1×MS agar plates and (a) photographs were taken 10 days after germination. (b) Close-up pictures of seedlings of Col-0, *sfr6-1*, *sfr6-1+35S*::*GUS* and *sfr6-1+35S*::*AtSFR6*.

3.2.2.3 *KIN2* expression in complemented lines

The restoration of *KIN2* transcripts after cold acclimation in the 3 transgenic lines (line #1, #2 and #6) were also tested after 6 h cold treatment at 4°C. All transgenic lines demonstrated *KIN2* expression levels similar to Col-0 (Figure 3.11). These results confirm further the necessity of *SFR6* for *COR* expression (Knight *et al*., 1999).

Figure 3.11

Restoration of *KIN2* expression in *sfr6-1* mutant by complementation with wild type *SFR6*.



Seven-day-old seedlings grown on MS agar plates were subjected to cold treatments at 4°C for 6 h and then samples were collected for RNA extraction. Data represented here are relative quantification (RQ) values for *KIN2* expression relative to the *KIN2* levels of cold treated Col-0. *ß-TUBULIN4* was used as an endogenous control to normalize expression values. Graph shows average of three biological replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

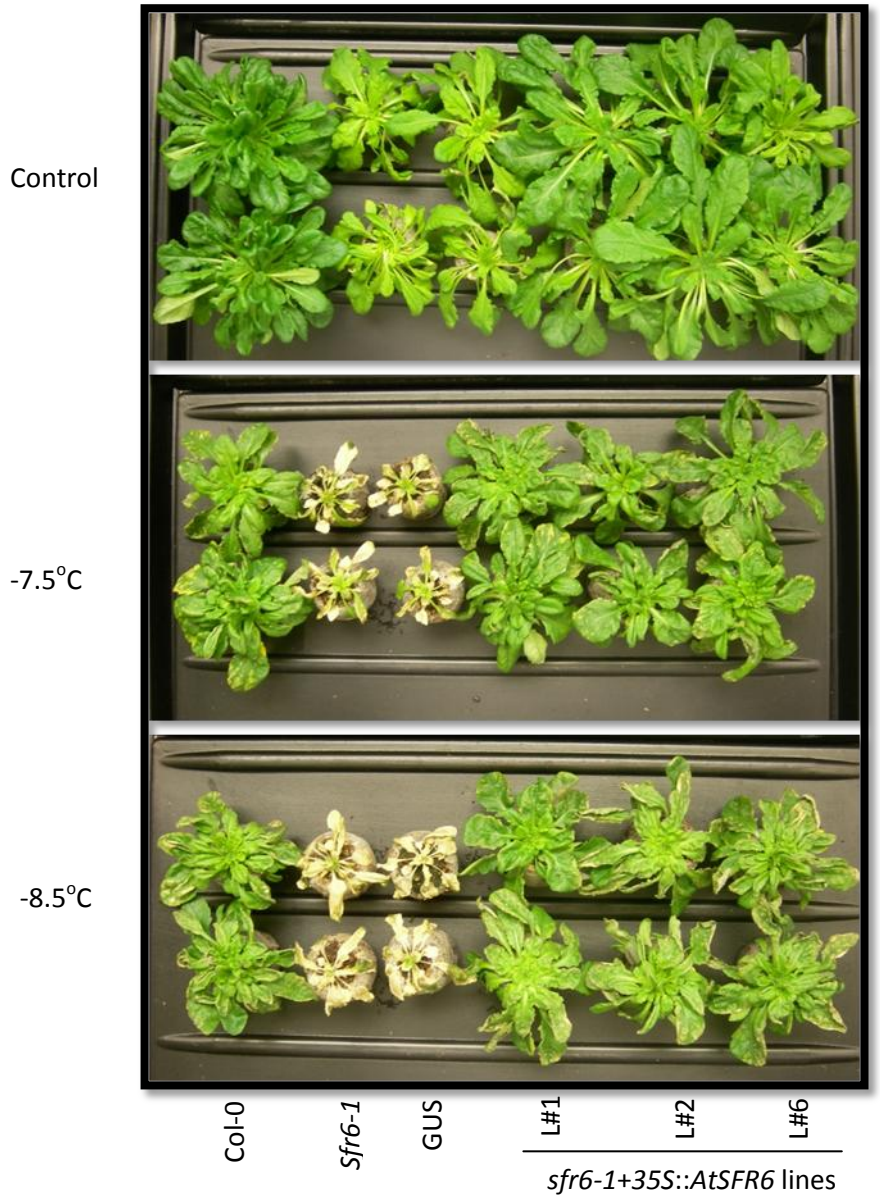
3.2.2.4 Freezing tolerance of complemented *sfr6-1* mutant lines

As *KIN2* expression was restored in *sfr6-1* + *35S*::*SFR6* complemented lines compared to *sfr6-1* freezing tolerance of these lines were tested. Three complemented lines (line #1, #2 and #6), Col-0, *sfr6-1* and *sfr6*-1+*35S*::*GUS* were grown on peat plugs under short day (8 h light and 16 h dark) conditions. After 5 weeks plants were cold acclimated for 11 days at 4^{o}C and the temperature ramped to freezing temperatures (-6.5 and -7.5^{o}C) within 3 h and plants maintained at these temperatures for 24 h. Then the temperature was returned to 20^{o}C and the development of freeze thaw-induced damage was monitored (Materials and method section 2.4.1.6) 5 days after thawing. At -7.5^{o}C *sfr6-1* and *sfr6-1+35S*::*GUS* transgenic plants showed severe damage after freezing and thawing but Col-0 and *sfr6-1* complemented lines were not damaged. At -6.5^{o}C the damage was not as clear as -7.5^{o}C.

Therefore, to confirm the results, the experiment was repeated but this time plants were exposed to -7.5 and -8.5^{o}C freezing temperatures. Figure 3.12 shows the photographs taken 5 days following freezing and thawing. The visible damage following freezing and thawing of the *sfr6-1* mutant and *sfr6-1*+ *35S*::*GUS* transgenic plants was very clear compared to Col-0 and the three *sfr6-1* complemented lines. These results further confirmed that SFR6 is necessary for cold acclimation and freezing tolerance in *Arabidopsis*.

Figure 3.12

Freezing tolerence of the *sfr6-1* complemented lines.



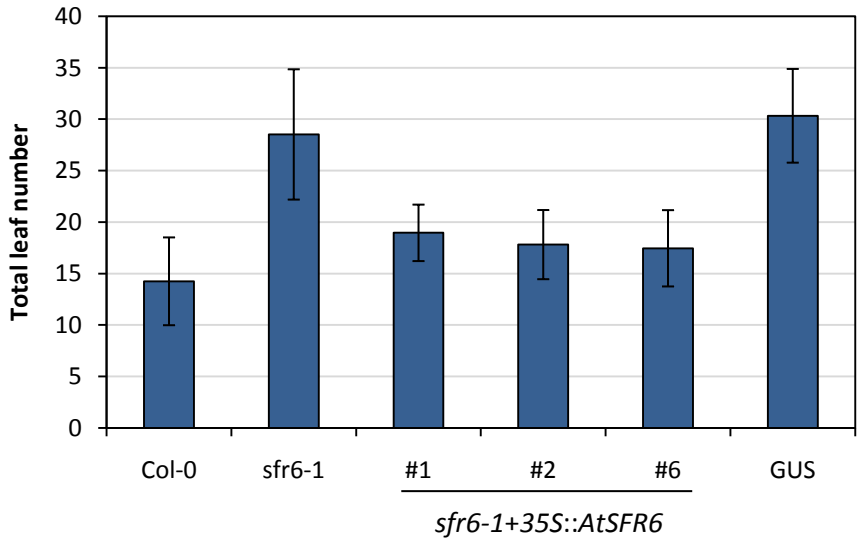
Photographs were taken 5 days after returning to 20°C. Five-week-old plants were cold acclimated at 4°C for 11 days then exposed to -7.5 and -8.5°C for 24 h. The *35S*::*SFR6* in *sfr6-1* mutant background (L#1, #2 and #6) were along with *35S*::*GUS* in the *sfr6-1* mutant, Col-0 and *sfr6-1* mutant as controls were used in this experiment. Ten plants from each line per each treatment were used.

3.2.2.5 Restoration of late flowering phenotype of *sfr6-1* mutant by *AtSFR6*

The *sfr6-1* mutant shows a late flowering phenotype (Knight *et al*., 2008). To determine whether complemented lines of the *sfr6-1* mutant by wild-type *SFR6* restore normal flowering phenotype, the flower induction of complemented lines was examined. Three *35S*::*AtSFR6* and one *35S*::*GUS* in *sfr6-1* background were used for this experiment. Col-0 and *sfr6-1* were also grown as controls. Plants were grown under a long day (16 h light and 8 h dark) photoperiod. Number of rosette and cauline leaves were counted when the inflorescence was approximately 1 cm long. *sfr6-1* mutants showed significantly higher number of leaves at flowering when compared to Col-0. The *35S*::*GUS* line also showed *sfr6-1* phenotype, bearing a higher number of leaves at flowering (Figure 3.13). *35S*::*AtSFR6* lines displayed slightly higher number of leaves at flowering than Col-0 but there was no significant difference. These results confirm that *AtSFR6* affects the flowering time of *Arabidopsis.*

Figure 3.13

Flowering time of *sfr6-1* complemented lines with *AtSFR6*



35S::*AtSFR6* and *35S*::*GUS* lines in *sfr6-1* background were grown under a long day photoperiod (16 h light and 8 h dark). Col-0 and *sfr6-1* were also grown as controls. Number of leaves were counted when flower shoot became approximately 1 cm long. Error bars are ±SD (n=25).

3.3 Discussion

3.3.1 SFR6 is necessary but not sufficient for *COR* gene expression of CBF pathway

Previous work identified *At4g04920* as the locus for *SFR6*; a gene required for *COR* gene expression and cold acclimation in *Arabidopsis* (Knight *et al*., 2009). This discovery led to the investigation of the effect of *SFR6* over-expression as a possible route to crop improvement in the future. Selected *SFR6* over-expressers showed higher expression levels of *SFR6* transcripts in ambient temperature; however *KIN2* expression did not increase in either ambient temperatures or after cold exposure. Restoration of all *sfr6-1* mutant phenotypes with the same over-expression construct confirms the *35S*::*AtSFR6* over-expression construct used for this study is functional. All these results indicate that levels of *SFR6* alone might not influence activation of target *COR* genes in *Arabidopsis*.

Previous studies also showed reduced *COR* gene expression in the *sfr6-1* mutant, not only after cold exposure, but also after osmotic stress induced by mannitol (Boyce *et al*., 2003). However, *KIN2* expression in *AtSFR6* over-expressing lines after osmotic stress did not alter compared to wild type *Arabidopsis*. These results further confirm SFR6 is necessary but not sufficient for *COR* gene expression, not only at low temperature, but also in response to osmotic stress.

Over-expression of *CBF* genes in the *sfr6-1* mutant did not alter *COR* gene expression (Knight *et al*., 2009). However, over-expression of *CBF* transcription factors in wild type *Arabidopsis* causes large increase in *COR* gene expression and freezing tolerance at ambient temperatures (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998; Kasuga *et al*., 1999; Gilmour *et al*., 2000). Therefore, the possible additive effect on *COR* gene expression due to an overabundance of *SFR6* and *CBF* was examined. The data showed that increasing both *SFR6* and *CBF1* had no effect on *COR* gene expression indicating that overabundance of both *SFR6* and *CBF1* has no additive effect on *COR* gene expression.

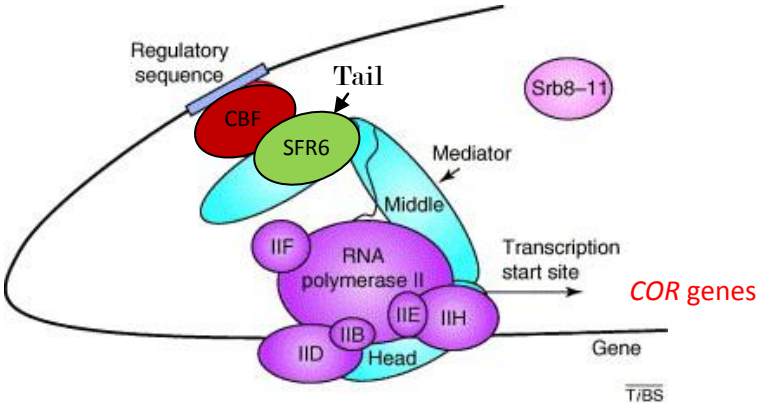
Previously, it was also observed that the expression of CBF proteins (translation and protein stability) was not defective in the *sfr6-1* mutant (Knight *et al*., 2009). Therefore, it was suggested that *SFR6* might affect CBF activity after CBF translation (Knight *et al*., 2009). Therefore, it might be possible that *SFR6* recruits CBF protein to the nucleus. However, the CBF1 localization data showed that SFR6 did not affect localization of CBF proteins either.

One explanation for the lack of effect when *SFR6* is over-expressed in wild type is that the SFR6 protein is required in stoichiometric proportions with one or more other proteins as a part of a complex and that an increase in the amount of any one of these individually cannot influence the amount of the complex as a whole. Supporting this hypothesis At4g04920 had already been identified as the MED16 subunit of the *Arabidopsis* mediator complex (Backstrom *et al*., 2007) before SFR6 had been mapped to the At4g04920 locus.

Mediator is the evolutionary conserved multi-protein complex that binds RNA polymerase II and controls transcription of genes (Flanagan *et al*., 1991). The possible mechanism suggested by Chadick and Asturias (2005), Bjorklund and Gustafsson (2005), and Haha (2004) for transcriptional activation by mediator was that the gene specific activators (transcription factors) recruits mediator to the transcription initiation site. Then, general transcriptions factors involved in gene transcription interact with the DNA-mediator complex to form a platform to bind RNA polymerase II. According to this mechanism when plants are exposed to low temperature CBF proteins activate and bind to the promoter of *COR* genes and recruits mediator complex to the promoter to build the RNA polymerase assembly platform. It is possible that MED16/SFR6 might directly bind with CBF (Figure 3.14) or with other mediator subunit which links with MED16 (Myers *et al*, 1999; Chadick and Asturias, 2005). Based on these results over-expression of a single gene of the mediator complex does not result in increased activity of the complex as a whole. Therefore, it is now unsurprising that over-expression of *SFR6/MED16* does not increase *COR* gene expression but over-expression of *CBF* does.

Figure 3.14

Model for activation of *COR* genes via SFR6 and CBF genes. Image reproduced from Bjorklund and Gustafsson (2005).



Under low temperature conditions CBF protein activate and bind to the promoter of *COR* genes and recruits mediator complex to the promoter to build the RNA polymerase assembly platform and once RNA polymerase II binds transcription of *COR* genes initiate. CBF, mediator interaction takes place via SFR6/MED16 tail subunit. IIB, IID, IIE, IIF and IIH are general transcription factors and Srb8-11 (MED12, MED13, Cdk8 and CycC respectively) form a module that is involved in negative regulation of transcription (refer to section 1.2.5 for more details of gene repression).

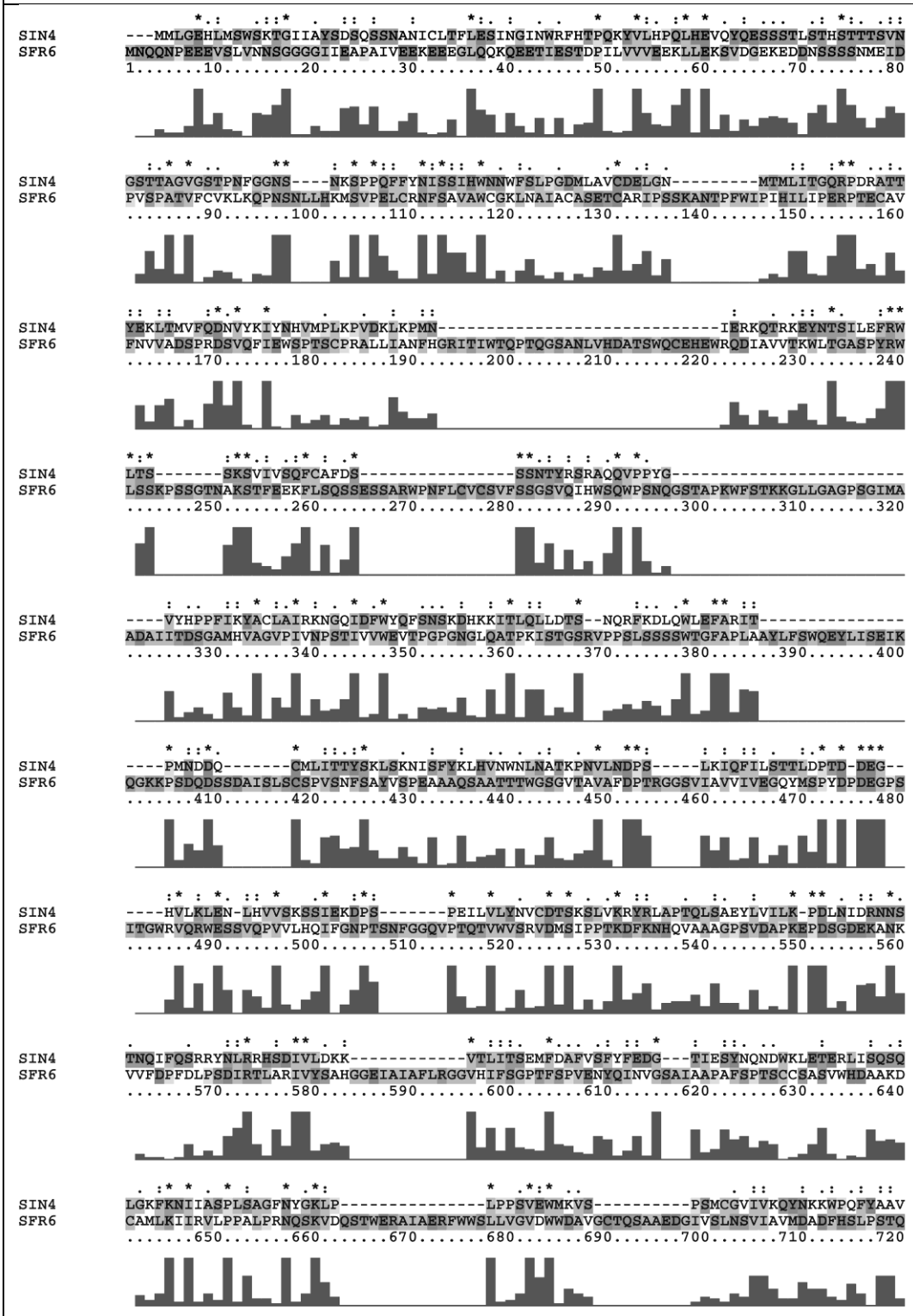
3.3.2 SFR6 has very low homology with yeast MED16 (SIN4)

In *S. cerevisiae* MED16 is also known as SIN4. Functional and structural studies of SIN4 showed that it has dual role in repression and activation of transcription (Myers and Kornberg, 2000). The deletion of MED16 (SIN4) from *S. cerevisiae* lacks the function of some of other tail subunits suggesting interaction of other subunits via SIN4 with other part of mediator complex (Myers *et al*., 1999; Chadick and Asturias, 2005). Therefore, it is possible that the low temperature sensitivity of *sfr6* mutants might be due to a loss of function of other tail subunits that interact with SFR6/MED16. However, the genetic interaction of other tail subunits with SFR6 in plants remains to be proved.

Arabidopsis mediator subunits show very low sequence similarity to yeast mediator subunits (Backstrom *et al*., 2007). Backstrom *et al*. (2007) have suggested that this divergence is because of the highest number of transcription factors specific to flowering plants. According to Backstrom *et al*. (2007) tail subunits have the lowest sequence similarities to other organisms compared to head and middle subunits. Consistent with these results the homology between protein sequences of yeast SIN4 and *Arabidopsis* SFR6 is extremely low (Figure 3.15). However, examining functional complementation of SFR6 with SIN4 would be interesting to characterize the role of SFR6 in transcriptional regulation in plants.

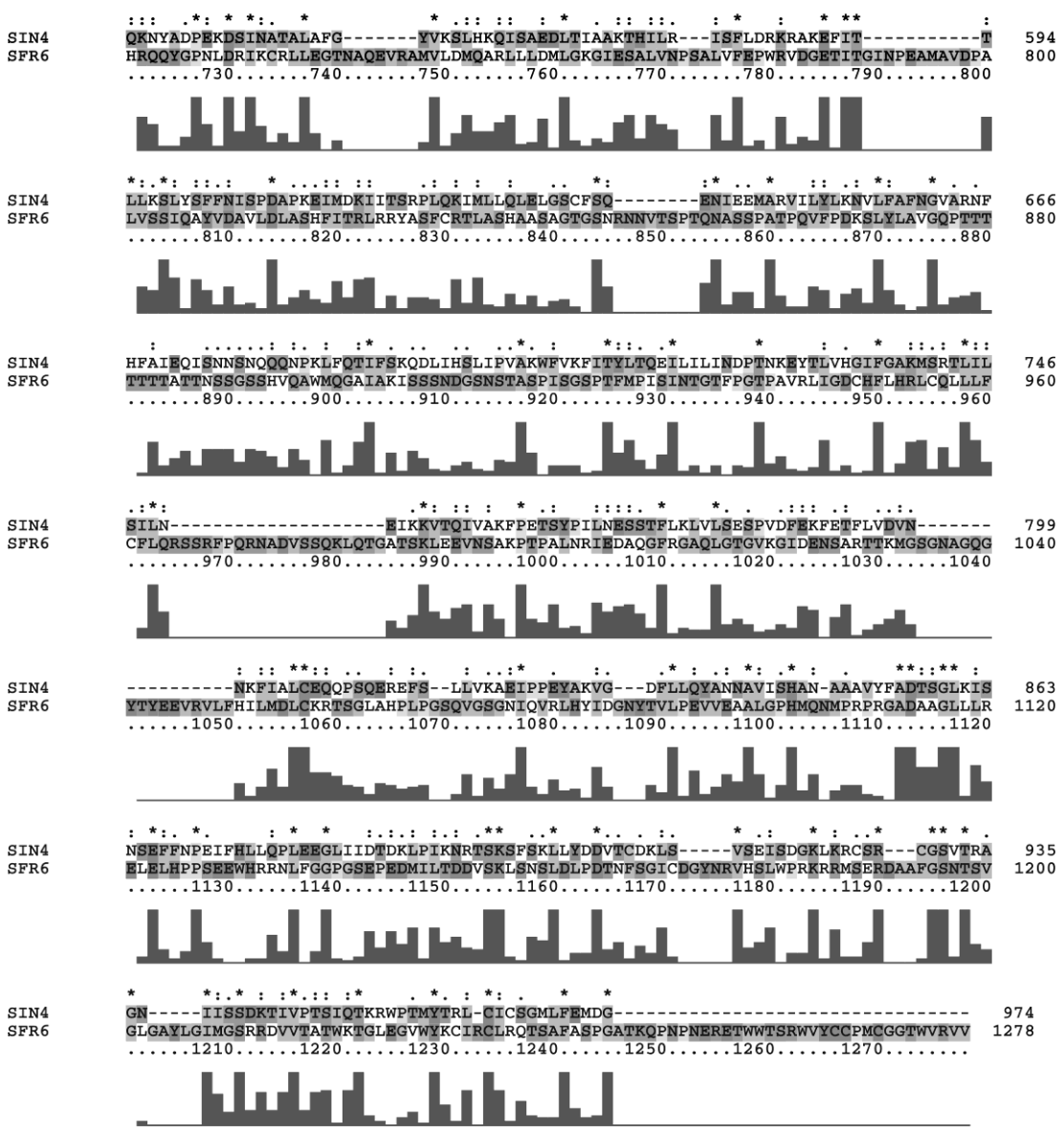
Figure 3.15

Comparison of SIN4 and SFR6 protein sequences.



(Figure continues to the following page)

Figure 3.15 *(Continued from the previous page)*



The alignment of the SIN4 in *S. cerevisiae* and *Arabidopsis* SFR6 protein sequences using T-Coffee multiple sequence alignment tool. "*" indicate fully conserved residue; ":" indicates highly conserved positions and "." Indicates the weakly conserved positions. Dashes indicate the gaps in the amino acid sequence. The columns show the score of the conservation of amino acids in each position.

3.3.3 LOS1 and LOS2

The mutant of *los1-1* and *los2* are the only other two mutants of *Arabidopsis* reported reduce expression of *COR* genes under cold stress and sensitive to freezing but not alter *CBF* expression (Guo *et al*., 2002; Lee *et al*., 2002). Both these mutants showed a wild type response to ABA, high salt or osmotic stresses demonstrating that these mutations specially affect low temperature responses (Guo *et al*., 2002; Lee *et al*., 2002). LOS1 encodes a translation elongation factor 2-like protein which is essential for protein synthesis by mediating translocation steps in peptide chain elongation (Guo *et al*., 2002). The authors suggested that the induction of CBF/DREB1 does not need new protein induction and LOS-1 is essential for induction of downstream cold genes (Guo *et al*., 2002). SFR6 also act downstream to CBF/DREB1 translation (Knight *et al*, 2009). Therefore, SFR6 and LOS-1 protein may interact to induce downstream cold gene expression under low temperature conditions.

The sequence of LOS2 indicates that it encodes an enolase that converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. The human homologue, MBP-1, also acts as a transcription factor that repress the expression of c-myc by binding the c-myc gene promoter (Lee *et al*., 2002). An electrolyte leakage assay of *los2* and WT *Arabidopsis* with and without cold acclimation showed that *los2* mutants are defective in cold acclimation (Lee *et al*., 2002). Although homologues of c-myc are not present in *Arabidopsis*, Lee *et al.* (2002) found a sequence similar to the c-myc MBP-1 binding site in the promoter of the putative transcriptional repressor STZ/ZAT10. STZ/ZAT10 expression in *los2* mutant is stronger and more sustained than in WT suggesting that LOS2 is a negative regulator of STZ/ZAT10 expression. These results coupled with the promoter sequence analysis suggest that LOS2 is positive regulator of *COR* gene expression by directly repressing STZ/ZAT10 expression. As LOS2 encodes a transcription factor and SFR6 has been identified as a regulator of transcription factor mediated expression, it is possible that SFR6 and LOS2 proteins might interact to promote *COR* gene expression. However, the role of these two genes in *COR* gene expression remains to be determined.

Chapter 4

Examining the response of *sfr6* mutant to different abiotic and biotic stresses

4.1 Introduction

The defective expression of genes in the *sfr6-1* mutant is not limited only to low temperature stress. Drought-responsive expression of the same group of *COR* genes is similarly effected (Knight *et al*., 1999; Boyce *et al*., 2003). In response to drought the expression of *COR* genes are controlled by DREB2 transcription factors (Liu *et al*., 1998; Shinwari *et al*., 1998). Another CBF transcription factor gene *CBF4* also induces *COR* genes in response to ABA and drought stimuli (Haake *et al*., 2002; Knight *et al*., 2004). Previous experiments also showed that SFR6 acts post-transcriptionally of CBF (Knight *et al*., 2009). In addition to the dramatic effect on the CBF regulon *sfr6-1* mutants also have pleiotropic phenotypes and mutation in SFR6 influences physical appearance, flowering time and circadian clock behaviour (Knight *et al*., 2008; Knight *et al*., 2009) (refer to sections 1.4.5 for more information). These results suggest that SFR6 may influence other signalling pathways activated in response to other environmental stresses.

Therefore, the specific hypothesis in this chapter is;

- Does the *SFR6* gene have a role in other abiotic and biotic stress signalling pathways in *Arabidopsis thaliana?*

This was tested by screening the susceptibility/tolerance of *sfr6-1* mutants to a variety of stresses such as drought, salinity, UV-C, heat, oxidative stresses and pathogen attack under different conditions (both soil and agar) and different developmental stages. Once clear difference to wild type was identified, two other *sfr6* alleles (*sfr6-2* and *sfr6-3*) were also tested under these particular stresses to determine whether *SFR6* is indeed needed to tolerate plants from those stresses. In this chapter the results of those screening tests are discussed.

4.2 Results

4.2.1 Response to drought

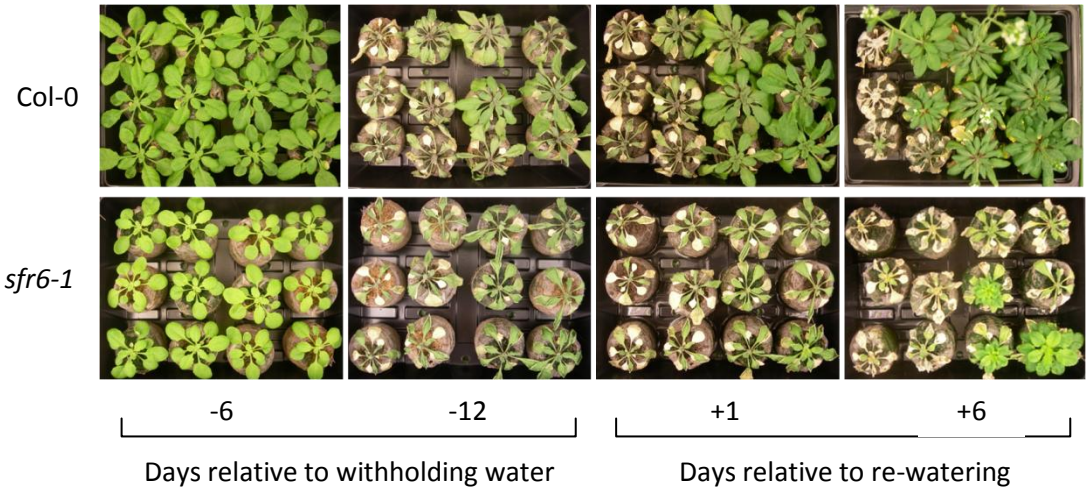
Boyce *et al*. (2003) have previously shown that the *sfr6-1* mutant is sensitive to osmotic stress by germinating seeds on mannitol supplemented plates. Therefore, the sensitivity of *sfr6* mutants to drought and/or osmotic stresses was investigated further at the seedling stage as well as at the mature plant stage by performing four types of assays. These were (1) analysis of *sfr6* for tolerance to drought induced by withdrawing water, (2) osmotic stress induced by watering mature plants with mannitol, (3) root reorientation and (4) elongation assays on mannitol supplemented 1×MS agar medium.

4.2.1.1 Withdrawing water

Three-week-old Col-0 and *sfr6-1* plants grown on peat plugs (with continuous watering) were then kept without water (see Materials and methods section 2.4.1.1.1) until plants withered. This took 10-12 days and the withering of *sfr6-1* leaves was more severe and earlier compared to Col-0. After 12 days plants were re-watered. Plant recovery from drought was measured by the number of plants that survived after a week of re-watering. Col-0 plants displayed a higher survival rate than the *sfr6-1* mutant as shown in Figure 4.1. This was repeated two times with the same results. To further confirm the results the experiment was repeated two times with *sfr6-2* and *sfr6-3* (Knight *et al*., 2009). The survival rate of *sfr6-2* and *sfr6-3* was compared with *sfr6-1* and Col-0. In this experiment plants did not exhibit such clear and strong differences as previously. Finally the same experiment was repeated under three different humidity (50, 60, and 70 %) conditions. The plants again did not display any difference in survival rate even between Col-0 and the *sfr6-1* mutant as observed previously.

Figure 4.1

Sensitivity of the *sfr6-1* mutant to drought induced by withdrawing water.



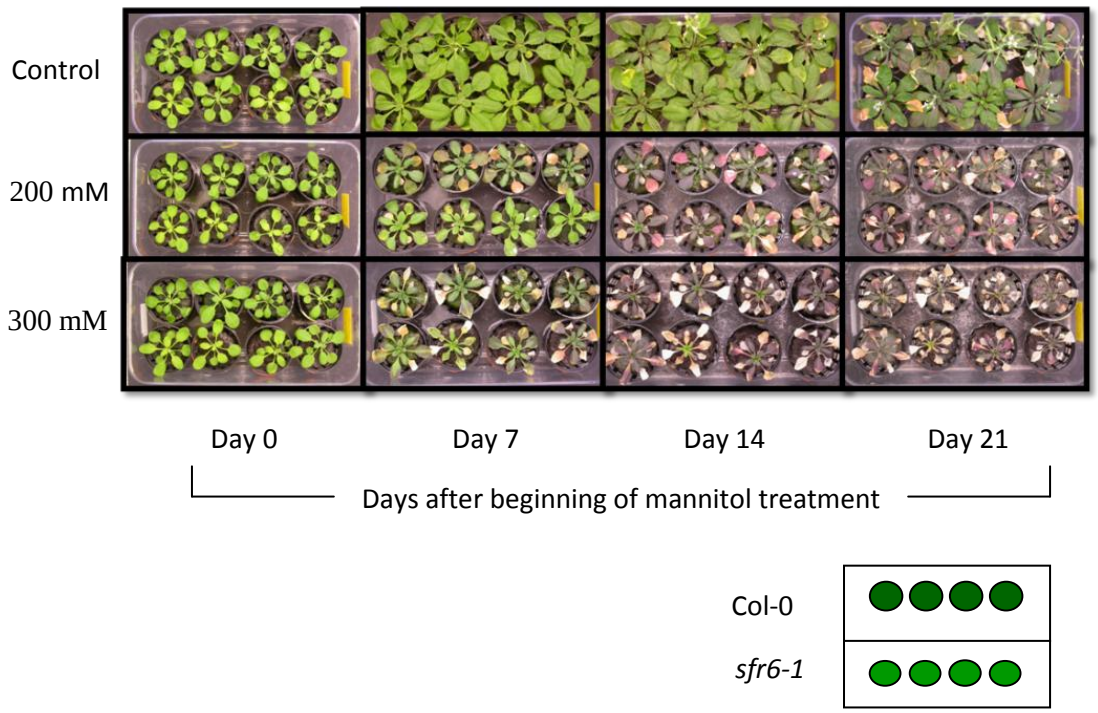
Results of the second experiment performed with only Col-0 and *sfr6-1*. Plants were grown for 3 weeks with normal watering then water was withheld for 12 days to allow dehydration then rewatered continuously. Photographs were taken 6 and 12 days after water was withheld and 1 and 6 days after rewatering. Twelve plants per line per treatmnet were used.

4.2.1.2 Watering mannitol

To examine further the sensitivity of *sfr6* to drought, three-week-old Col-0 and *sfr6-1* plants were grown on peat plugs and watered with different concentrations (0, 50, 100, 150, 200, 300 mM) of mannitol (see Materials and methods 2.4.1.1.2). Both the *sfr6-1* and Col-0 plants accumulated anthocyanin and the rate of accumulation was quicker in wild type plants. However, *sfr6-1* and wild-type plants did not display clear visual differences in bleaching of leaves or growth retardation (Figure 4.2). This was repeated two times and the same results observed. The experiment was repeated with the two higher concentrations of mannitol (200 and 300 mM) (Figure 4.2) and the results were similar to the previous two times.

Figure 4.2

Sensitivity of *sfr6-1* and Col-0 plants to watering mannitol.



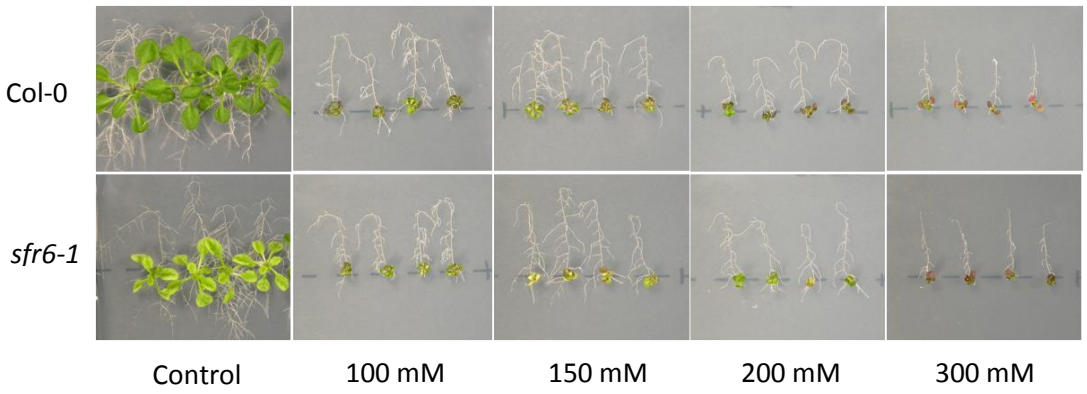
Three-week-old plants grown on peat plugs were subjected to osmotic treatment by watering of 200 and 300 mM mannitol. Control plants were continuously watered with same amount of water. Pictures were taken weekly until three weeks post treatments. Eight plants per line per treatment were used.

4.2.1.3 Root reorientation assay

Five-day-old seedlings were transferred to vertical plates supplemented with different concentrations of mannitol (0, 100, 150, 200 and 300 mM) (see Materials and methods section 2.4.1.1.3). Plates were turned 180^{o} and grown for 14 days to examine root growth. The growth of seedlings was drastically reduced even at low concentrations of mannitol and the leaves of both *sfr6-1* and Col-0 plants accumulated anthocyanin (Figure 4.3). Higher concentrations of mannitol completely stopped root growth of both *sfr6-1* and Col-0. However, no differences were observed between *sfr6-1* compared to Col-0 plants in this assay.

Figure 4.3

Comparison of root reorientation of Col-0 and the *sfr6-1* mutant under osmotic stress induced by mannitol.



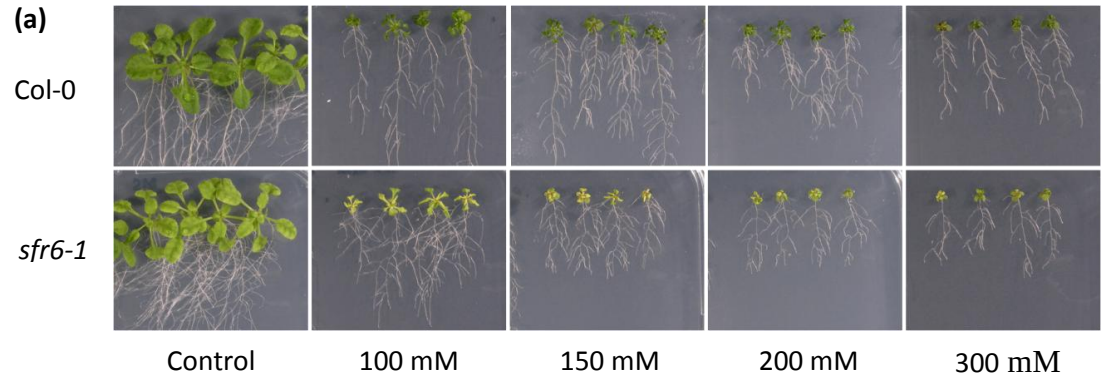
Five-day-old seedlings grown on 1×MS media were transferred to 1×MS medium or 1×MS media supplemented with different concentrations of mannitol (0, 100, 150, 200 and 300 mM) and plates were turned 180^{o} (plants upside down). Plates were placed vertically in a growth room. Photographs were taken 12 days post treatment. One out of three independent experiments with similar results is shown in this figure. Twelve plants per line per treatment were used.

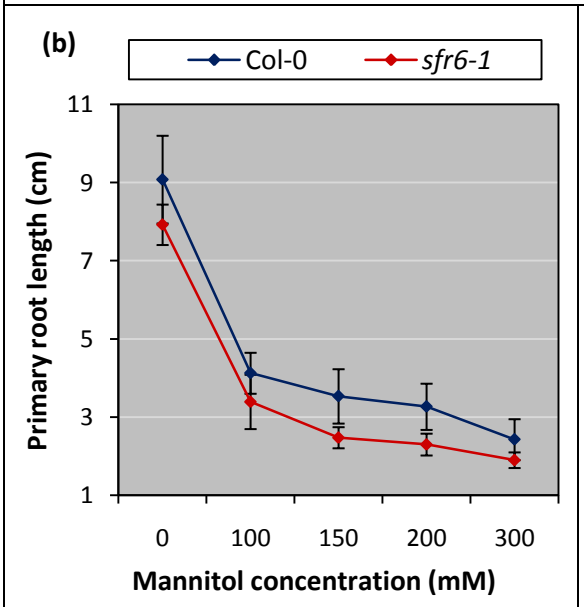
4.2.1.4 Root elongation assay

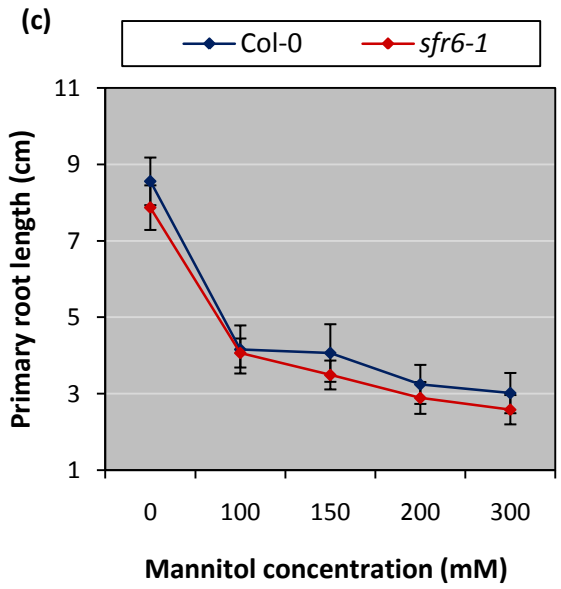
Five-day-old seedlings were transferred to vertical plates supplemented with different concentrations of mannitol (0, 100, 150, 200 and 300 mM), but this time plates were not turned through 180^{o} (see Materials and methods section 2.4.1.1.4). The primary root length was marked every two days up to two weeks to determine whether *sfr6-1* showed higher sensitivity to osmotic stress induced by mannitol. Both the Col-0 and *sfr6-1* showed a significant decrease in root elongation in the presence of mannitol (Figure 4.4), but there were no significant difference between wild type and *sfr6-1*.

Figure 4.4

Comparison of osmotic sensitivity induced by mannitol in Col-0 and the *sfr6-1* mutant by root elongation assay.







Five day old seedlings grown on MS medium were transferred to 1×MS medium or 1×MS media supplemented with different concentrations of mannitol and plates were kept in growth room vertically. Twelve plants per line per treatment were used. (a) Growth of Col-0 and *sfr6-1* seedlings plants 12 days post treatment. (b & c) Root length of Col-0 and *sfr6-1* 12 days after exposure to mannitol. Graphs (b) and (c) represent the results of replicate 1 and 2 respectively. Root lengths are presented in mean ±SD (n=12).

4.2.2 Response to salt

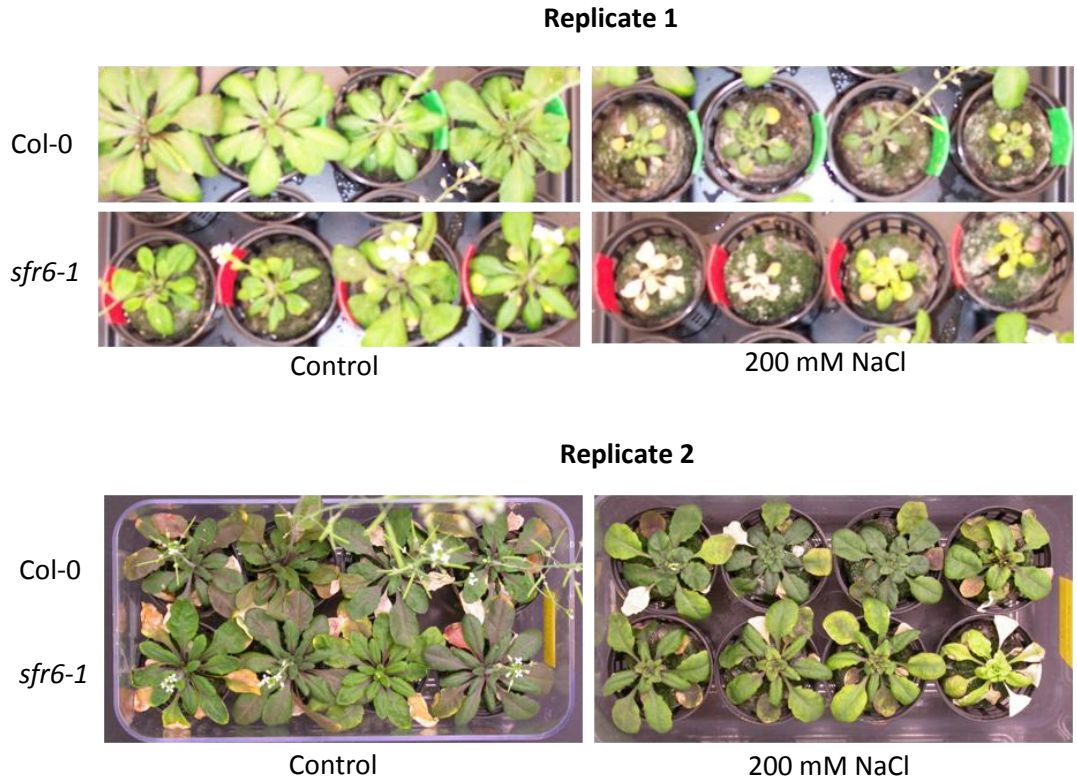
Analysis of sensitivity of the *sfr6* mutants to salinity was also performed by exposing plants to salt stresses induced by a range of methods at both the seedling and mature plant stage as well as the effect of salt on seed germination. Four types of assays were performed, firstly by testing sensitivity of *sfr6* to salt stress induced by watering NaCl onto mature plants. Secondly by examining the response of seedlings to salt stress, through root reorientation and elongation on NaCl supplemented 1×MS agar medium. Finally, the effect of salt to seed germination was examined by germinating seeds on 1×MS agar medium supplemented with various concentrations of NaCl.

4.2.2.1 Watering NaCl

The susceptibility of *sfr6-1* to salt was examined by continuously watering 200 mM NaCl solution onto three week old plants grown on peat plugs (Material and methods section 2.4.1.2.1). In the first experiment *sfr6-1* plants exhibited enhanced sensitivity to salt compared to Col-0 (Figure 4.5) with greater yellowing and bleaching 3 weeks post treatment. However, this result was not obtained in subsequent experiments (Figure 4.5).

Figure 4.5

Sensitivity of *sfr6-1* and Col-0 to salt through watering.



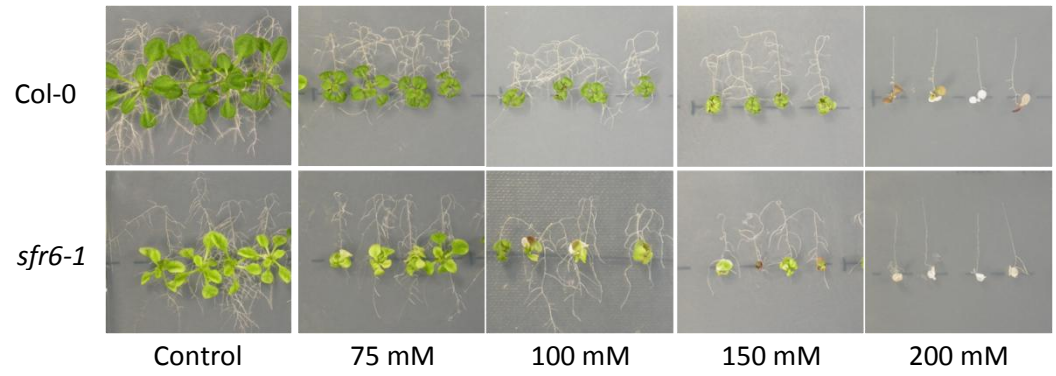
Plants were continuously watered with 200 mM NaCl from three weeks after germination onwards. Eight plants per line per treatment were used. Images are shown the growth of plants 21 days post-treatment.

4.2.2.2 Root reorientation assay

Five-day-old seedlings of Col-0 and *sfr6-1* were transferred to vertical plates containing different concentrations of NaCl (0, 75, 100 and 200 mM), and plates were turned 180^{o} as described previously for mannitol stress in section 4.2.1.3 (Materials and methods section 2.4.1.2.2). Plants showed relatively small responses in growth in the presence of low concentrations of NaCl, but the growth of seedlings was completely inhibited at high concentrations (Figure 4.6). However, *sfr6-1* and wild type did not show substantial differences in their root growth in this assay.

Figure 4.6

Comparison of salt sensitivity in Col-0 and the *sfr6-1* mutant by root reorientation assay.



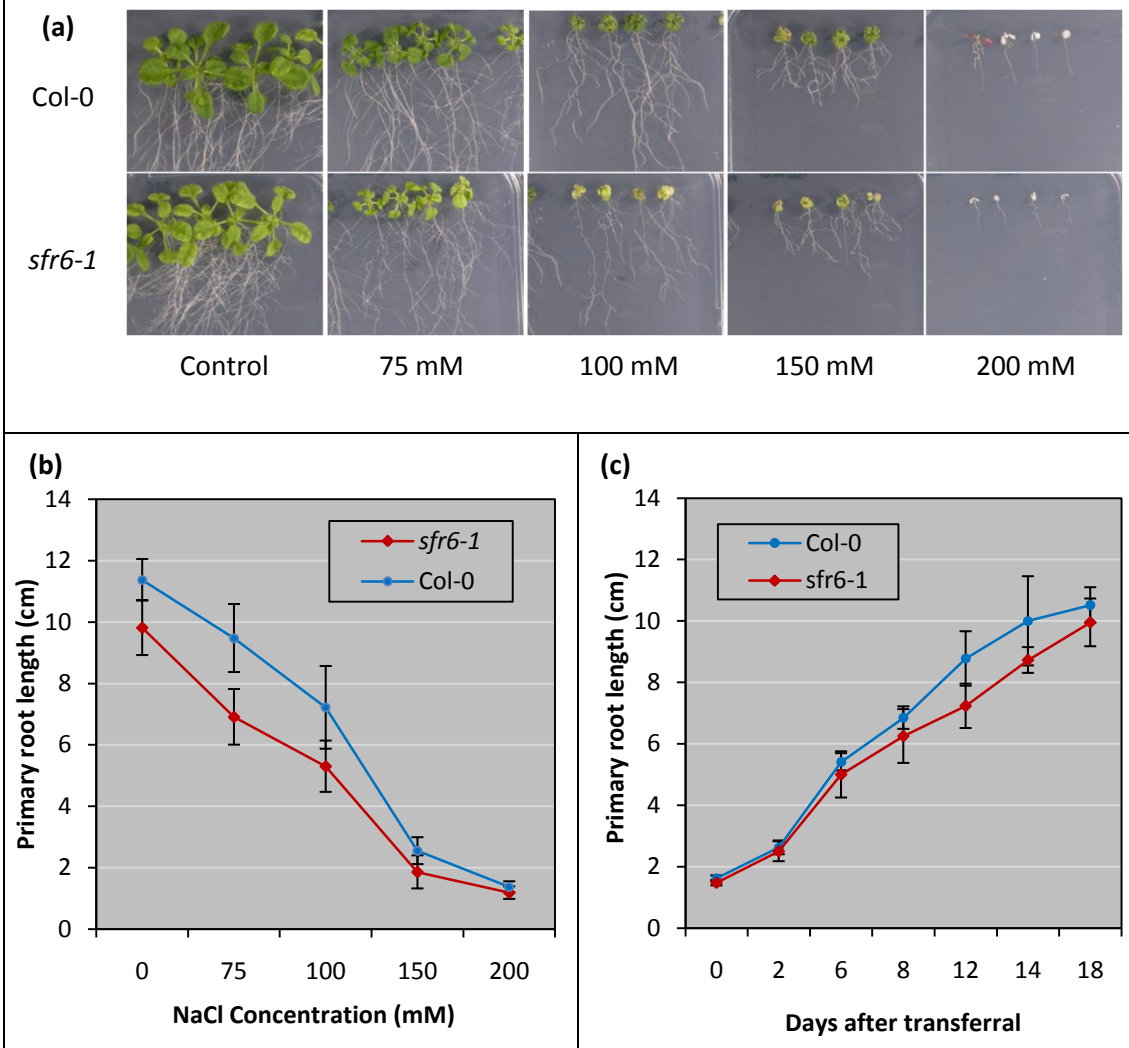
Five-day-old seedlings grown on 1×MS media were transferred to 1×MS media supplemented with different concentrations of NaCl (0, 75, 100, 150 and 200 mM) and plates were turned 180^{o} (plant upside down). Plates were placed vertically in a growth room. Twelve plants per line per treatment were used. Photographs were taken 14 days post treatment. One out of three independent experiments with similar results is shown.

4.2.2.3 Root elongation assay

As described in section 2.4.1.2.3 for osmotic stress induced by mannitol, root elongation assay was performed to examine the sensitivity of *sfr6-1* to salt. Both Col-0 and *sfr6-1* showed substantial reduction in primary root elongation in the presence of NaCl in their growth medium. s*fr6-1* showed a small but significant increase in sensitivity compared to the wild type control at 75 mM NaCl (Figure 4.7). Experiments were repeated with only 75 mM NaCl and root length measured every other day up to 18 days after transferral (Figure 4.7). However, plants did not display the significant difference observed earlier.

Figure 4.7

Comparison of salt sensitivity in Col-0 and the *sfr6-1* mutant by root elongation assay.



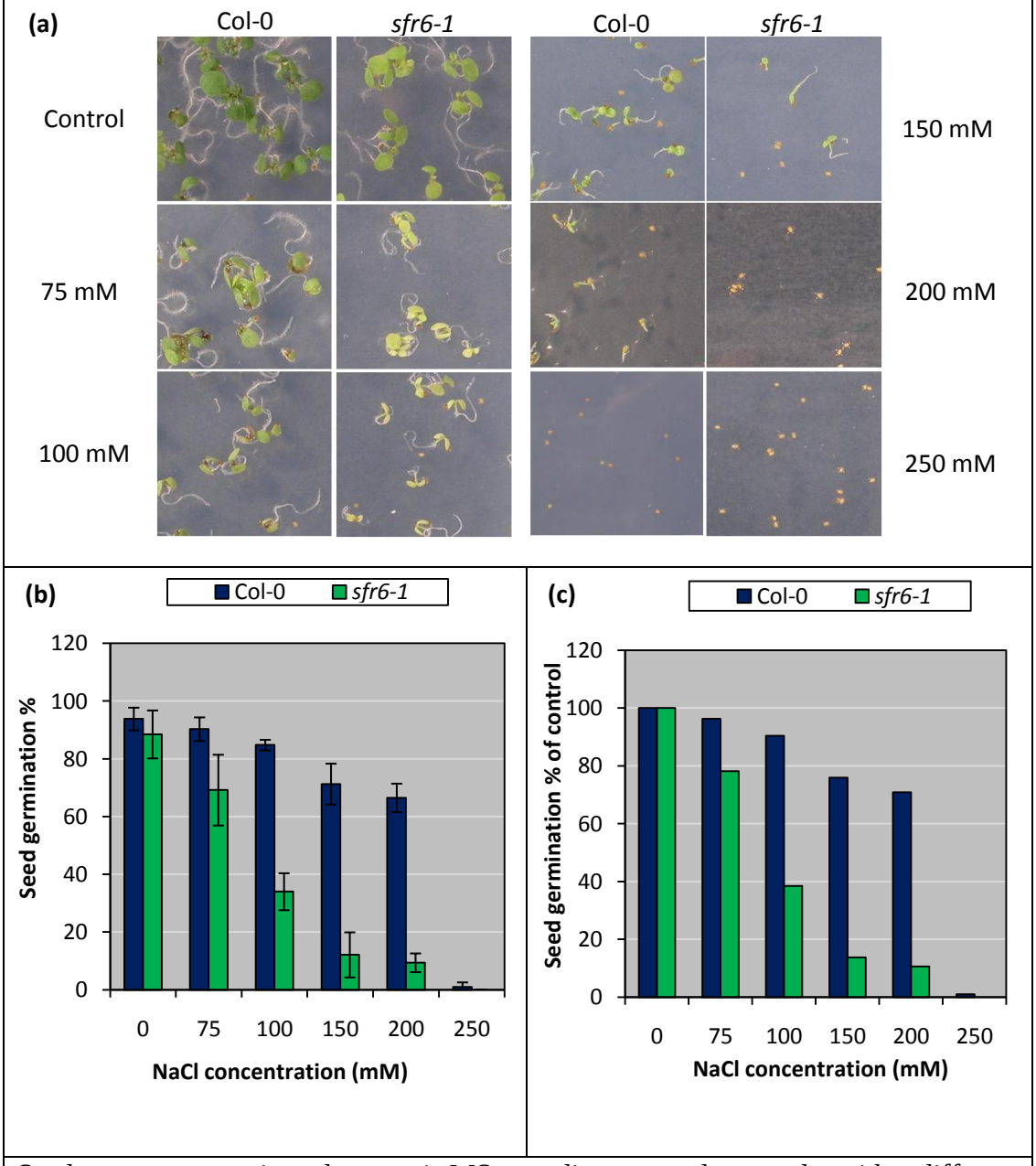
Five-day-old seedlings grown on 1×MS medium were transferred to 1×MS medium or 1×MS media supplemented with different concentrations of NaCl and plates were kept in growth room vertically. Twelve plants per line per treatment were used. (a) Growth of plants after 12 days of seedling transferral (b) Root growth of seedlings grown on NaCl supplemented plates for 12 days. (c) Root growth of seedlings grown on 1×MS agar medium supplemented with 75 mM NaCl. Root length is represented by the mean ±SD (n=12).

4.2.2.4 Seed germination assay

The effect of salt on seed germination of *sfr6-1* mutant seeds was analysed by germinating both Col-0 and *sfr6-1* seeds on 1×MS agar medium supplemented with a range of NaCl concentrations (75, 100, 150, 200, 250 mM) as described in Materials and methods section 2.4.1.2.4. Seven days after sowing, the number of seeds that had germinated were counted (seeds with visible radicals were counted as successfully germinated seeds). The germination percentage of both Col-0 and *sfr6-1* seeds reduced in a dose-dependent manner. *sfr6-1* exhibited hypersensitivity to salt compared to Col-0 at all concentrations. The germination percentage of untreated seeds of Col-0 and *sfr6-1* did not differ. As shown in Figure 4.8 the germination percentage of Col-0 was more than 70 % up to 150 mM NaCl concentration. At 200 mM germination of Col-0 seeds reduced to around 65 % and germination was completely stopped at 250 mM NaCl. 100 mM or greater NaCl concentration significantly decreased seed germination of *sfr6-1* . At 200 mM *sfr6-1* germination was reduced to 10% and was completely inhibited at 250 mM. This experiment was repeated three times and each time *sfr6-1* showed significantly reduced seed germination compared to Col-0. Therefore, to further confirm whether SFR6 has a role in seed germination under salt stress conditions, we examined the seed germination percentage of *sfr6-2* and *sfr6-3* in the presence of a range of NaCl concentrations.

Figure 4.8

Comparison of seed germination of Col-0 and *sfr6-1* mutant on NaCl supplemented agar plates.

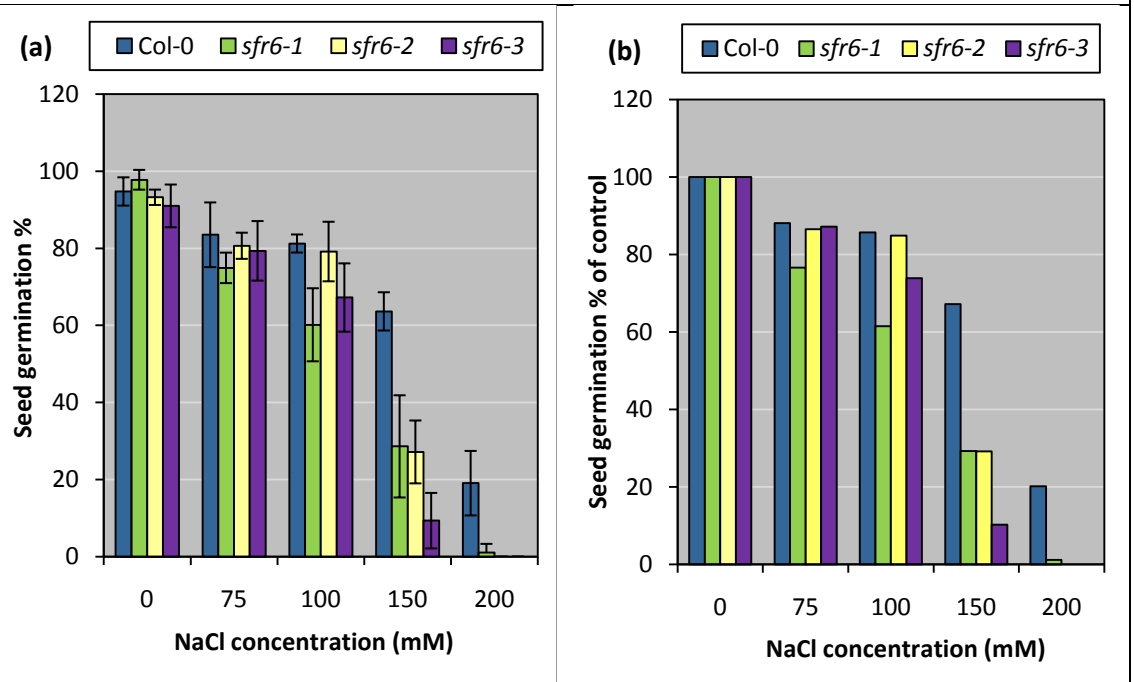


Seeds were germinated on 1×MS medium supplemented with different concentrations of NaCl. Number of seeds germinated was counted on day 7. (a) The appearance of seedlings after 7 days. (b) Seed germination percentage of Col-0 and *sfr6-1* on medium supplemented with different concentrations of NaCl for 7 days (c) Percentage of seed germination is presented as a percentage relative to the germination percentage on 1×MS medium without salt. Germination % was calculated as mean ±SD (n=3 plates-30-40 seeds per plate).

sfr6-2 and *sfr6-3* alleles displayed a high germination % up to 100 mM NaCl concentration and the germination percentage was reduced thereafter (Figure 4.9). Although *sfr6-1* still showed significant difference compare to the germination rate of Col-0, it displayed a higher germination rate in general than observed in the previous experiments. All *sfr6* alleles showed less than 50 % germination percentage in 150 mM NaCl concentration while Col-0 showed 55-70 % germination. This experiment was conducted three times and the germination of *sfr6* seeds always displayed hypersensitivity at 150 mM NaCl relative to Col-0. Because of this modest difference this was not further investigated.

Figure 4.9

Comparison of salt sensitivity on seed germination among Col-0 and three *sfr6* mutant alleles.



Seeds were germinated on 1×MS medium supplemented with different concentrations of NaCl. Number of seeds germinated was counted on day 7. (a) Seed germination percentage after 7 days on medium with different concentrations of NaCl. (b) Percentage seed germination after 7 days is presented as a percentage relative to the germination percentage on 1×MS medium. Error bars represented mean ±SD (n=3 plates, each plate consisted 30-40 seeds).

4.2.3 Responses to oxidative stress

Oxidative stresses are caused by an imbalance in the generation and removal of reactive oxygen species (ROS) which include free radicals and peroxides. These ROS are generated by normal cellular metabolism and plants have evolved mechanisms to rapidly remove them. Several environmental stresses such as UV irradiance, pathogen infection, and severe dehydration increase the production of ROS. In these cases plant defence mechanisms are unable to metabolize excess ROS and the natural balance is altered leading to oxidative stress. To examine the sensitivity of the *sfr6-1* mutant to oxidative stresses the following types of experiment were performed with both seedling and mature plants. Firstly, the sensitivity of *sfr6* to oxidative stress was analysed by spraying H_2O_2 on 3-week-old plants. Secondly, seedlings were grown on 1×MS medium supplemented with different concentrations of methyl viologen (MV; paraquat) a herbicide known to be an inducer of oxidative stress. Finally, 10 day old seedlings and detached leaves from 3-week-old plants were also immersed in solutions of different MV concentrations.

4.2.3.1 Spraying H_2O_2

To assess sensitivity to H_2O_2 three-week-old *sfr6-1* and Col-0 plants were sprayed with 100 mM H_2O_2 solution (see Material and methods section 2.4.1.3). Plants were observed at three weeks post treatment but no noticeable difference was observed between *sfr6-1* and Col-0. Spotty bleaching on leaves could be observed in both lines.

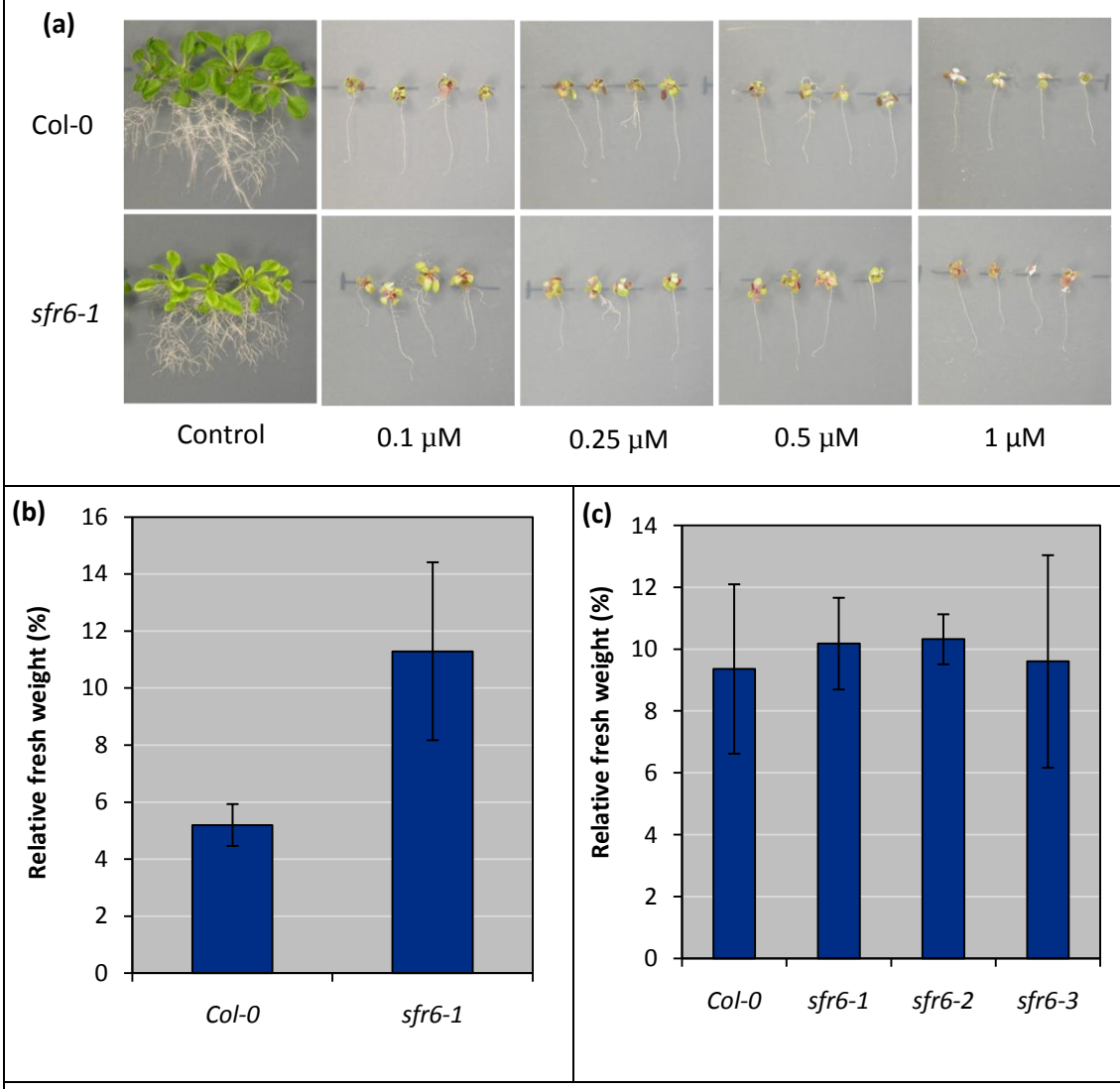
4.2.3.2 Growing on MV supplemented agar medium

MV is a well known herbicide widely used to induce oxidative stress in plants as it affects photosynthesis and under light conditions it can take electrons from photosystem 1 (PSI) and produce ROS. Therefore, the effect of MV on growth of *sfr6-1* and Col-0 seedlings using a series of MV doses was examined. Five-day-old

plants were transferred to 1×MS agar plates supplemented with different concentrations (0, 0.1, 0.25, 0.5, 1 µM) of MV (see Materials and methods section 2.4.1.3). As shown in Figure 4.10 *sfr6-1* and wild type plants were highly affected even at lowest concentrations of MV displaying severe growth inhibition. Leaves of both lines displayed chlorophyll degradation. A slight difference was observed on 0.1 µM paraquat supplemented plates *i.e. sfr6-1* displayed slightly greater biomass than did Col-0. This experiment was repeated independently two further times and similar results were obtained. To further quantify these data fresh weight of control and 0.1 µM paraquat treated plants were measured after 14 days of treatment to calculate relative weights to support visual observations. Interestingly, *sfr6-1* showed a significantly higher relative fresh weight compared to Col-0 plants (Figure 4.10). The experiment was repeated with *sfr6-1*, *sfr6-2* and *sfr6-3* but the previous results were not reproducible (Figure 4.10). Interestingly, Col-0 showed higher value for the relative fresh weight (8) than previous experiment but sfr6 mutants showed similar responses.

Figure 4.10

Growth of *sfr6* and Col-0 on agar medium supplemented with MV.



Five day old seedlings grown on 1×MS medium were transferred to 1×MS medium or 1×MS media supplemented with different concentrations of MV. Seedlings were allowed to grow on vertical plates for two weeks under long day conditions. (a) Appearance of seedlings after 2 weeks growth on MV supplemented MS medium. (b) Relative fresh weight of *sfr6-1* and Col-0 after two weeks growth on 0.1 µM MV concentration. (c) Relative fresh weight of the three *sfr6* mutant alleles and Col-0 after two weeks on 0.1 µM MV. Data presented in mean ±SD (n=12).

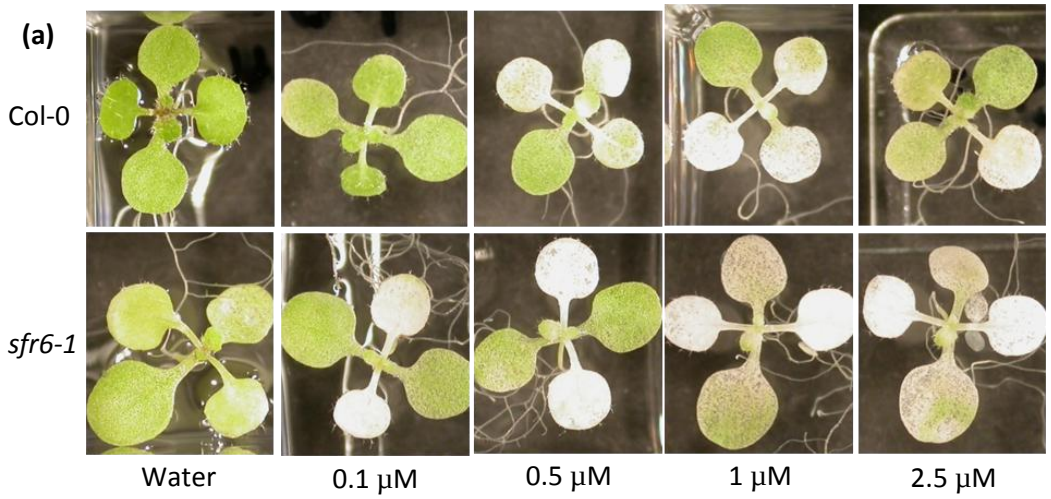
4.2.3.3 Immersion of tissues in MV solutions

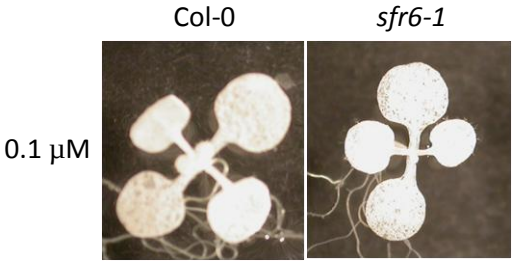
Ten-day-old *sfr6-1* and Col-0 seedlings and the leaves of 4 weeks old plants were submerged in solutions of different MV concentrations (0, 0.1, 0.5, 1 and 2.5 µM) as described in Materials and methods section 2.4.1.3. *sfr6-1* seedlings displayed modest hypersensitivity 24 h after treatment with all solutions of different MV concentrations (Figure 4.11a). However, whole seedlings were bleached in both *sfr6-1* and Col-0 backgrounds after 48 h (Figure 4.11b). The difference in bleaching of 4 weeks old leaves after 24 and 48 h were also not distinctive (Figure 4.12).

As *sfr6-1* has substantially less chlorophyll than Col-0, a chlorophyll assay was performed using leaves of 4 weeks old plants to quantify observed differences in MV sensitivity. Leaves of 4 week old plants were excised and submerged in solutions of different MV concentrations (0, 0.1, 0.25, 0.5 and 2.5 µM) and chlorophyll assays conducted after 48 h (see Materials and methods section 2.4.1.3). According to the results *sfr6-1* contains less chlorophyll than Col-0 and is more resistant to low levels of MV (Figure 4.12b). However this experiment was not repeated to confirm these results. Together these results indicate that the *sfr6* mutation does not affect the ability of plants to tolerate oxidative stresses. However further research are needed to conclude these results.

Figure 4.11

Immersion of *sfr6-1* and Col-0 seedlings in solutions of different MV concentrations

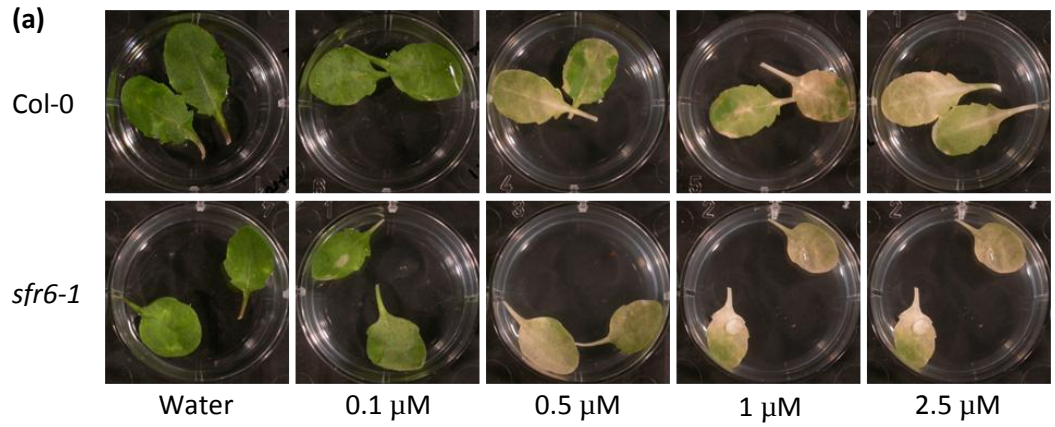


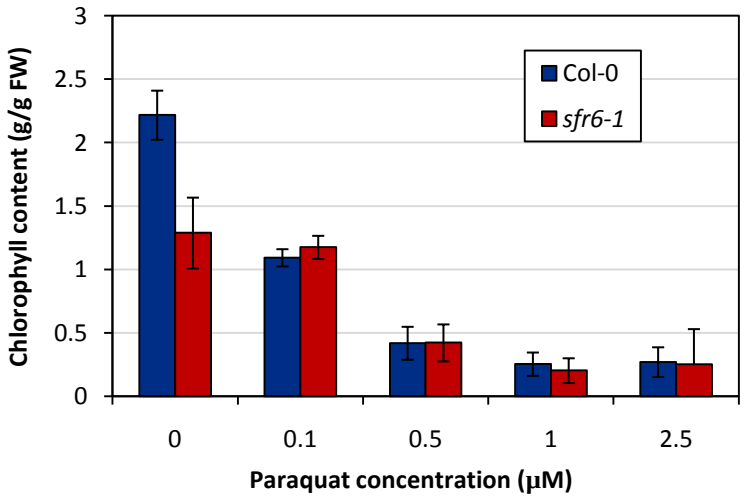


Ten-day-old seedlings grown on 1×MS agar medium were submerged in sterile wells containing 3 ml of water or 0.1, 0.5, 1 and 2.5 µM MV solutions. One seedling was transferred to each well and returned to growth chamber. Photographs were taken 24 and 48 h post treatment.

Figure 4.12

Immersion of leaves of *sfr6-1* and Col-0 seedlings in solutions of different MV concentrations





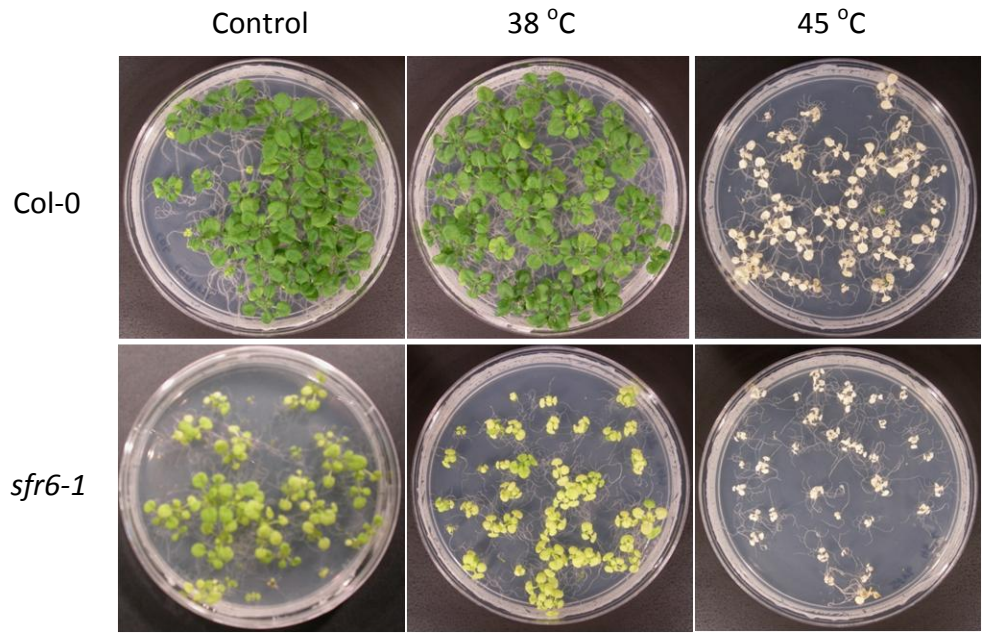
Leaves detached from 3 week old plants were submerged in sterile wells containing 3 ml of water or 0.1, 0.5, 1 and 2.5 µM MV solutions. Two leaves were transferred to each well. (a) Appearance of leaves after 48 h. (b) Measurement of chlorophyll content of 3 week old *sfr6-1* and WT leaves 48 h after submerging in solutions of different MV concentrations. Error bars indicate ±SD (n=3).

4.2.4 Heat stress

An experiment was designed to examine the susceptibility of the *sfr6-1* mutant to heat stress. Fourteen-day-old seedlings grown on 1×MS agar plates were used for as described in Materials and methods section 2.4.1.4. Survival rate after exposure to 38^{o}C for 90 min and 45^{o}C for 120 min were determined. The leaves of both *sfr6-1* and Col-0 plants exposed to 45 ^{o}C for 120 min were totally bleached and were all dead 7 days post treatment, whilst the *sfr6-1* and Col-0 plants treated with 38 ^{o}C for 90 min totally survived after 7 days (Figure: 4.13). These data indicated that the sfr6 mutation does not confer differential sensitivity to heat stress.

Figure 4.13

Sensitivity of *sfr6-1* to heat stress.



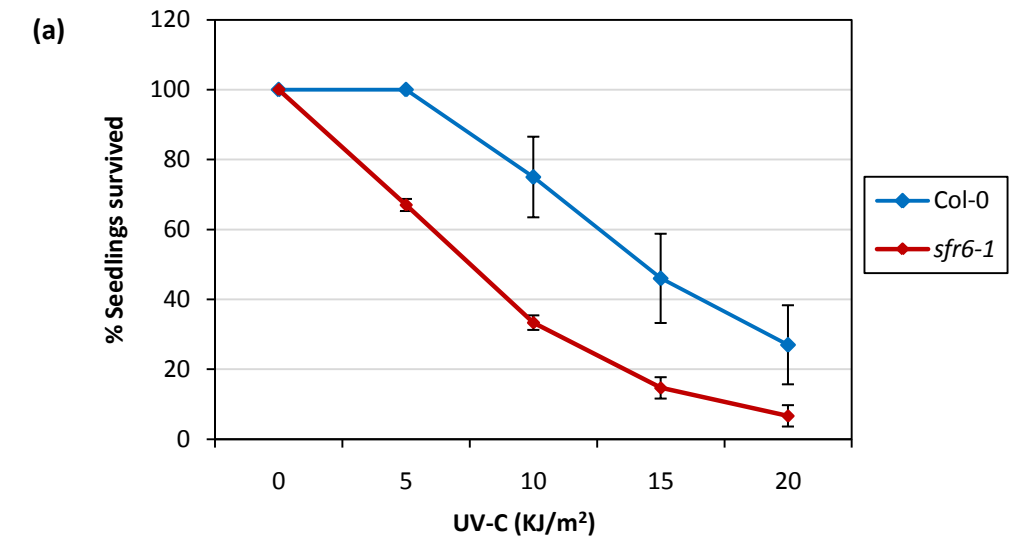
Appearance of Col-0 and *sfr6-1* seedlings 7 days after heat treatment. Fourteen-day-old seedlings grown on 1×MS agar plates were treated with two heat regimes as described in Materials and methods section 2.4.1.4. Photographs were taken 7 days post treatment. Thirty to 40 seedlings per plate and 3 plates per treatment were tested.

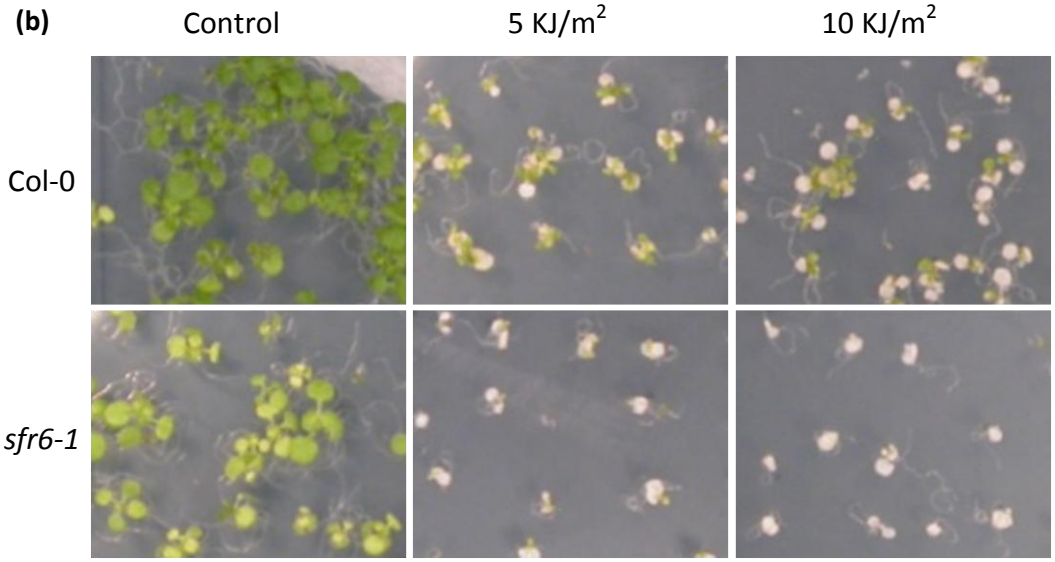
4.2.4 UV stress

To determine whether or not SFR6 plays a role in tolerating UV irradiance induced stress, 7 day old seedlings grown on 1×MS agar medium were exposed to different doses of UV-C irradiance (0, 5, 10, 15 and 15 KJ/m^2) as described in Materials and methods section 2.4.1.5. Survival of the seedlings was estimated visually (seedlings with a growing green coloured meristem were considered to have survived) 10 days post treatments. The percentage survival of seedlings reduced in a dose dependent manner in both Col-0 and *sfr6-1*. Overall *sfr6-1* plants were visibly more damaged than Col-0 plants (Figure 4.14) at all doses of UV-C irradiance. For instance wild type seedlings showed 100 % survival after ten days of treatment of 5 KJ/m^2 UV-C irradiance, whilst *sfr6-1* showed only 65-70 % survival. The reproducibility of this strong phenotype of *sfr6-1* mutant to UV-C irradiance was tested by repeating the experiment 3 times and similar results were obtained.

Figure 4.14

Sensitivity of *sfr6-1* to stress induced by UV-C irradiance.





Seven-day-old plants grown on 1×MS agar medium were exposed to different concentrations of UV-C irradiance. Twenty to 30 seedlings per plate and 3 plates per each treatment were used. (a) No. of seedlings survived were counted 10 days post treatment. (b) Photographs were taken 10 day post treatment.

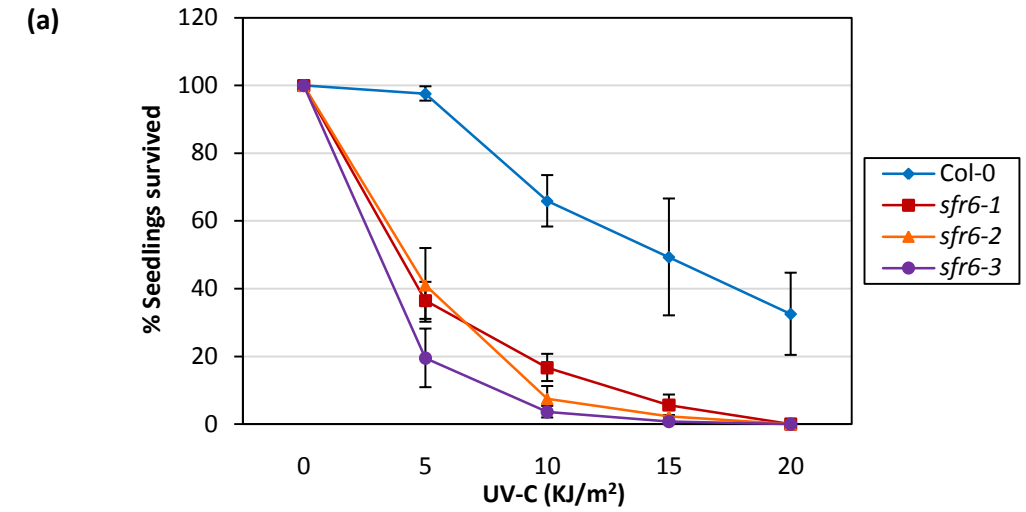
In order to verify whether the phenotype we observed could be ascribed to loss of function of the *SFR6* gene the experiment was repeated with two other *sfr6* alleles,

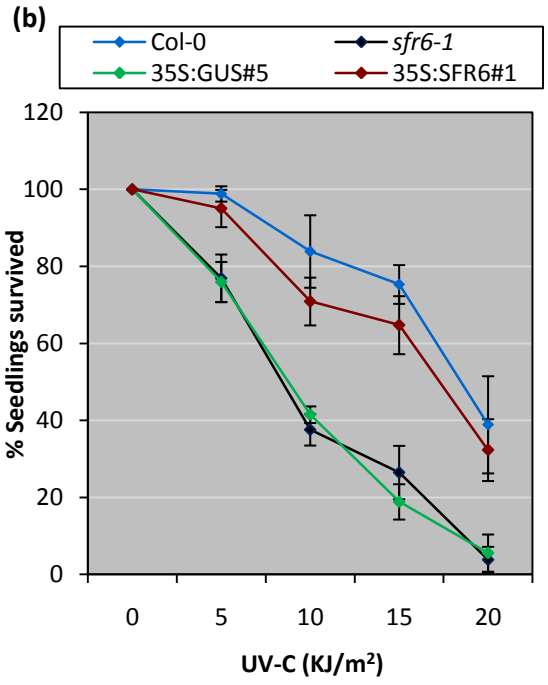
sfr6-2 and *sfr6-3*. As shown in Figure 4.15 the damage on the three *sfr6* alleles were all greater than Col-0. To clearly establish whether the UV susceptibility of *sfr6* mutants is due to the loss of function of *SFR6* gene, two lines of *35S*::*AtSFR6* (line #1 and #6) in *sfr6-1* background were tested for the tolerance to UV-C irradiance. *35S*::*AtSFR6* in *sfr6-1* lines showed reduced sensitivity to UV-C irradiance compared to the original *sfr6-1* parental line. Two lines of *35S*::*GUS* in *sfr6-1* were also used as a control line and showed sensitivity similar to original *sfr6-1* mutant.

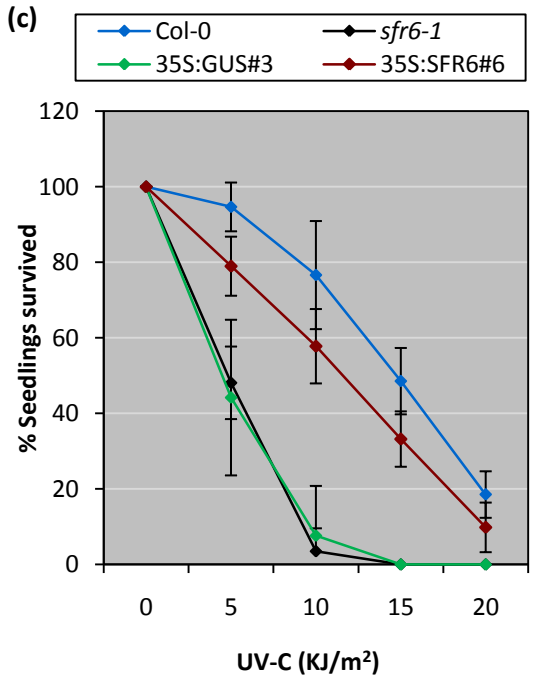
Together these experiments demonstrate that SFR6 is required for UV-C tolerance. Because of this clear and strong phenotype of *sfr6* to UV-C irradiance, UV induced gene expression in *sfr6* mutants was analysed and those results are described in Chapter 5.

Figure 4.15

Effect of UV-C irradiance on other 2 *sfr6* alleles and *sfr6-1* complemented with wild type *AtSFR6*.







Seedlings (7 days old) were treated with 0, 5, 10, 15 and 20 KJ/m^2 of UV-C irradiance. Surviving plants were counted 10 days post treatment. (a) Number of seedlings surviving for Col-0 and 3 *sfr6* alleles. This experiment was repeated three times with same results and graph shows data of one experiment as an example. (b) and (c) effect of UV-C irradiance on 2 *sfr6-1* complemented lines and 2 lines of *sfr6-1* mutant transformed with *35S*::*GUS* construct. Error bars represent ±SD (n=3 plates).

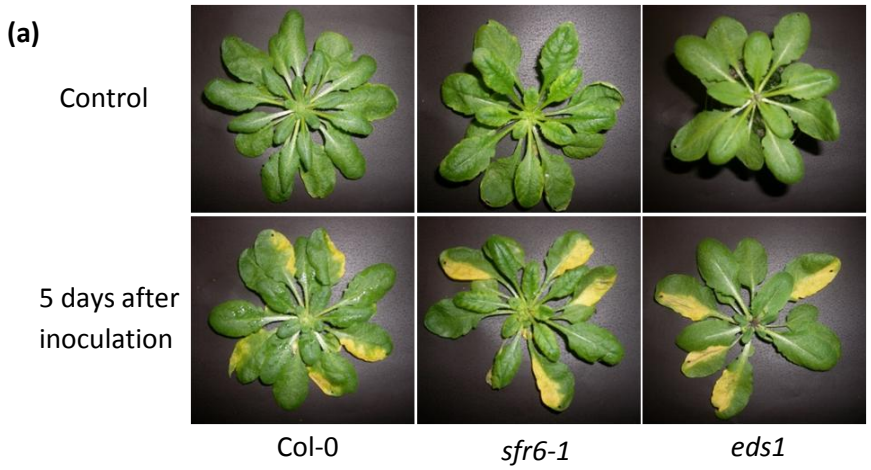
4.2.5 Biotic stress

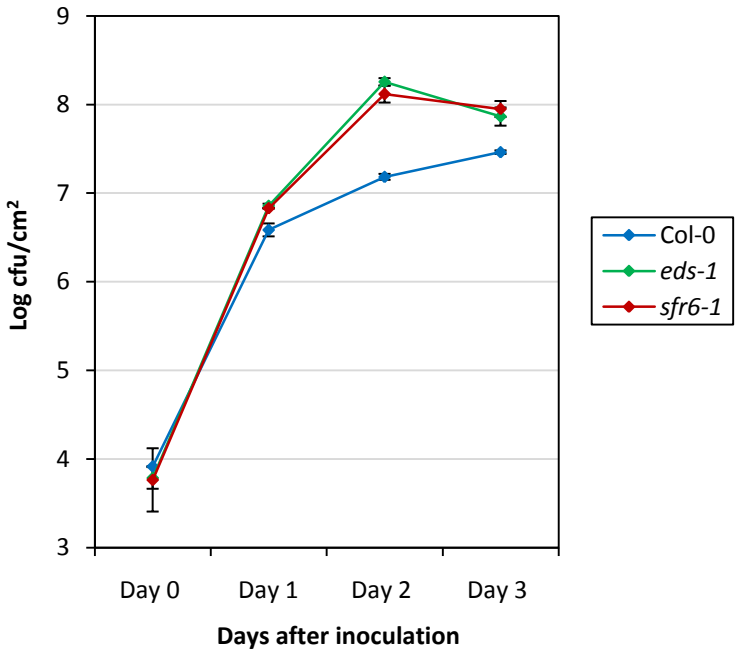
To examine the involvement of SFR6 in plant disease resistance plants were inoculated with *Pseudomonas syringae* pv tomato (Pst) DC3000, *P. syringae* DC3000-lux-avrRpm1 and *P. syringae* DC3000-lux strains (see Materials and methods section 2.4.1.7). *Pseudomonas syringae* pv tomato (Pst) is a bacterial pathogen that is virulent against a number of plant species. The artificially derived *Pseudomonas* strain DC3000 has been established as a virulent pathogen of *Arabidopsis* accession Col-0 (Whalen *et al*., 1991) and shows the production of grey brown lesions with chlorosis spreading out from the site of infection. *P. syringae* DC3000-lux-avrRpm1 and *P. syringae* DC3000-lux strains have been made by inserting the luxCDABE operon from *Photorhabdus luminescens* into the *P. syringae* chromosome under the control of a constitutive promoter (Fan *et al*., 2008). This allows bacterial growth to be measured by bioluminescence assay.

The *eds-1* mutant can be used as a positive control for *P. syringae* infection as this mutant demonstrates hypersensitivity in response to infection (Parker *et al*., 1996). Five-week-old plants of Col-0, *eds-1* and *sfr6-1* grown under 12:12 h dark light cycle was used for *Pseudomonas* infiltration. Leaves were infiltrated with *Pseudomonas* as described in Materials and methods section 2.4.1.7. Leaf bacterial number was determined at 0, 1, 2, and 3 days after inoculation as cfu (colony forming units). The *sfr6-1* mutant plants infiltrated with *P. syringae* DC3000 displayed increased disease lesions and increased bacterial numbers (Figure 4.16) compared with Col-0. The growth of bacteria was slower in Col-0 plants from day 1 after inoculation whilst the bacterial growth in *sfr6-1* and *eds-1* mutants continued at a high level.

Figure 4.16

Increased suceptibility of the *sfr6-1* mutant to *Pseudomonas syringae* DC3000.



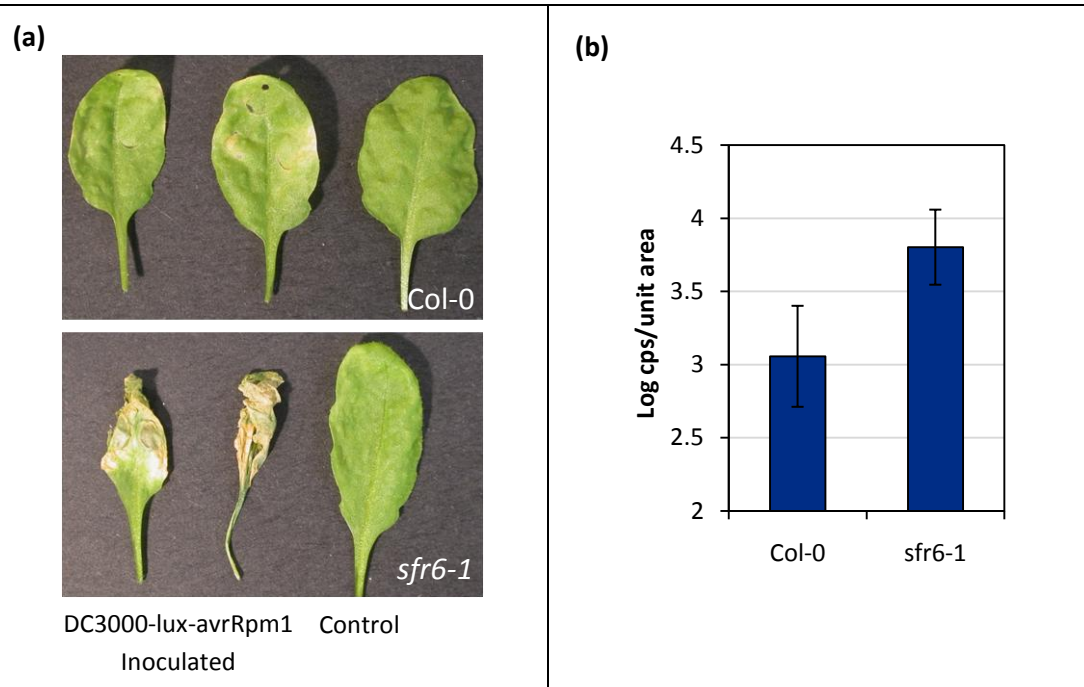


The leaves of 5-week-old plants were inoculated with *P. syringae* DC3000 virulent strain. Inoculated half of the leaves were marked by punching out a small hole. Refer to Materials and methods section 2.4.1.7 for details of the method for bacterial infection. (a) Comparison of the spread of lesions caused by *P. syringae* in *sfr6-1*, *eds1* and wild type Col-0 leaves. Photographs were taken 5 days after inoculation. (b) The growth of the strain was determined by scoring colony forming units (cfu) in plate assays using the leaf disc extraction method. For each time point leaf discs were taken from three plants. Data presented here are mean ±SD (n=3). Values plotted are $\log_{10}$ cfu. This experiment was repeated three times with similar results.

To examine the susceptibility of *sfr6-1* to the *P. syringae* avirulent strain, plants were infiltrated with *P. syringae* DC3000-lux-avrRpm1. The procedure of bacterial infection onto plants was similar to bacterial infection by *P. syringae* DC3000 strain. However, chlorosis of wild type Col-0 leaves was less compared to the leaves infected with *P. syringae* DC3000 strain (Figure 4.17a). All these results suggested that SFR6 function is required for wild type level of resistance to pathogen infection in *Arabidopsis.*

Figure 4.17

Increased suceptibility of the *sfr6-1* mutant to *Pseudomonas syringae* pv. tomato DC3000-lux-avrRpm1.



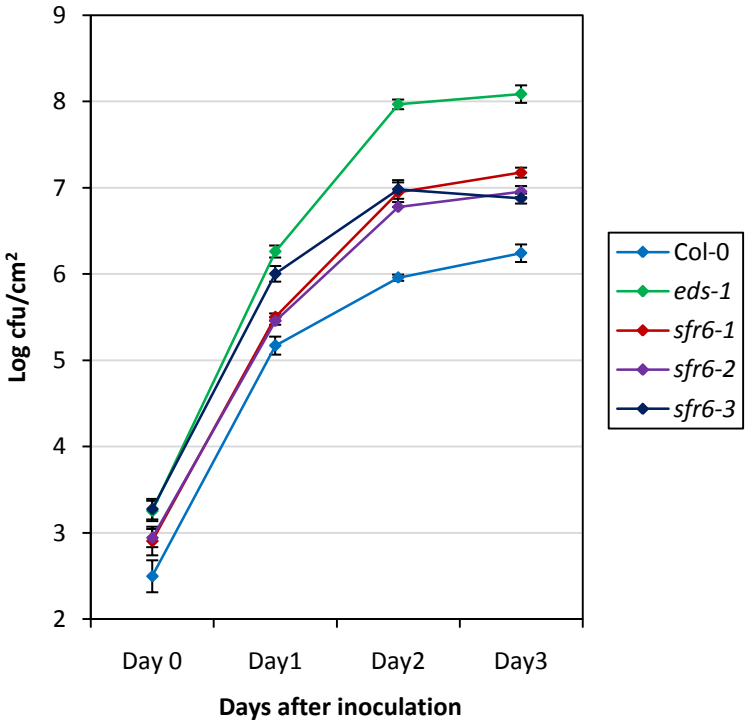
Leaves of 5-week-old plants were inoculated with *P. syringae* DC3000-lux-avrRpm1. Refer to Materials and methods section 2.4.1.7 for details of the method of bacterial infection. (a) Comparison of the spread of DC3000-lux-avrRpm1in *sfr6-1* and wild type Col-0 leaves. Photographs were taken 5 days after inoculation. (b) Bacterial growth in Col-0 and *sfr6-1* leaves were compared by bioluminescence 2 days after inoculation. Bacterial growth were determined by cps (counts per second). Three leaves from individual plants were used for this assay and error bars represent ±SD (n=3).

In order to verify that the increased susceptibility to pathogen attack was attributable to loss of SFR6 function the experiment was repeated with two additional mutant alleles. After day 2 and 3 following infection *sfr6* alleles showed significantly higher cfu values than Col-0 (Figure 4.18) indicating that SFR6 is required for resistance against *P. syringae* infection. Figure 4.18 shows the increased level of chlorotic lesions seen in all 4 mutant lines examined 5 days post inoculation when compared with Col-0 wild type.

Figure 4.18

Increased suceptibility of three *sfr6* mutant to *Pseudomonas syringae* DC3000.

(a)



(b)

Col-0 *sfr6-1* *sfr6-2* *sfr6-3* *eds-1*

Plants were inoculated with *P. syringae* DC3000 virulent strain by syringe infiltration into the leaves as described in legend for Figure 4.16. (a) The bacterial growth on Col-0, *eds-1* and three *sfr6* alleles is represented as $\log_{10}$ cfu for 0, 1, 2 and 3 days post inoculation. Means are the average of 3 replicates. Error bars are ±SD (n=3). (b) Close-up photographs of leaves of the 3 mutant alleles, Col-0 and *eds-1* 5 days post inoculation. This experiment was repeated twice with similar results.

To further demonstrate that the pathogen-sensitive phenotype was due to loss of function of the SFR6 gene the experiment was repeated with DC3000-lux strain in two complemented lines of *sfr6-1* (line #2 and 6#: lowest and highest level of over-expression of *SFR6* transcripts respectively. Refer to Figure 3.9). *sfr6-1* mutant seedlings expressing *35S*::*SFR6* showed levels of resistance similar to those of wild type (Figure 4.19a). Together these results demonstrate that SFR6 function is required for normal wild type level of tolerance to *P. syringae* infection. Significantly higher levels of bacterial growth were observed in *sfr6-1* mutant compared to Col-0. The level of bacterial growth at day 2 is presented in Figure 4.19b. Five days after inoculation severe chlorosis was observed in *sfr6-1* leaves.

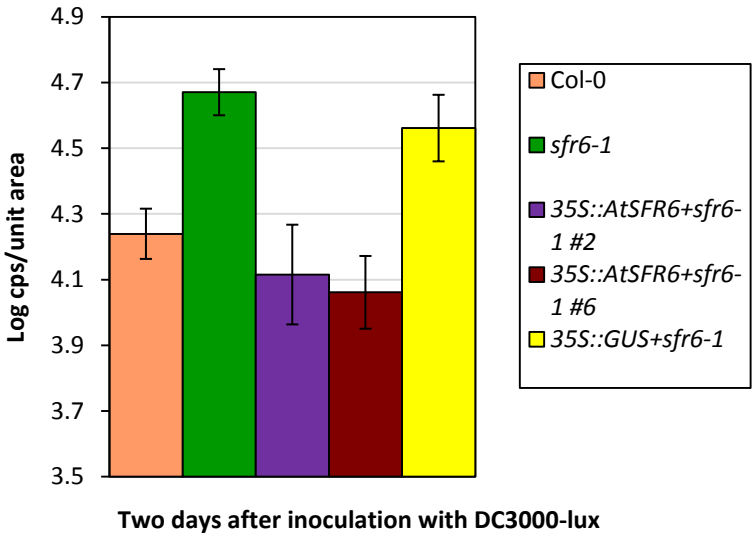
Figure 4.19

Comparision of the growth of *Pseudomonas syringae* pv. Tomato DC3000-lux in leaves of *sfr6-1* mutant plant over-expressing *35S*::*AtSFR6*.

(a)

Col-0 | *35S::AtSFR6+ sfr6-1*#2 | *35S::AtSFR6+ sfr6-1*#6 | *sfr6-1* | *35S::GUS+ sfr6-1*#6

(b)



Plants were innoculated with *P. syringae* DC3000-lux strain by syringe injection into the leaves as described in legend for Figure 4.16 and bacterial growth was compared by bioluminescence (a) appearance of leaves 5 days after inoculation. (b) Bacterial growth, 2 days after inoculation in *sfr6-1* plant overexpressing *35S::AtSFR6* (2 lines were used), Col-0, *sfr6-1* and *sfr6-1* plants over-expressing *35S::GUS*. Three leaves from individual plants were used for this assay and error bars represent ±SD (n=3).

The clear hypersensitive phenotype of *sfr6* mutants to *P. syringae* prompted examination of the expression of some pathogen-induced marker genes (*PR1*, *EDS5* and *ICS1*) after *P. syringae* DC3000 inoculation to gain further insight into the involvement of SFR6 in defence gene expression. These results are described in Chapter 5.

4.3 Discussion

4.3.1 SFR6/MED16 plays a role in protection against UV-C irradiance and pathogen infection

The survey of the responses of *sfr6* mutants to a range of environmental stresses described in this Chapter were performed to examine whether SFR6 might have a role other stress response pathways. This was performed because, as described in section 4.1, previous studies showed that SFR6 is involved in gene expression of other signalling pathways such as drought, flowering time, circadian clock function, *etc.*

The results presented in this Chapter showed that in addition to known roles, SFR6 also has a role in protecting against UV irradiance (see section 4.2.4) and pathogen infection in *Arabidopsis* (see section 4.2.5).

The hypothesis that SFR6 is involved in several signalling pathways is supported by the hypersensitivity of *sfr6* mutants to UV irradiance and pathogen infection. Identification of *At4g04920* as the *SFR6* locus places SFR6 as the MED16 subunit of the plant mediator complex (Backstrom *et al*., 2007). Several observations suggest that plant mediator controls a range of gene expression responses through multiple interactions between its subunits with specific transcription factors (Jiang and Stillman, 1992; Cerdan and Chory, 2003; Kidd *et al*., 2009) consistent with the above hypothesis. As an example the plant mediator subunit MED25 also known as PFT1 (PHYTOCHROME AND FLOWERING TIME1) was recently identified as a regulator of the jasmonate-dependent defence response in *Arabidopsis* (Kidd *et al*., 2009). *pft1* mutants showed hypersensitivity to leaf infecting necrotrophic fungal pathogens and reduced expression of jasmonate-dependent defence genes such as *PDF1.2*, *HEL*, *CHIB* etc. (Kidd *et al*., 2009). It is important to note that PFT1/MED25, like SFR6/MED16, is also involved in the control of flowering time through FT gene expression (Cerdan and Chory, 2003; Knight *et al*., 2008).

After identification of yeast mediator in 1991 (Flanagan, 1991) mediator was identified in filamentous fungi and higher eukaryotes but, until the paper by

Backstrom *et al.* (2007) was published, it had not been identified in plants. This was most probably due to the very low sequence homology between *Arabidopsis* and other eukaryotic mediator subunits (Backstrom *et al*., 2007). Therefore, the specific functions of different subunits of plant mediator have not been well studied. However, mediator of yeast and other eukaryotes has been well described. Research upon the function of yeast mediator suggested the possibility of involvement of mediator subunits in the regulation of different pleiotropic phenotypes by physical interactions with other subunits of the mediator complex. Therefore, loss of one subunit resulted in loss of function of several subunits (Jiang and Stillman, 1992; Chadick and Asturias, 2005). According to the function of yeast MED16 (SIN4), SFR6/MED16 physically interacts with other tail subunits such as MED2, MED3, MED14 and MED15. Consistent with this a mutation in yeast MED16 also shows loss of function of some of these tail subunits (Jiang and Stillman, 1992). Therefore, posible physical interations among subunits raised the possiblility that the hypersensitivity to UV irradiance and *P.syringae* infection might be a result of loss of fuction of other tail subunits in *sfr6* mutants. It will be worth addressing this hypothesis in future for a proper understanding of transcription regulation by SFR6/MED16 in plants.

No mutant has previously been reported to be sensitive to freezing, UV irradiance and pathogen infections. However, activation of the same defence pathways in *Arabidopsis* following UV irradiance and pathogen attack has been demonstrated by several research groups. For examples, Nawrath *et al*. (2002) observed the activation of SA-mediated defence gene expression following UV-C irradiation and Kilian *et al*. (2007) also observed the expression of a large number of genes annotated to be involved in pathogen signal transduction and defence response following UV-B irradiance on the Affymetrix ATH1 microarray. These authors also observed biphasic expression kinetics typical of early-induced pathogen related genes following UV-B irradiance. Therefore, these authors suggested that UV light induces an oxidative burst in plant cells similar to that of pathogens. Accumulation of ROS has also been observed in response to various abiotic stresses such as extreme temperatures, high light levels, drought etc. (reviewed in Mahalingam and Fedoroff, 2003). Moreover, Fujibe *et al*. (2004) also reported that the *Arabidopsis* mutant *rcd1*

(*radical-induced cell death1*) that is resistant to methyl viologen was also resistant to UV-B irradiation and showed increased freezing tolerance. These previous results suggest the possible convergence of the signalling pathways activated by freezing, UV light and pathogen infections. Results of this study suggest SFR6 as a possible control point in the regulation of these signalling pathways.

4.3.2 Cross-talk between low temperature, drought and salt stress

Significant progress in studying the cellular and molecular responses of plants to environmental stresses have been made in recent years (Xiong *et al*., 2002; Shinozaki *et al*., 2003). These studies show several common aspects in response to environmental stresses such as the accumulation of second messenger molecules like cytoplasmic calcium (Knight and Knight, 2001; Xiong *et al*., 2002) and reactive oxygen species (ROS) (Mahalingam and Fedoroff, 2003) and the expression of a set of common genes (Seki *et al*., 2001; Chen *et al*., 2002; Seki *et al*., 2002; Kilian *et al*., 2007). A large number of examples of cross-talk between low temperature, drought and salt stresses have been published (Xiong *et al*., 2002; Shinozaki *et al*., 2003), many more than for cross-talk between responses of low temperature, UV irradiance (Kilian *et al*., 2007) and pathogen infection. As discussed and described in section 4.1 previous studies have shown altered expression of drought induced expression of *COR* genes in the *sfr6-1* mutant (Boyce et al., 2003). Therefore, this study was extended to examine the sensitivity of *sfr6* mutants to drought and salt stresses. Various drought and salt stress induced treatments were tested on different developmental stages throughout this study (section 4.2.1 and 4.2.2). However, hypersensitivity of *sfr6-1* mutants to drought or salt stress was not observed. It is, however, important to note that the significant decrease in seed germination of *sfr6-1* on MS agar plate supplemented with different concentrations of salt is similar to the results observed by Boyce *et al*. (2003) for *sfr6-1* seed germination on mannitol supplemented agar medium. However, this difference could only be observed for the *sfr6-1* allele. For the other two alleles there was no significant difference compared to wild type control.

Microarray analysis performed by Seki *et al*. (2002) identified 40 transcription factor genes amongst the cold, drought and salt stress inducible genes suggesting the existence of many transcriptional regulatory mechanisms in the signal transduction pathways of these three stresses. Upon stress stimuli, mediator subunits may bind with specific transcription activators or repressors and with other components of the pre-initiation complex to increase or decrease stress responsive gene expression (Bjorklund and Gustafsson, 2005; Bourbon, 2008). SFR6/MED16 is only one such tail subunit of the plant mediator complex (Backstrom *et al*., 2007). So far SFR6 has been identified with its effect on CBF/DREB1 transcription factor mediated expression of a group of *COR* gene which contain the CRT/DRE promoter sequence motif. It is unlikely, therefore, that down-stream gene expression of all these transcription factors observed by Seki *et al*. (2002) are controlled directly by SFR6/MED16. Therefore, unaltered resistance to drought and salt stress of *sfr6* mutants observed in this study might be due to the regulation of most of the drought and salt stress induced transcription factors via other subunits of the plant mediator complex than SFR6/MED16.

Chapter 5

UV and pathogen induced gene expression in *sfr6* mutants

5.1 Introduction

sfr6 mutants shows reduced tolerance to UV irradiance and pathogen infection (Chapter 4). The reduced tolerance of *sfr6* mutants to freezing stress is due to reduce expression of low temperature induce genes (Knight et al., 2009). Therefore it was proposed that the reduced tolerance to UV irradiance and pathogen infection of *sfr6* mutants may be due to reduced expression of genes important in mediating tolerance to these stresses. Plant defence responses activated upon exposure to UV-C light and pathogen infection are briefly discussed below.

5.1.1 Plant defence responses

Due to their sessile nature plants have evolved multiple defence mechanisms to protect themselves against biotic and abiotic stresses. Pathogen-induced defence mechanisms are studied by a great many research groups. The number of research articles published about the different components involved in these pathways reflects the complexity of plant pathogen induced defence mechanism.

All plants contain structural defences such as wax, cuticle and dense epidermal cell layers on the surface of leaves and stems to prevent invasion of pathogens. In addition cell wall compounds such as cellulose, hemicelluloses, pectin, lignin and some chemical compounds such as antimicrobial peptides also act as effective barriers against pathogen invasion (Heath, 2000). However, when pathogens overcome these physical barriers various defence mechanisms activate including the production of ROS (Hématy *et al*., 2009). Plant susceptibility or resistance is dependent on how fast plants are able to recognize pathogen invasion and activate defence responses. If plants are unable to recognize pathogens or the activation of defence responses is too slow the plant is susceptible and the pathogen is described

as virulent. The pathogen is able to colonize and spread into the plant and cause disease. This is known as a compatible host-pathogen interaction. On the other hand if the plant can prevent the spread of pathogen infection, the plant is resistant and the plant pathogen interaction is incompatible (Jones and Dangl, 2006).

The first line of defence responses activated in susceptible plants to virulent pathogens is called basal resistance. Basal resistant initiates with the recognition of pathogen derived molecules such as pathogen associated molecular patterns (PAMPs) and effector proteins that are secreted into plant cells by pathogens (He, 1998; Thordal-Christensen, 2003). Basal resistance includes the production of peroxidases, phenolic compounds and proteins rich in proline, hydroxyproline and the activation of defence genes (antimicrobial proteins- PATHOGENESIS RELATED-PR proteins) at the site of pathogen infection (Klement *et al*., 2003). The second line of defence that activates in plants in response to pathogen infection is called gene-for-gene resistance. Gene for gene resistance activates at the site of infection by plant resistant genes (R) recognizing pathogen avirulent (*avr*) gene products (Heath, 2000). This defence mechanism is known as the hypersensitive response (HR) and restricts pathogen growth at the site of infection by rapid localized host cell death (Heath, 2000). HR can also activate systemic acquired resistance (SAR) which initiates defence responses in distal parts of the site of pathogen infection (reviewed in Durrant and Dong, 2004). SAR requires phytohomones such as SA, JA and ET which accumulate and activate a set of genes including *PR* and plant defencins to increase plant resistance against subsequent pathogen attack (reviewed in Durrant and Dong, 2004). The accumulation of SA after pathogen infection correlates with the levels of *PR* gene expression indicating the importance of SA for plant pathogen resistance (reviewed in Durrant and Dong, 2004).

5.1.2 Salicylic acid-mediated defence signalling

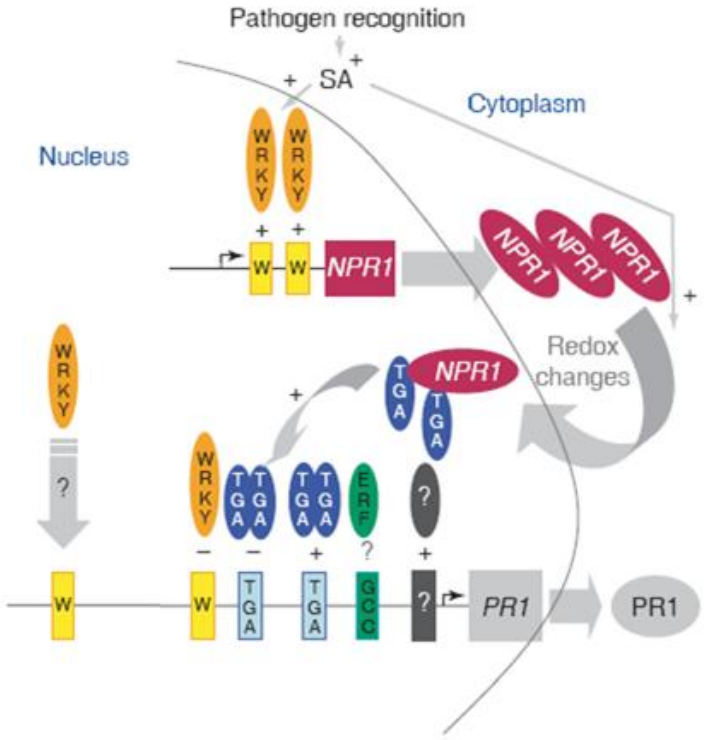
Salicylic acid is a key signalling molecule involved in the activation of plant defence responses following pathogen attacks (reviewed in Dempsey *et al*., 1999). An

increase in endogenous SA level after a pathogen infection has been identified in many plant species. Moreover *Arabidopsis* mutants defective in accumulation of SA show more sensitivity to pathogen infections due to an inability to express *PR* genes (Delaney *et al*., 1994). Furthermore, tobacco and *Arabidopsis* plants expressing bacterial *nahG* gene which encodes the SA-degrading enzyme salicylate hydroxylase enhances disease susceptibility and suppresses *PR* gene expression (Delaney *et al*. 1994).

NPR1 (*NON EXPRESSOR OF PR1*) plays a major role in SA-mediated *PR* gene expression in plant defence (Cao *et al*., 1994; Shah *et al*., 1997). In the *npr1* mutant the accumulation of SA is similar to wild type but expression of *PR* genes is very low causing increased levels of susceptibility to pathogen infections (Cao *et al*., 1997). The cloning of NPR1 using map-based approaches revealed that it is a protein with ankyrin repeats and is not a DNA binding protein (Cao *et al*., 1997). Over-expression of the *NPR1*/*NIM1* gene in *Arabidopsis* enhances resistance to a wide range of pathogen infections (Cao *et al*., 1997; Friedrich *et al*., 2001). However, Cao *et al*. (1997) observed that the over-expression of *NPR1* did not result in constitutive expression of *PR* genes. More recently, the function of *NPR1* in SAR of *Arabidopsis* and some other plants has been studied by several research groups who found interesting results regarding the activity of NPR1 (Dong, 2004). Elevated SA upon pathogen infection increase *NPR1* transcription via WRKY factors binding to two W boxes in the UTR of NPR1 (Eulgem, 2005; Yu *et al*., 2001). Mou *et al*. (2003) also found that in unstressed *Arabidopsis* cells NPR1 is present as an oligomer but with the alteration of cellular redox state associated with SAR induction due to pathogen infection a conformational change of NPR1 occurs to produce the active monomer. The NPR1 protein then translocates to the nucleus where it is involved in the SA-dependent activation of defence genes (Dong, 2004). In the nucleus NPR1 interacts with bZIP family transcription factors called TGA/OBF (ocs-element binding factor) to activate *PR* gene expression (Zhang *et al*., 1999; Despres *et al*., 2000). Several TGA transcription factors have been identified in *Arabidopsis* and some of them acts as repressors of *PR1* expression (reviewed in Eulgem, 2005). The model for SA dependent *PR1* gene expression via NPR1 according to the Eulgem *et al*. (2005) is presented in Figure 5.1.

Figure 5.1

Model for SA dependent *PR1* gene expression via NPR1 (adapted from Eulgem *et al*., 2005).



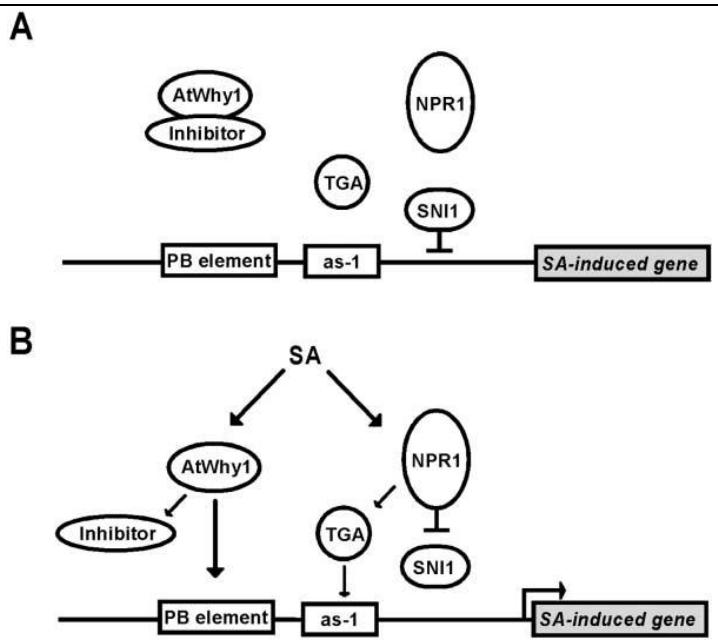
Upon pathogen infection elevated SA levels increase *NPR1* expression via WRKY factor. In resting cells NPR1 oligomers are sequestered to the cytoplasm and upon pathogen invasion biphasic changes in the cellular redox environment convert NPR1 oligomer into monomer which re-localises to the nucleolus where it activate binding of TGA factor to TGA boxes in the *PR1* promoter. The GCC box likely to interact with ERF transcription factors. The w-box and one TGA box likely to repress *PR1* expression. Additional putative W-boxes also found upstream of *PR1* promoter (adapted from Eulgem *et al*., 2005).

Although NPR1 plays a key role in *PR* gene expression in the SA-dependent plant defence signalling pathway, there was some evidence that an NPR1-independent pathway also affects *PR* gene expression. This hypothesis was proved by the observation of low levels of *PR* gene expression in pathogen-infected *NahG* plants (Delaney *et al*., 1994) and *PR1* gene expression in pathogen infected *npr1* mutant (Shah *et al*., 1997). Furthermore, the *AtWhyl* factor has also been identified as an essential component for full basal and systemic disease resistance responses

functioning in a SA-dependent but NPR1 independent, pathway (Desveaux *et al*., 2004). The single mutation of *AtWhyl* showed strong susceptibility to pathogen infections and reduced expression of *PR1* gene similar to *npr1* mutant. However, SA induced expression of *AtWhyl* in *npr1* mutant is not altered suggesting NPR1 independent manner of AtWhy1 activity (Desveaux *et al*., 2004). *AtWhyl* is a transcription factor bind to PB element (GTCAAAA/T) of the promoter of *PR* genes (Desveaux *et al*., 2004). These results suggest simultaneous action of AtWhy1 and NPR1 is required for *PR* gene expression (Figure 5.2). Meanwhile, a screen for suppressors of the *npr1-1* mutant was found SNI1 (SUPPRESSOR OF NPR1-1, INDUCIBLE 1) as negative regulator of *PR1* gene expression (Li *et al*., 1999). However, the exact mechanism of suppression of *PR1* gene expression by SNI is not known.

Figure 5.2

Proposed model for the activity of AtWhy transcription factor to activate maximal SA induced gene expression (adapted from Desveaux *et al*., 2004).



(A) Both AtWhy1 and NPR1 involve in SA-induced gene expression. In uninfected tissues AtWhy1 DNA binding activity is repressed by an inhibitor, while SNI inhibit SA-induced gene expression. (B) Upon SA treatment, both AtWhy1 and NPR1 activities are induced and removed the SNI1 repression. AtWhy1 bind to the PB element and NPR1 activate TGA factor to bind with *as-1* like elements to induce SA-dependent gene expression (adapted from Desveaux *et al*., 2004).

DTH9 (*DETACHMENT 9*) also shows NPR1-independent SAR regulation (Mayda *et al*., 2000). A *dth6* mutant exhibit increased disease susceptibility but normal *PR* gene expression and endogenous SA accumulation (Mayda *et al*., 2000). Moreover, exogenous application of SA also increase PR gene expression in *dth6* mutant similar to wild type plants but failed to compensate for its SAR phenotype (Mayda *et al*., 2000). Mayda *et al*. (2000) therefore suggested DTH6 might act upstream to the well known SA mediated signalling pathway or in a parallel pathway to SA (Mayda *et al*., 2000).

The many positive and negative regulatory genes of the SA-dependent defence response pathway have been published. *PAD4* (*PHYTOALEXIN DEFICIENT 4*) (Zhou *et al*., 1998), *EDS1* (*ENHANCED DISEASE SUCEPTIBILITY 1*) (Falk *et al*., 1999) and *SID2*/*ICS1* (*SALICYLIC ACID INDUCTION DEFICIENT 2*/ *ISOCHORISMATE SYNTHASE* 1) (Wildermuth *et al*., 2001) play major roles as positive regulators upstream of SA production. Another disease-susceptible mutant, *eds5*, shows less accumulation of *EDS1* and *PAD4* indicating that *EDS5* acts upstream of *EDS1* and *PAD4* (Nawrath *et al*., 2002). *CAD1* (*CONSTITUTIVELY ACTIVATED CELL DEATH 1*) (Tsutsui *et al*., 2008), *ACD6* (*ACCELERATED CELL DEATH6*) (Rate *et al*., 1999) and *CPR1* (*CONSTITUTIVE EXPRESSION OF PR*) (Bowling *et al*., 1994) were identified as genes negatively regulating the SA-mediated signalling pathway as these mutants constitutively express *PR* genes independent of *NPR1*.

The biosynthesis of SA has been shown to occur mainly via two pathways. Lee *et al*. (1995) showed that SA accumulates through phenylpropanoid pathway. Later the isochorismate pathway of SA synthesis was identified (Wildermuth *et al*., 2001). In this pathway chorismate is converted into isochorismate via ISOCHORISMATE SYNTHESE1 (ICS1) then isochorismate is used to generate SA. The *sid2* mutant has mutation in the *ICS1* gene and shows very low levels of SA accumulation after pathogen infections indicating that the isochorismate pathway is the main pathway of SA synthesis in plants (Wildermuth *et al*., 2001). However the *sid2* mutant is not completely defective in accumulation of SA. Therefore, Wildermuth *et al*. (2001) suggested there is another pathway involved in SA synthesis independent to the

ICS1. They have suggested that it could be via another *ICS* gene or thought the other SA biosynthesis pathway like the phenylpropanoid pathway.

The accumulation of both free SA and SA-O-ß-glucoside (SAG) has been investigated after pathogen infection. The enzyme SA glucosyltransferase (SAGT) convert free SA in to SAG (Dean *et al*., 2005). The *Arabidopsis* mutant *pbs3* which is more susceptible to both virulent and avirulent *P. syringae* DC3000 exhibits significantly higher level of free SA but significantly lower level of SAG (Nobuta *et al*., 2007). *PBS3* has been identified as a member of GH3 family of acyl adenylase forming enzyme (Nobuta *et al*., 2007). Nobuta *et al*. (2007) suggested that PBF3 plays a major role in SA-acid mediated plant defence responses acting upstream of SA, directly on SA or on a competitive inhibitor of SA.

5.1.3 Jasmonic-acid mediated plant defence

In 1996 Penninckx *et al*. reported an SA and NPR1-independent pathogen-resistance pathway which is characterized by induction of the plant defensin *PDF1.2*. *PDF1.2* was induced after treatment with methyl jasmonate but not with SA. They also observed accumulation of JA both in pathogen-infected and non infected leaves of the same plant. The induction of plant defensin was unaffected in *nahG*, *npr1* or *cpr1* *Arabidopsis* mutants but *PDF1.2* expression was blocked in the *ein2* ethylene and *coi1* (*coronatine insensitive1*) JA insensitive mutants, (Penninckx *et al*., 1996). They reported that induction of plant defensin upon pathogen infection is independent of SA but requires components of the JA response. More recently, several lines of evidence showed that JA is as important a signalling molecule in plant defences as SA. Jasmonic acid is synthesized by oxidation of α-linolenic acid by lipoxigenase enzymes via the octadecenoid pathway and the JA-mediated defence pathway is activated in *Arabidopsis* in response to infection with necrotrophic pathogens (reviewed in Vasyukova and Ozeretskovskaya, 2009). *PDF1.2* is used as a marker gene of the JA mediated defence pathway. *Arabidopsis* mutants insensitive to JA treatments such as *coi1* and *jar1* (*jasmonic acid response1*) are more susceptible to

pathogens (reviewed in Lorenzo and Solano, 2005) indicating the importance of plant defensin and other JA mediated defence genes for defence against pathogens.

5.1.4 Cross-talk between SA and JA-mediated plant defence signalling pathways

The cross-talk of the two major plant defence signalling pathways, SA and JA, has been described in many research articles. Both synergistic and antagonistic relationships between these two pathways have been discussed. However, most of the studies have identified antagonistic cross-talk between these two signalling pathways. Most *Arabidopsis* mutants unable to accumulate SA or downstream components of the SA response defence pathway demonstrate enhanced expression of JA-responsive genes. As an example Spoel *et al*. (2003) showed that the *npr1* mutant of *Arabidopsis* exhibits increased expression of JA-induced marker genes such as *LOX2*, *PDF1.2* and *VSP*. Few studies have identified synergistic interactions between these two signalling pathways. One example is a microarray analysis of SA and JA induced gene expression which identified expression of 55 genes in common between these two pathways (Schenk *et al*., 2000). *NPR1* has been suggested to be involved in SA-mediated suppression of JA signalling (Spoel *et al*., 2003). In contrast, there is evidence that SA-induced *NPR1* present in the cytosol is involved in suppressing JA-response gene expression (Lorenzo and Solano, 2005). However, constitutive expression of both PR1 and *PDF1.2* observed in *Arabidopsis* mutants *cpr5*, *cpr6* (*constitutive PR gene expression 5 and 6*) and *acd2* (*accelerated cell death2*) suggest that these two signalling pathways are connected probably in early steps of the pathways (reviewed in Pieterse and van Loon, 1999).

5.1.5 Programmed cell death pathway mediated by metacaspase

Apoptosis is the well-described programmed cell death (PCD) pathway in animal systems. In animal systems when cells have been damaged caspases are activated and initiate cell death by degrading essential proteins. Although several caspase-like activities have been detected in plant systems, the orthologues of animal caspase

sequences have not been detected (Rotari *et al*., 2005). However, with the identification of paracaspases and metacaspases distant relatives of animal caspase-like proteases (Uren *et al*., 2000), the involvement of plant metacaspases as plant caspase like activities has been suggested. Plant metacaspases are sub-divided into two groups as type I and type II based on their structure and sequence: type I have an N-terminal prodomain which is not present in type II (He *et al*., 2008). The *Arabidopsis* genome has 9 metacaspse genes but all of these are not expected to be involved in the regulation of PCD (He *et al*., 2008). It has been observed that the expression of metacaspases increases upon UV-C irradiance and pathogen infection. As an example, the rapid increase of tomato metacaspase1 (*LeMCA1*) mRNA levels was observed upon *Botrytis cinerea* infection (Hoeberichts *et al*., 2003) and increased levels of *Arabidopsis* metacaspse 8 (*AtMCA8*) has been observed upon oxidative stress induced by UV-C, H_2O_2 and methyl viologen (He *et al*., 2008). However, the function of plant metacaspases has not yet been revealed.

5.1.6 Aim

The aim of this Chapter is to examine;

- Whether SFR6 regulate UV-C and pathogen-induced gene expression.

This was tested by examining the expression of genes (*PR1*, *EDS5*, *ICS1*, *NPR1*, *PDF1.1* and *MCA8*) induced after UV-C irradiance and inoculation with *P. syringae*. In 1994 Yalpani *et al*. showed accumulation of SA and *PR* genes after exposure to UV-C light and ozone fumigation in tobacco leaves. The induction of SA-dependent defence responses after pathogen infection also widely studied (see section 5.1.2). More recently, in 2002 Nawrath *et al*. showed that UV-C light is a good inducer of SA biosynthesis in *Arabidopsis*. *EDS5* (Nawrath *et al*., 2002) and *ICS1* (Wildermuth *et al*., 2001) genes were identified as essential components of the SA-mediated defence pathway. Several research groups demonstrated that accumulation of SA is essential for *PR1* gene expression and therefore, *PR1* is used as a marker gene to analyse SA dependent defence gene expression. *MCAs* (*MTACASPASEs*) are

caspase-like proteases identified in plants as functional homologues of animal caspases which are key components of animal apoptosis (Uren *et al*., 2000). Nine *MCA* genes were identified in *Arabidopsis* and *AtMCA8* has been identified as a gene strongly induced by UV-C light and H_2O_2 (He *et al*., 2008). *NPR1* is the central regulator of plant defence responses including SAR and SA/JA cross-talk (Durrant and Dong, 2004). The plant defence pathway induced by JA is also as important as the SA-induced defence pathway (reviewed in Vasyukova and Ozeretskovskaya, 2009). Antagonistic and synergistic cross-talk between these two pathways has also been documented (see section 5.1.4). Therefore, in this study I assayed the transcript levels of *MCA8*, *ICS1*, *EDS5*, *PR1*, *NPR1 and PDF1.1* after UV-C irradiance and *P.syringae* infiltration.

5.2 Results

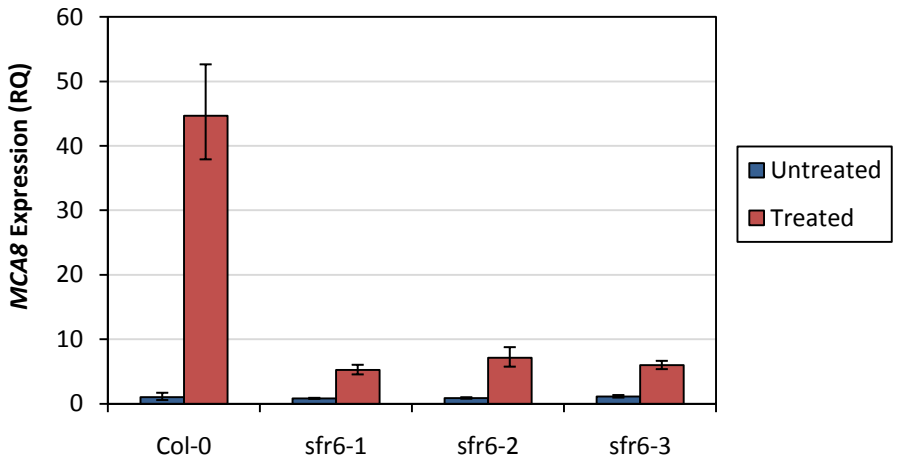
5.2.1 Expression of stress-inducible genes in response to UV-C irradiance

Seven-day-old seedlings grown on agar plates were used to measure transcript levels of selected genes. Samples were collected 1, 6 and 24 hours after UV treatment (5 KJ/m^2; 254 nm). These time points were selected as peak expression time points according to the published data on gene expression assays of wild type plants after exposure to UV-C light and personal communication with R. Ingle (University of Cape Town, South Africa). Samples collected 1 h post treatment was used to analyse the expression of *AtMCA8* (He *et al*., 2008) while samples collected 6 h after post treatment were used for *EDS5* (Nawrath *et al*., 2002) and *ICS1* (R. Ingle pers. and Zimmermann *et al*., 2004) gene expression and 24 h post treatment for *PR1* gene expression. (Nawrath *et al*., 2002). Control samples were also collected without UV-C irradiance in parallel at these time points. Measurement of gene expression was performed using qRT-PCR and expression level of gene of interest was normalized to expression of *At4G26410*, a gene with stable expression levels that are not altered by UV treatment.

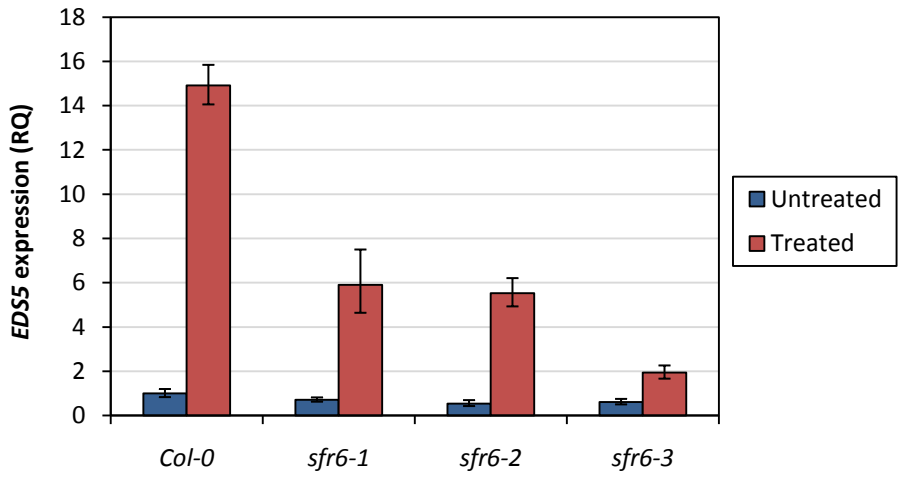
Figure 5.3

Analysis of UV-C induced gene expression in *sfr6* mutants

(a)



(b)



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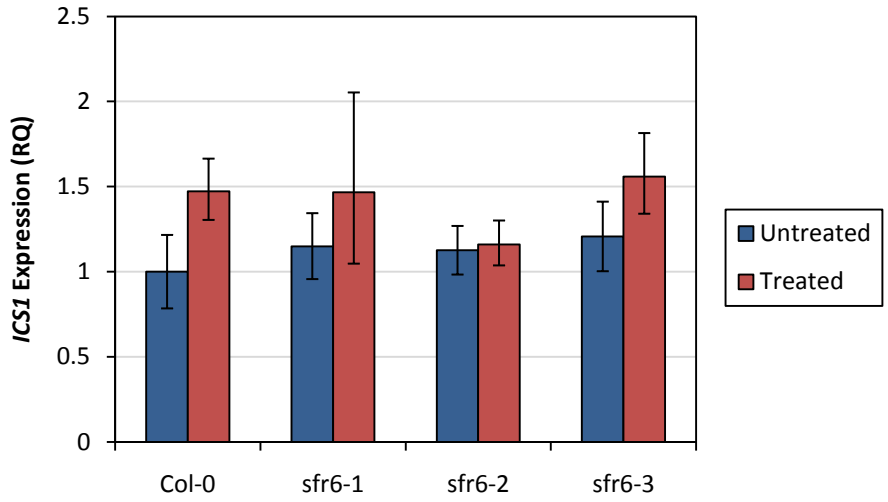
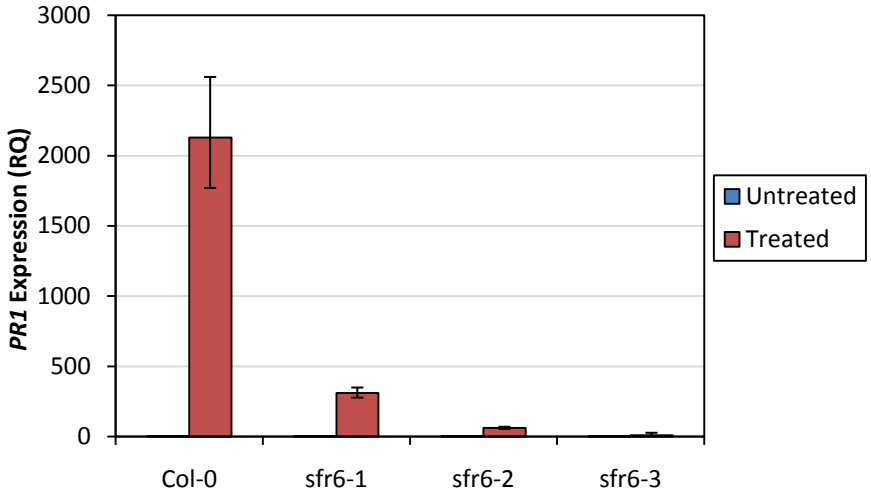
(c)



(d)



UV-C induced gene expression was measured in Col-0, *sfr6-1*, *sfr6-2* and *sfr6-3* in 7-day-old seedlings treated with 5 kJ/m^2 UV-C irradiance or control treatment. Expression of (a) *MCA8* (b) *EDS5* (c) *ICS1* and (d) *PR1* is shown after normalization to *At4G26410* using qRT-PCR. Graphs show the relative quantification (RQ) values relative to the expression levels of un-stressed Col-0 plants. Un-stressed samples from each line were tested as controls. Each value is the mean of three technical replicates. The experiment was repeated three times with similar results. This example shows the data of one replicate. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test.

UV-inducible expression of *MCA8, EDS5* and *PR1* lower in all *sfr6* mutant alleles compared to wild type Col-0 (Figure 5.3). However, in contrast to these results the expression of *ICS1* was not significantly increased after UV treatment both in mutant alleles and Col-0 and there was no significant difference between *sfr6* alleles and wild type plants both in treated and untreated conditions (Figure 5.3c). These results indicate that although *ICS1* is a key component of SA induced defence pathway (Wildermuth *et al*., 2001) it is not induced by UV-C irradiance. The expression of the *MCA8* gene in the mutant was 40-fold lower than in Col-0 (Figure 5.3a) indicating that SFR6 might be involved in PCD regulated by caspase-like proteases. As *EDS5* is involved upstream of SA synthesis (Nawrath *et al*., 2002) the reduced accumulation of transcripts of this gene suggest that *sfr6* mutants may synthesise less SA and explaining why the marker gene *PR1* shows comparatively reduced transcript levels after UV-C irradiance (Figure 5.3d). Together these results indicate that *SFR*6 is essential for accumulation of *PR1* after UV-C irradiance.

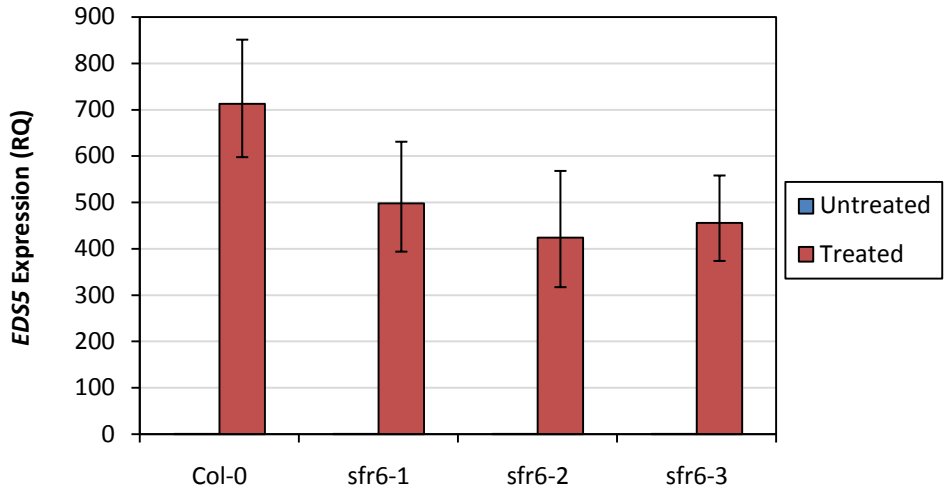
5.2.2 Expression of stress inducible genes in response to virulent *P. syringae* inoculation of *sfr6* mutants.

Five-week-old plants were used for Pseudomonas infiltration and samples were collected at time points chosen to visualize the peak of expression of each gene based on previous data (Nawrath *et al*., 2002) and personal communication with R. Ingle (University of Cape Town, South Africa). As expected all three genes were strongly induced after infection (Figure 5.4). The expression of *EDS5* 24 h post inoculation was significantly lower in *sfr6-2* and *sfr6-3* mutants than in Col-0 (Figure 5.4a). However, even though the *sfr6-1* allele showed lower average expression of *EDS5* levels than Col-0, there was no statistically significant difference. All three *sfr6* alleles showed significantly reduced levels of *PR1* gene expression 48 h post inoculation. On the other hand levels of *ICS1* gene expression were induced approximately 100 fold in *sfr6* alleles after infection with *P. syringae* and there was no statistically significant difference compare to wild type plants. *ICS1* and *EDS5* act upstream of SA induction after *Pseudomonas* inoculation (Wildermuth *et al*., 2001; Nawrath *et al*., 2002).

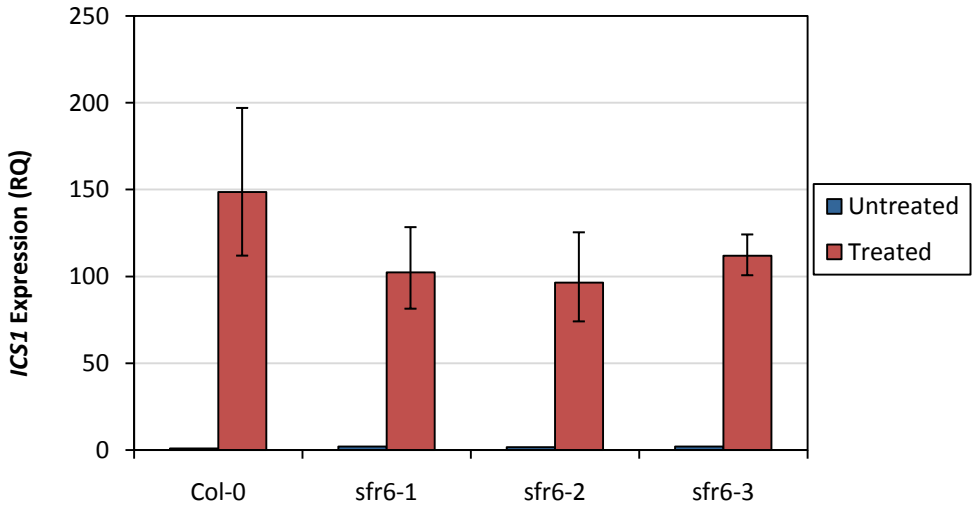
Figure 5.4

Analysis of *EDS5*, *ICS1* and *PR1* expression in *sfr6* alleles in response to *Pst DC3000* infection.

(a)



(b)



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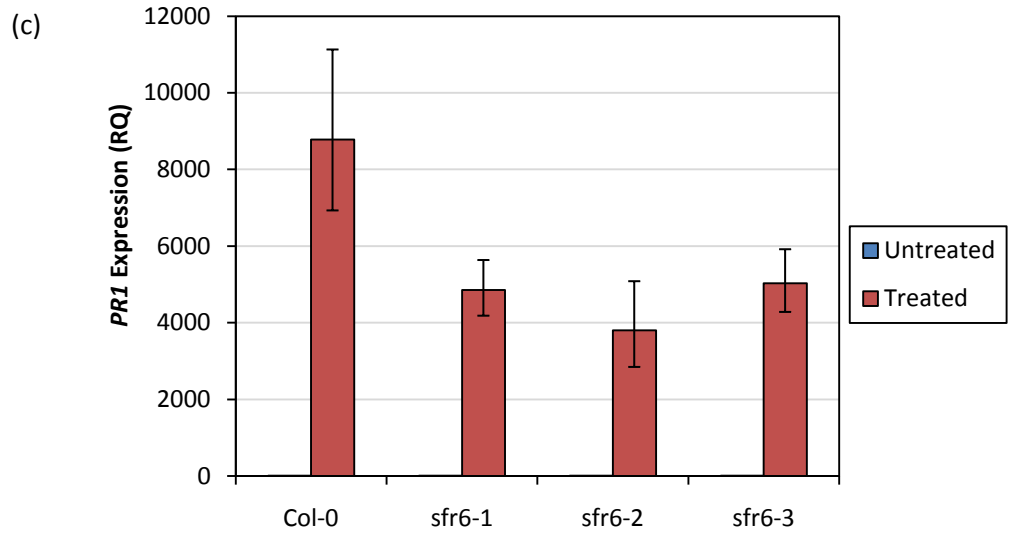


The expression of (a) *EDS5* (b) *ICS1* (c) *PR1* is shown after inoculation with *P. syringae*. The leaves of 5-week old plants were inoculated with *P.syringae DC3000* virulent strain as described in Materials and methods section 2.4.1.7. *ß-TUBULIN*4 gene was used as endogenous control. Leaves innoculated only with sterilized water were tested as controls. Graphs show the relative quantification (RQ) values relative to the expression levels of un-stressed (water control) Col-0 plants. Un-stressed samples from each line were tested as controls. Each value is the mean of three technical replicates and is representative of 3 biological repeat experiments. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's *t* test.

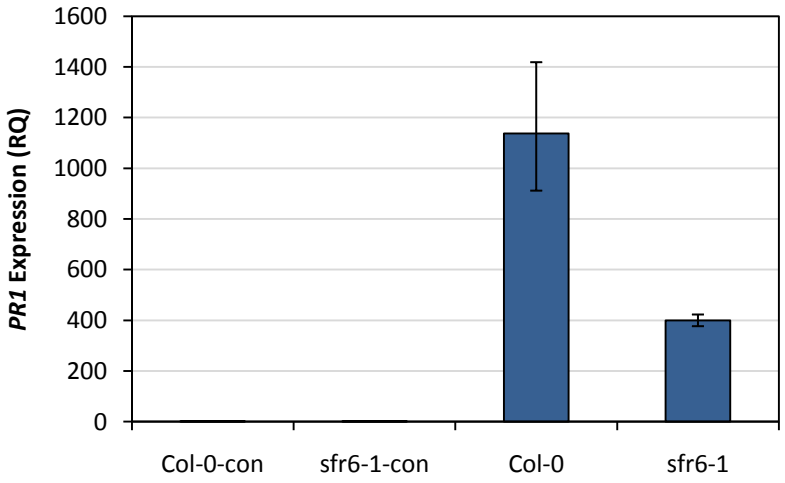
5.2.3 Expression of *PR1* after exogenous application of SA to the *sfr6-1* mutant

According to the above results the reduction of *PR1* gene expression after UV-C treatment and *Pseudomonas* DC3000 inoculation was drastic compare to the reduction of *ICS1* and *EDS5* expression (Figures 5.3 and 5.4). Therefore, the reduced expression of *PR1* in *sfr6* mutants after UV-C irradiance and *Pseudomona*s inoculation might be a result of reduced accumulation of SA. Therefore, to test this assumption *PR1* expression was analyzed after endogenous application of SA. Three-

week-old *sfr6-1* and Col-0 plants were dipped in 1 mM sodium salicylate solution (Materials method section 2.4.2.5) and samples were collected 24 h post treatment. As presented in Figure 5.5 the accumulation of *PR1* was drastically reduced in the *sfr6-1* mutant compared to Col-0 suggesting *SFR6* might affects gene expression downstream of SA accumulation. This experiment was repeated twice with similar results.

Figure 5.5

PR1 expression after exogenous application of Sodium salicylate.



Three-week-old plants were dipped in 1 mM Sodium salisylate solution with 0.01 % Silwet and leaves were harvested 24 h post treatment for RNA extraction. Graph shows the relative quantification (RQ) values for *PR1* gene expression relative to un-stressed Col-0 plants. *At4G26410* was used as endogenous control. Leaves harvested from plants dipped only in 0.01 % Silwet were tested as controls. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

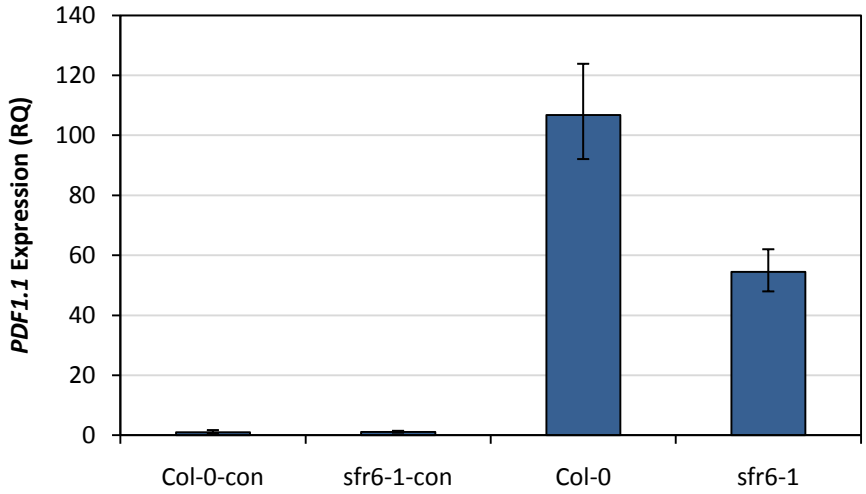
5.2.4 Expression of stress induced genes controlled by JA in the *sfr6-1* mutant

To examine the effect of *sfr6* mutation on expression of plant defensins the expression of *PDF1.1* was tested in the *sfr6-1* mutant after UV treatment, *Pseudomonas* DC3000 infection and exogenous application of 100 µM methyl jasmonate. *PDF1.1* was expressed after UV-C irradiance and exogenous application of JA (Figure 5.6a and 5.6b). However, *PDF1.1* was not expressed 48 h after *Pseudomonas* infection (Figure 5.6c). Significantly reduced expression of *PDF1.1* was observed in *sfr6-1* mutant after UV stress and exogenous application of JA.

Figure 5.6

Analysis of stress induced *PDF1.1* gene expression in the *sfr6-1* mutant.

(a) UV irradiance



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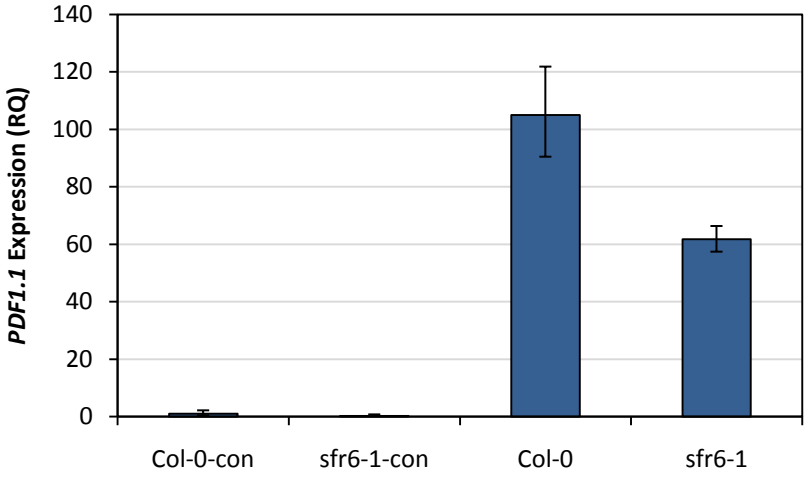
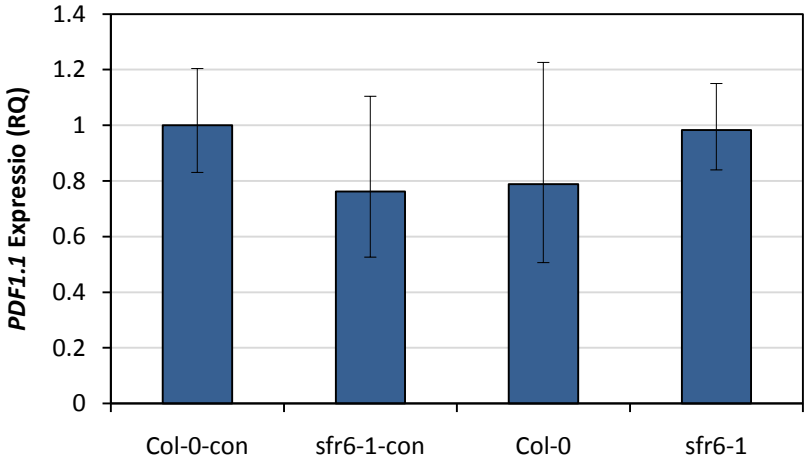
(b) Exogenous application of methyl jasmonate



(c) *P. syringae* infiltration



PDF1.1 gene expression (a) 24 h after 5 kJ/m2 UV-C irradiance (b) 24 h after exogenous application of 100 µM methyl jasmonate (c) 48 h after inoculation of *P. syringae* DC3000. Graph shows the relative quantification (RQ) values for *PDF1.1* gene expression relative to un-stressed Col-0 plants. *PEX4* was used as endogenous control for data presented in Figure 5.4b and 5.4c, *At4g26410* used to normalize *PDF1.1* levels after UV irradiance. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

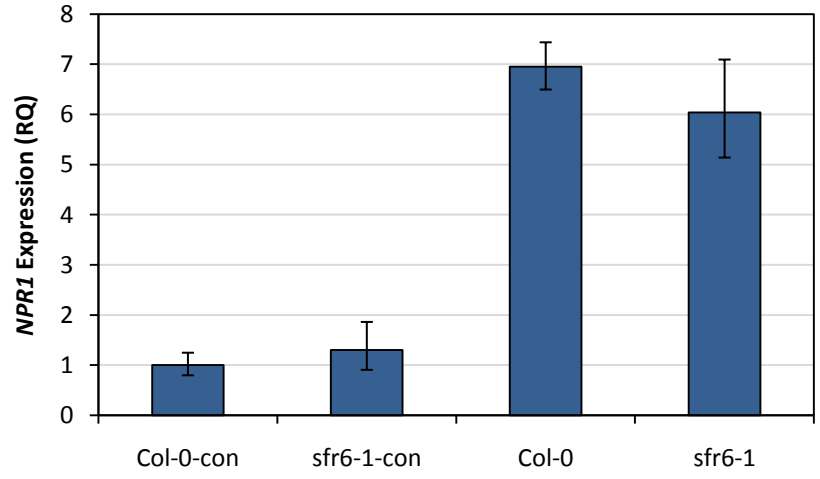
5.2.5 Expression of *NPR1* after treatment with SA, UV-C treatment and inoculation with *P. syringae* in the *sfr6-1* mutant

NPR1 is the central regulator of plant defence responses including SAR and SA/JA cross-talk (Durrant and Dong, 2004). *PR1* expression after pathogen infection in the *npr1* mutant was very low (Shah *et al*., 1997). Therefore, the expression of *NPR1* in the *sfr6-1* mutant was examined using qRT-PCR.

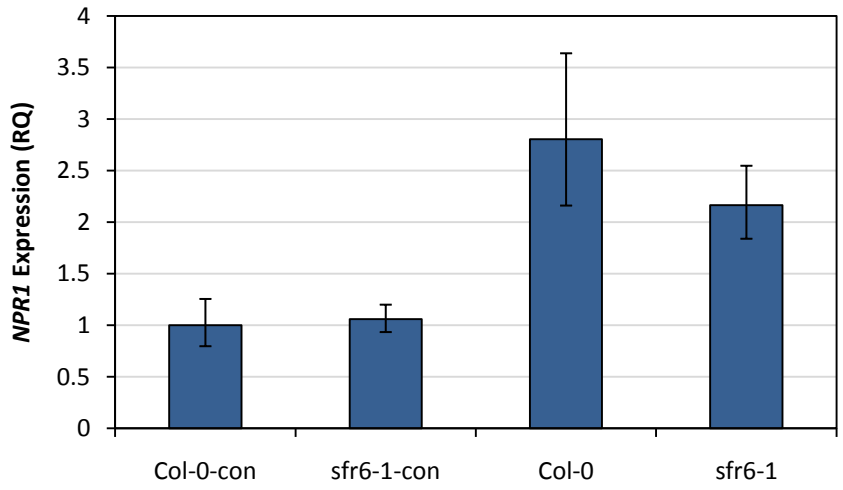
Figure 5.7

Analysis of *NPR1* expression after infection of *P. syringae* DC3000 strain, UV-C irradiance and exogenous application of SA in *sfr6-1* mutant.

(a) *P. syringae* infiltration



(b) Endogenous application of sodium salicylate



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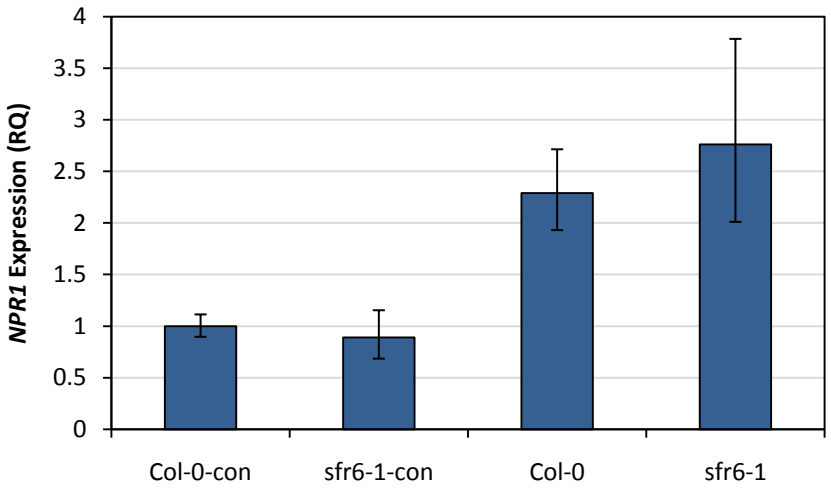
(c) UV-C irradiance



NPR1 gene expression (a) 48 h after inoculation of *P. syringae* DC3000 (b) 24 h after exogenous application of 1 mM sodium salicilate (c) 24 h after 5 kJ/m2 UV-C irradiance. Graph shows the relative quantification (RQ) values for *NPR1* gene expression relative to un-stressed Col-0 plants. *PEX4* was used as endogenous control. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

NPR1 expression increased in response to all of these treatments both in *sfr6-1* and wild type Col-0 plants and there was no significant difference between *sfr6-1* and Col-0. However, only 2-3 fold induction of *NPR1* was observed after exogenous application of SA and UV-C treatment relative to non-stressed wild type plants. In contrast 6-7 fold induction of *NPR1* observed after *P. syringae* infection.

5.3 Discussion

5.3.1 SFR6/MED16 involved in defence-related gene expression

5.3.1.1 SA-mediated defence gene expression

In Chapter 4 it was shown that the *sfr6* mutants were more susceptible to UV-C irradiance and *P. syringae* infection than wild type plants. The results presented in this chapter show that the expression of UV-C irradiance-induced genes *PR1* and *EDS5* were significantly reduced in *sfr6* mutants compared to Col-0 wild type control. Similarly, I observed significantly reduced expression of *PR1* and *EDS5* in *sfr6* mutants compared to Col-0 subjected to *P. syringae* DC3000 infiltration. *PR1* and *EDS5* have previously been identified as UV-C and pathogen induced genes and also as components of SA mediated defence pathway (Nawrath *et al*., 2002). These results indicate that *SFR6* has a role in SA mediated defence related gene expression. *ICS1* was also identified as an important gene involved in SA biosynthesis upon pathogen infection (Wildermuth *et al*., 2001). However, it is interesting to note that the expression of *ICS1* upon pathogen infection and UV-C irradiance behaved unlike the other genes tested. The expression of *ICS1* increased after pathogen infection but there was no significant difference between mutants and wild type plants. These results suggest *ICS1* might be activated by different transcription factors than those that affect *PR1* and *EDS5* expression. SFR6, as a part of mediator, may not have an effect on the transcription factor which is involved in *ICS1* expression. These results are similar to defective expression of cold induced genes in *sfr6-1* mutant; all cold induced genes were not mis-regulated in the mutant only *COR* genes controlled by CBF/DRE transcription factor that contain the CRT/DRE motif were affected (Knight *et al*., 1999; Knight *et al*., 2009).

Upon UV-C radiation increased expression of *ICS1* was not observed in this study. Furthermore, there is no documented evidence that *ICS1* is induced upon UV-C irradiance. However, increased transcript levels have been observed in response to 15 kJ/m^2 UV-B irradiance (GENEINVESTIGATOR database; Zimmermann *et al*., 2004). Therefore, these results suggested that although *ICS1* is an important component in *PR* gene expression via SA dependent defence pathway (Wildermuth

et al., 2001) it is not induced by UV-C irradiance or it may need higher concentrations of UV-C irradiance (5 kJ/m^2 was used for this study).

5.3.1.2 Accumulation of SA is probably not affected in the sfr6-1 mutant

ICS1 has an important role in SA dependent plant defence responses (Wildermuth *et al*., 2001). Upon pathogen infection the co-accumulation of *ICS1*, SA and *PR1* has been observed by Wildermouth *et al*. (2001). They also observed that SA is synthesized from chorismate by means of *ICS* and this pathway is required for local responses and SAR. The strong correlation of SA accumulation and the expression of *PR1* upon UV-C irradiance have also been shown by Nawrath *et al*. (2002). However, the synthesis of SA upon pathogen infection does not occur only via the isochorismate pathway as *ics1*/*sid2* mutants of *Arabidopsis* showed certain level of SA accumulation (Wildermuth *et al*., 2001). Therefore, it has been suggested that SA accumulates via several pathways such as the phenylalanine pathway which produce SA precursors. Therefore, it is possible that the reduced accumulation of *PR1* transcript in *sfr6* mutants after pathogen infection and UV-C irradiance might be due to reduced accumulation of SA via other pathways independent of isochorismate. The un-induced levels of *ICS1* and significant reduction of *PR1* accumulation upon UV-C irradiance is further support for this hypothesis. Moreover, SA has been identified as a regulator of flowering time in non stressed plants and it has also been shown that SA accelerates the transition from vegetative to reproductive phase in stressed plants (Martinez *et al*., 2004). Most of the SA deficient *Arabidopsis* genotypes such as *nahG*, *eds5*, *sid2* show a late flowering phenotype and the exogenous application of SA accelerated flowering of these mutants except *nahG* which is unable to accumulate SA (Martinez *et al*., 2004). Further supporting this hypothesis is the fact that *sfr6* mutants also show a late flowering phenotype (Figure 3.13 and Knight *et al*., 2008). However, exogenous application of sodium salicylate did not complement the reduced levels of *PR1* accumulation in *sfr6-1* mutants (Figure 5.3). These results suggest the accumulation of SA might not be altered in *sfr6-1* mutants and SFR6 might not be involved in SA accumulation upon pathogen infection and UV-C irradiance.

5.3.1.3 Stress-induced expression of NPR1

NPR1 has been identified as a factor playing a pivotal role in *PR* gene expression induced by SA dependent defence response downstream of SA accumulation in *Arabidopsis* (Cao *et al*., 1994; Shah *et al*., 1997). As exogenous application of SA did not compensate reduced *PR1* levels of *sfr6-1* mutant it was hypothesized that the reduced level of *PR1* expression might be due to defective expression of *NPR1*. However, the expression of *NPR1* was not altered by UV-C, SA or *P. syringae* infiltration in the *sfr6-1* mutant compared to wild type Col-0. These results suggest that SFR6/MED16 acts downstream of *NPR1* transcription and these results are similar to the defective expression of *COR* genes in *sfr6* mutants with normal levels of expression of CBF transcription factors which regulate *COR* gene expression upon low temperature exposure (Knight *et al*., 1999; Knight *et al*., 2009). However, *NPR1* is not a DNA binding protein but it facilitates nuclear localization of some DNA binding transcription factors involved in *PR* gene expression (Cao *et al*., 1997; Dong, 2001; Eulgem, 2005). Elevated SA upon biotic and abiotic stresses changes the redox state of *NPR1* and reduces its condition to a monomeric form able to relocalize to the nucleus (Mou *et al*., 2003) where it activates TGA transcription factors to bind with TGA boxes in the promoter of *PR* genes (reviewed in Eulgem, 2005). The physical interaction of NPR1 with TGA factors is essential to activate the DNA binding ability of TGA factor and subsequent *PR* gene expression (reviewed in Eulgem, 2005). According to these results SFR6 might recruit TGA transcription factors to the promoter of *PR* genes. Knockout mutant analysis showed at least three TGAs; TGA2, TGA5 and TGA6 evolved redundant and essential function for positive regulation of SAR induction (Zhang *et al*., 2003). Therefore, it will be interesting to examine the effect of *sfr6* on TGAs expression to understand the molecular mechanism of SFR6/MED16 in stress induced gene transcription.

5.3.1.4 SFR6/MED16 is involved in plant defensin expression

There was a significant reduction in *PDF1.1* expression after UV-C irradiance and exogenous application of methyl jasmonate in the *sfr6-1* mutant compare to Col-0

suggesting that SFR6 might have a role in expression of plant defensin *via* the JA-mediated defence pathway. However, *PDF1.1* expression was not increased when tested at 48 h after pathogen infection. Most studies have identified antagonistic relationships between SA and JA mediated defence gene expression. As an example the *npr1* mutant of *Arabidopsis* shows increased accumulation of JA mediated defence genes such as *LOX2, PDF1.2* and *VSP* indicating *NPR1* mediates cross-talk between these two pathways (Spoel *et al*., 2003). Therefore, the un-induced levels of *PDF1.1* after pathogen infection might be due to the suppression of JA mediated defence gene expression by increased expression of SA mediated defence gene. Similar to the data presented here, Mackerness *et al*. (1999) observed that UV-B irradiance increased expression of *PR1* and *PDF1.2*. Recently another mediator subunit, PFT1/MED25, was identified as a regulator of the JA mediated defence response in *Arabidopsis* (Kidd *et al*., 2009). The expression of *PR1* was also reduced in the *pft1* mutant of *Arabidopsis* suggesting involvement of the same mediator subunit in mediating different plant defence responses. Moreover, these results suggest involvement of different mediator subunits to fine tune plant defence response induced by UV-C irradiance and pathogen infections.

5.3.1.5 Metacaspase induced programme cell death pathway

Nine metacaspase genes have been identified in the *Arabidopsis* genome and only *AtMCA8* has been observed to be strongly up-regulated by oxidative stress caused by UV-C, H_2O_2 and methyl viologen (He *et al*., 2008). The data presented here also show strong up-regulation of *AtMC8* upon UV-C irradiance in wild type Col-0 plants but significantly reduced expression was observed in *sfr6* mutants (Figure 5.3a). The molecular pathway lead to activation of *AtMCA8* gene has not yet been identified (He *et al*., 2008). However, our data suggest that SFR6 plays a role in the regulation of *AtMC8* expression upon UV-C irradiance.

Chapter 6

Functional analysis of rice *SFR6* (*OsSFR6*)

6.1 Introduction

The CBF cold response pathway is an important component of cold acclimation in *Arabidopsis* (Thomashow, 2001). SFR6 was identified as an essential component for cold-induced expression of the *CBF* regulon (Knight *et al*., 1999; Boyce *et al*., 2003). The ultimate objective of studying stress response genes is decreasing yield losses by improving stress tolerance of agricultural crops. Therefore, when studying the role of stress response genes, it is important to identify and characterize orthologues of particular genes in different species, especially in commercially important crops.

Rice is a model monocot plant due to its small genome size (~430 Mb) and diploid nature (2n=24) (Ito *et al*., 2006). Rice is also an important staple crop for over half of the world's population, and is closely related to other important cereal crops such as wheat, sorghum and barley (www.forestry.sciencesapplied.com/scope-of-rice-genome-project/). Rice is sensitive to a variety of abiotic stresses, including salinity, drought, submersion and cold (chilling temperatures) (Lafitte *et al*., 2004). Therefore, studying molecular mechanisms of stress tolerance in rice is important not only to improve stress tolerance of rice but also to improve stress tolerance of monocot crops in general.

Great effort has been made to understand the mechanisms involved in rice abiotic stress responses and manipulating these traits to produce stress-tolerant rice varieties. Although rice is a chilling sensitive plant it has functional *DREB1*/*CBF* genes (Dubouzet *et al*., 2003; Ito *et al*., 2006). In 2003 Dubouzet *et al*. isolated five *DREB* type homologues: OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D and OsDREB2A. Expression of these genes in *Arabidopsis* induced over-expression of target stress inducible genes and increased stress tolerance of *Arabidopsis* (Dubouzet *et al*., 2003). Similarly, over-expression of *OsDREB1* genes and *AtDREB1* in rice

also induced target stress inducible genes and resulted in stress tolerance (Ito *et al*., 2006). Therefore, the fundamental question which arises when considering these results is whether rice has a functional *SFR6* gene regulating its CBF activity.

Therefore, the specific considered hypothesis in this chapter is,

- Rice does have a functional orthologue of *AtSFR6*.

The work performed to test this hypothesis is described in this Chapter under three main sections.

Section 1: Functional complementation of *sfr6-1* mutant by AtSFR6 homologue from rice.
Section 2: Generation of OsSFR6 knockdown rice.
Section 3: The effect of OsSFR6 on promoters containing CRT/DRE elements.

6.2 Results

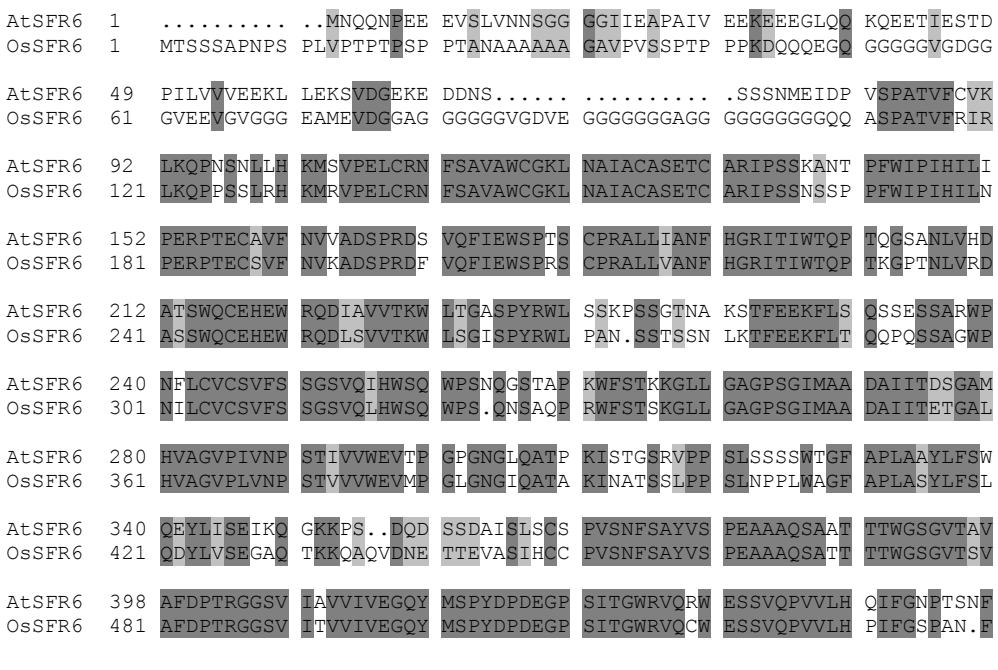
6.2.1 Identification of a SFR6 homolog in rice

A rice genomic database (http://www.tigr.org) was searched to find rice protein sequence homologues to the AtSFR6 protein sequence. Figure 6.1 shows the only protein (Os10g35560) that is anything like AtSFR6 in the rice genome and will be referred to as OsSFR6. There is quite a lot of homology between the sequences, but there are also areas where the homology is not so good. However, approximately 72 % of the protein sequence identity was observed between AtSFR6 and OsSFR6 protein sequences (Figure 6.1).

Figure 6.1

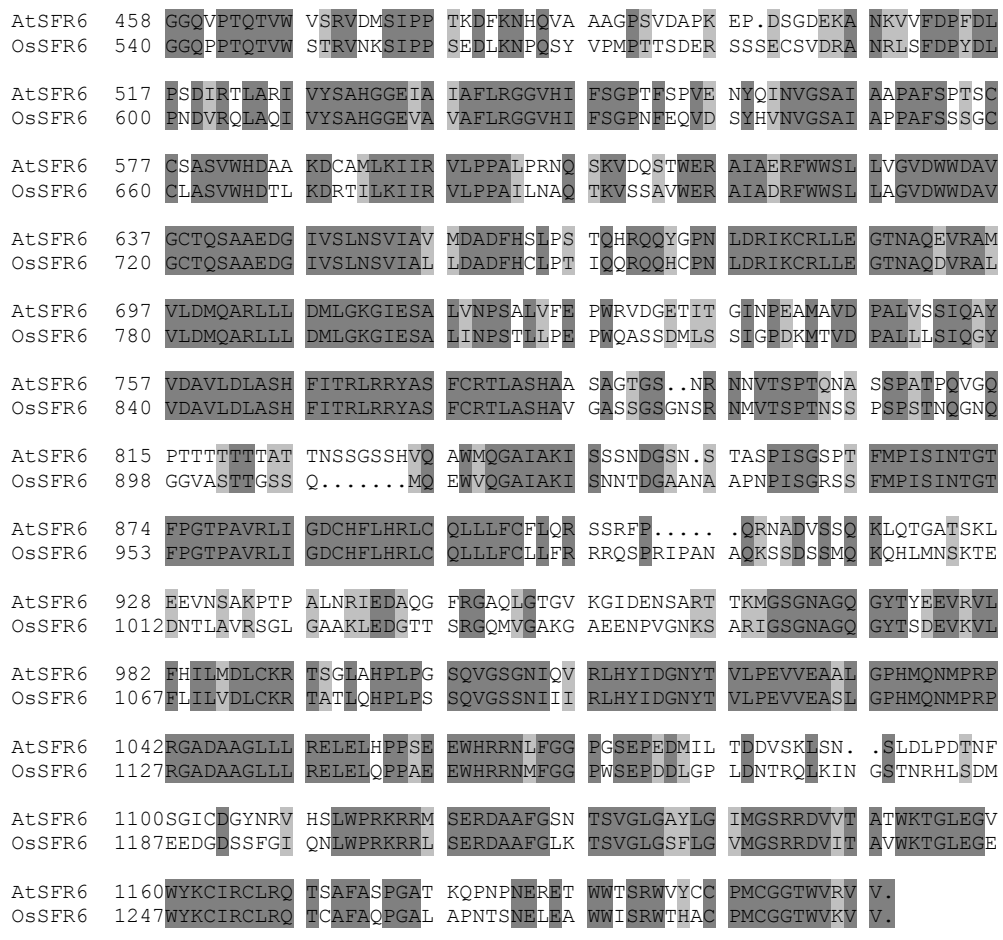
Amino acid sequence comparison of OsSFR6 (Os10g35560) of cultivar Japonica and AtSFR6 (At4g40920)

(Link: http://www.tigr.org/tigr-scripts/euk_manatee/shared/ORF_infopage.cgi?db=osa1r5&orf=12010.m06380)



(Figure continues to the following page)

Figure 6.2 *(Continued from the previous page)*



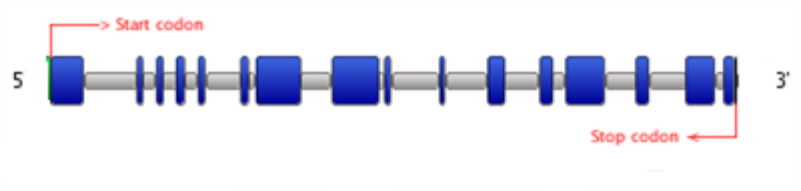
Alignment of AtSFR6 with SFR6-like protein from rice by Vector NTI software http://www.invitrogen.com. Fully conserved residues and highly conserved positions of amino acid sequences are indicated in dark and light coloured boxes respectively. "." indicates gaps in the amino acid sequences.

At the genomic level the *AtSFR6* gene is composed of 16 exons and 15 introns (Knight *et al*., 2009) (refer to Figure 1.8a for structural map of *AtSFR6*) and similarly the *OsSFR6* gene is also composed of 16 exons and 15 introns (Figure 6.2). The length and position of these introns and exons of *AtSFR6* and *OsSFR6* is fairly similar to each other. According to http://bioinformatics.psb.ugent.be/plaza there are 18 homologues of SFR6 in 7 species, *i.e. Arabidopsis thaliana* (1 gene), *Populus trichocarpa* (9 genes), *Oriza sativa* (1 gene), *Vitis vinifera* (2 genes), *Physcomitrella patens* (1 gene), *Sorghum bicolour* (1 gene) and *Carica papya* (3 genes). The predicted protein of OsSFR6 encodes 1301 amino acids (the length of AtSFR6

protein is 1268; Knight *et al*., 2009). Genomic sequence length is 8927 bp (*AtSFR6*; 7060 bp) and CDS length is 3906 bp (*AtSFR6*; 3807 bp).

Figure 6.2

Structure of *OsSFR6* gene.



Representation of the *OsSFR6* genomic coding sequence. Gray and blue coloured boxes represent introns and exons respectively. Adapted from http://bioinformatics.psb.ugent.be/plaza/genes/view site.

6.2.2 Cloning of the *OsSFR6* homolog from rice

First, rice cDNA was synthesized (see Materials and methods section 2.6.2.1) by reverse transcriptase polymerase chain reaction (RT-PCR) using total RNA extracted (see Materials and methods section 2.6.1) from leaves of rice (*Oryza sativa* cv. lemont) seedlings. Then, 2 primers were designed to amplify the whole *OsSFR6* coding sequence but this was not successful. Therefore, 3 reverse primers were designed; (1) starting right at the end of the *OsSFR6* gene, (2) starting a few nucleotides before the stop codon and (3) starting a few nucleotides beyond the stop codon, and several forward primers starting from different positions of the *OsSFR6* gene (refer to appendix C.3 for the sequence of these primers and the positions of them in *OsSFR6* coding sequence). The following primer pair produced the longest PCR fragment (3513 bp) of the *OsSFR6* coding region named *OsSFR6**. Phusion polymerase was used for this PCR amplification.

1. 5'-CCGGTACCCCCGGGGATGCGCGTGCCCGAGCTCTGCAGGAACTT-3'
2. 5'-GGGCGGGGGCGGCCGATCCCGTCAAATTCAAACGACTTTCAC-3'

All efforts to amplify the ~390 bp long N-terminus region (indicated in blue coloured letters in Figure 6.3) were not successful and this might be due to high G and C

content in this region of the *OsSFR6* gene or they are not expressed. Therefore, further studies were carry on using *OsSFR6** fragment. The *OsSFR6** was cloned into *pENTR1A* gateway entry vector using the Sma1 site within the ccdB region and sequenced (DBS, Durham University) to make sure of lack of mutations. Nucleotide sequences of the isolated cDNA and the predicted nucleotide sequence were aligned using the Blast2 sequencing programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and 7 mismatches were revealed. However, 2 of them were found to be polymorphisms from variety Indica and another 2 mutations did not change protein sequence of OsSFR6. Thus, only 3 mismatches remained to be accounted to be. Therefore, *OsSFR6** was re-amplified from rice cDNA to make sure whether these three mismatches are mutations due to the fragment being amplified by PCR or due to errors of public data bases. The 2 primers used earlier were redesigned this time by adding enzyme restriction sites and ATGCGC sequence to forward primer in purpose of adding start codon to resulting *OsSFR6**. Subsequently *OsSFR6** was cloned into the *Kpn1* and *Not1* sites of *pENTR1A* gateway entry vector (Figure 6.3) and sequenced again (DBS, Durham University). The 3 mismatches of nucleotide sequence were the same as previous mismatches. Therefore, these mismatches were considered as errors of public data bases.

Figure 6.3

cDNA sequence of *OsSFR6* gene.

http://bioinformatics.psb.ugent.be/plaza/genes/view

```
   1  ATGACCTCTT CCTCCGCCCC AAACCCTAGC CCCCTCGTCC CCACCCCCAC CCCCTCTCCC
      CCCACCGCCA ACGCCGCCGC CGCCGCCGCC GGAGCCGTGC CCGTCTCCTC GCCGACGCCG
 121  CCGCCCAAGG ATCAGCAGCA GGAGGGCCAG GGGGGTGGAG GAGGAGTGGG GGATGGGGGT
      GGAGTGGAGG AGGTGGGCGT AGGGGGAGGC GAGGCCATGG AGGTGGATGG TGGCGCGGGA
 241  GGTGGTGGTG GGGGGGTTGG GGATGTGGAG GGGGGTGGGG GTGGTGGTGG TGCGGGGGGA
      GGAGGAGGTG GAGGTGGAGG TGGGCAGCAG GCGTCGCCGG CGACCGTGTT CCGGATCCGG
 361  CTCAAGCAGC CGCCCTCGAG CCTCCGCCAC AAGATGCGCG TGCCCGAGCT CTGCAGGAAC
      TTCAGTGCAG TTGCTTGGTG CGGGAAGCTC AATGCAATTG CATGCGCATC AGAGACTTGT
 601  GCACGCATAC CAAGCTCTAA TTCAAGCCCA CCATTTTGGA TTCCCATACA CATTCTAAAT
      CCAGAGAGAC CAACAGAATG TTCTGTTTTC AATGTGAAAG CAGATTCTCC ACGCGACTTT
 721  GTTCAATTCA TTGAATGGTC TCCTCGATCA TGCCCTCGTG CATTACTGGT GGCAAATTTT
      CATGGAAGGA TTACTATATG GACACAGCCA ACTAAGGGTC CTACTAATCT TGTACGTGAT
 841  GCCAGTTCCT GGCAATGTGA ACACGAATGG CGTCAAGATC TTTCGGTGGT GACTAAGTGG
      TTGTCAGGAA TTTCTCCGTA TAGATGGCTT CCTGCAAACT CTAGTACTTC ATCAAACTTG
 961  AAAACCTTTG AGGAAAAGTT CCTTACCCAG CAGCCTCAAA GTTCGGCTGG GTGGCCAAAC
      ATTCTATGTG TCTGTTCAGT TTTTTCATCG GGTTCTGTTC AGCTTCATTG GTCACAATGG
1081  CCTTCTCAAA ACTCAGCACA ACCTAGATGG TTTTCTACTA GCAAAGGGCT TTTAGGAGCA
      GGGCCAAGCG GCATAATGGC TGCTGATGCT ATTATTACTG AAACTGGAGC ATTACATGTT
1201  GCTGGTGTTC CCCTTGTTAA TCCATCTACT GTAGTGGTTT GGGAGGTGAT GCCAGGCCTT
      GGCAATGGTA TTCAGGCAAC TGCAAAGATA AATGCAACAA GCTCTCTTCC TCCATCACTA
1321  AATCCCCCAC TCTGGGCTGG TTTTGCTCCA CTTGCATCTT ACCTCTTCTC TTTGCAAGAC
      TACCTTGTTT CCGAGGGCGC ACAGACAAAA AAACAGGCAC AGGTAGATAA TGAGACCACT
1441  GAGGTAGCAT CGATCCATTG TTGTCCAGTT TCCAACTTTT CAGCTTACGT CAGTCCTGAA
      GCTGCTGCCC AGTCAGCCAC TACCACAACA TGGGGATCTG GGGTTACCTC AGTTGCTTTT
1561  GATCCCACTC GAGGGGGATC AGTTATTACA GTTGTAATAG TTGAAGGGCA GTACATGTCT
      CCTTATGATC CTGATGAAGG ACCTTCCATC ACTGGATGGA GAGTCCAGTG CTGGGAATCT
1681  TCAGTCCAAC CTGTTGTTCT TCATCCAATA TTTGGAAGCC CTGCAAACTT TGGTGGACAG
      CCACCTACAC AGACTGTTTG GTCCACAAGA GTTAACAAAA GCATCCCACC ATCTGAGGAC
1801  CTTAAGAACC CTCAATCATA TGTTCCAATG CCAACAACTT CAGATGAGCG GAGTTCTTCT
      GAGTGCAGTG TTGACAGGGC GAACCGACTT AGCTTTGACC CTTATGATCT TCCAAATGAT
1921  GTCAGACAAT TGGCCCAAAT AGTTTATTCT GCTCATGGTG GTGAGGTTGC AGTTGCATTC
      CTGCGTGGAG GTGTGCACAT TTTTTCAGGT CCAAACTTTG AACAGGTTGA TAGCTATCAT
2041  GTCAATGTTG GCTCAGCAAT TGCTCCACCA GCCTTCTCCT CCAGTGGTTG TTGCTTGGCA
      TCAGTATGGC ATGACACACT CAAAGATCGA ACCATACTAA AGATAATACG TGTGCTTCCT
```

(Figure continues to the following page)

Figure 6.3 *(Continued from the previous page)*

```
2161 CCTGCAATTC TTAATGCTCA GACAAAGGTT AGCTCAGCTG TTTGGGAACG AGCAATAGCA
     GATAGATTTT GGTGGAGTCT ATTGGCTGGT GTGGATTGGT GGGATGCTGT TGGCTGCACA
2281 CAAAGTGCTG CTGAAGATGG TATTGTCTCA CTGAACAGTG TGATAGCTTT GCTGGACGCG
     GACTTCCATT GTCTTCCAAC TATACAACAG AGGCAACAAC ACTGTCCTAA TCTTGATAGG
2401 ATAAAGTGTA GATTGTTGGA AGGAACAAAT GCTCAAGATG TCAGAGCACT TGTGTTGGAC
     ATGCAAGCAA GATTGCTTCT GGATATGCTT GGCAAGGGAA TTGAGTCTGC CCTGATAAAT
2521 CCATCAACTC TGCTACCTGA ACCGTGGCAA GCTTCCAGTG ACATGTTATC TAGCATTGGG
     CCTGACAAAA TGACTGTTGA CCCAGCTCTA CTTTTAAGCA TCCAGGGGTA CGTTGATGCT
2641 GTTCTAGATT TAGCGTCACA TTTTATCACA CGCTTGCGAC GCTATGCGAG CTTCTGCCGA
     ACTTTGGCTA GCCATGCAGT TGGAGCATCT TCTGGTTCAG GCAATTCTAG GAATATGGTT
2761 ACAAGTCCAA CCAACAGTTC TCCTTCACCT TCAACTAACC AAGGTAATCA AGGTGGAGTA
     GCGTCTACAA CAGGGAGCTC ACAAATGCAA GAGTGGGTCC AAGGTGCCAT TGCTAAGATT
2881 AGTAACAATA CTGATGGTGC TGCAAATGCT GCACCAAATC CAATTAGCGG GAGGTCATCA
     TTCATGCCTA TTAGCATAAA TACGGGAACA TTCCCTGGCA CACCAGCTGT TAGACTTATT
3001 GGGGACTGCC ATTTCCTTCA TAGATTATGT CAGCTGTTGC TATTTTGTTT GCTTTTTCGG
     AGAAGGCAAT CTCCAAGGAT ACCTGCAAAT GCACAAAAAA GTTCTGATTC TAGCATGCAG
3121 AAACAACACT TGATGAACAG TAAGACAGAG GATAATACTT TGGCAGTCAG ATCTGGTCTA
     GGTGCTGCCA AATTGGAAGA TGGCACAACT TCACGTGGAC AGATGGTTGG AGCAAAGGGT
3241 GCTGAAGAAA ATCCAGTGGG CAACAAATCT GCTAGGATAG GTTCTGGCAA TGCTGGCCAA
     GGTTATACTT CAGACGAGGT GAAAGTCCTT TTTCTCATAT TAGTTGACCT ATGTAAACGG
3361 ACTGCAACCT TGCAACATCC GTTGCCTTCT TCTCAGGTTG GTTCGAGCAA TATTATTATA
     AGGCTGCATT ACATCGATGG CAATTACACT GTGCTCCCTG AGGTAGTGGA AGCATCTCTT
3481 GGCCCTCATA TGCAGAATAT GCCTCGTCCA CGTGGAGCTG ATGCTGCTGG CCTTCTACTT
     CGAGAATTAG AACTGCAGCC CCCTGCTGAA GAATGGCATA GACGCAACAT GTTTGGTGGG
3601 CCATGGTCAG AACCAGATGA TCTTGGTCCA TTGGATAATA CGCGACAGCT AAAAATCAAT
     GGCTCTACCA ATCGCCACTT ATCGGACATG GAAGAGGATG GCGACAGCTC CTTTGGGATT
3721 CAAAATCTTT GGCCAAGAAA GCGCCGGTTG TCTGAAAGAG ATGCAGCATT TGGTCTGAAA
     ACATCCGTGG GGCTGGGATC TTTTCTAGGT GTGATGGGTT CTCGGAGAGA TGTTATTACA
3841 GCTGTGTGGA AAACAGGCCT CGAAGGTGAA TGGTACAAGT GCATACGATG TTTGAGGCAA
     ACCTGTGCAT TTGCTCAGCC TGGTGCTCTA GCTCCGAACA CGTCGAATGA GCTTGAGGCA
3961 TGGTGGATCA GCCGATGGAC CCATGCTTGC CCAATGTGCG GTGGGACATG GGTGAAAGTC
     GTTTGACGGG ATCCGGCCGC
```

Whole cDNA sequence of *OsSFR6* gene according to the public data bases is presented in this figure. Blue coloured region represent the region which is not included in *OsSFR6**. Red coloured boxes showed where mismatches were found in *OsSFR6** compare to putative cDNA sequence of *OsSFR6*. G, G, A, C, A, T and C has changed to A, A, C, T, G, C and T respectively according to our sequencing results. Nucleotides marked with blue coloured stars were found as polymorphism to Indica and nucleotides marked with green coloured stars are not change predicted OsSFR6 protein sequence.

6.2.3 Section 1: OsSFR6 functions in *Arabidopsis*

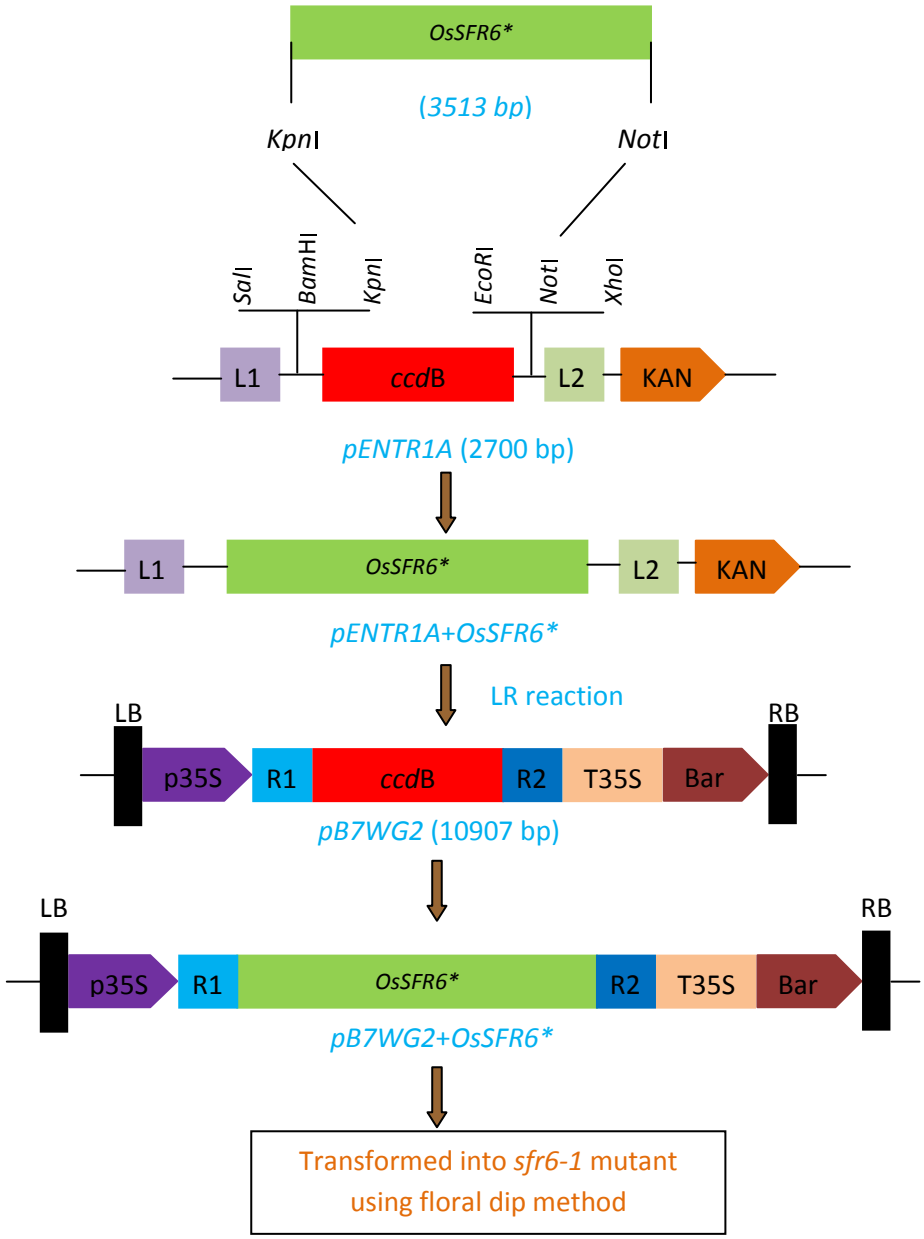
To compare the function of OsSFR6 with that of AtSFR6, transgenic *Arabidopsis* plants expressing *35S::OsSFR6* in *sfr6-1* and Col-0 background were generated. Following selection for the transgenic *Arabidopsis* expressing *35S::OsSFR6*, the effect of *OsSFR6* in *Arabidopsis* on stress-induced gene expression and restoration of the different phenotypes of the *sfr6-1* mutant by expression of *35S::OsSFR6*, including freezing sensitivity, was assessed.

6.2.3.1 Generation of transgenic *sfr6-1* expressing *OsSFR6**

The complete *OsSFR6** coding sequence in *pENTR1A* (section 6.2.2) was cloned into the *pB7WG2* gateway destination vector (Karimi *et al*., 2002), which contains the *35SCaMV* promoter upstream, by LR reaction to generate a binary vector construct (Figure 6.3). Finally the binary vector was introduced in to *A. tumefaciens* C58C1 (Materials and methods section 2.10.3) and transformed into the *sfr6-1* mutant using the floral dip method (Materials and methods section 2.11.1). Transformed plants (T0) were selected on soil with the herbicide "Basta - glufosinate ammonium" (250 mg/L) which was sprayed onto plants three times at three day intervals after one week of seed germination. Following this, plants that survived were transferred individually to peat plugs and grown in long day conditions (Materials and methods section 2.2.4). Ten transgenic lines were obtained after "Basta" selection.

Figure 6.4

Schematic representation of cloning *OsSFR6** into *pB7WG2* gateway destination vector.



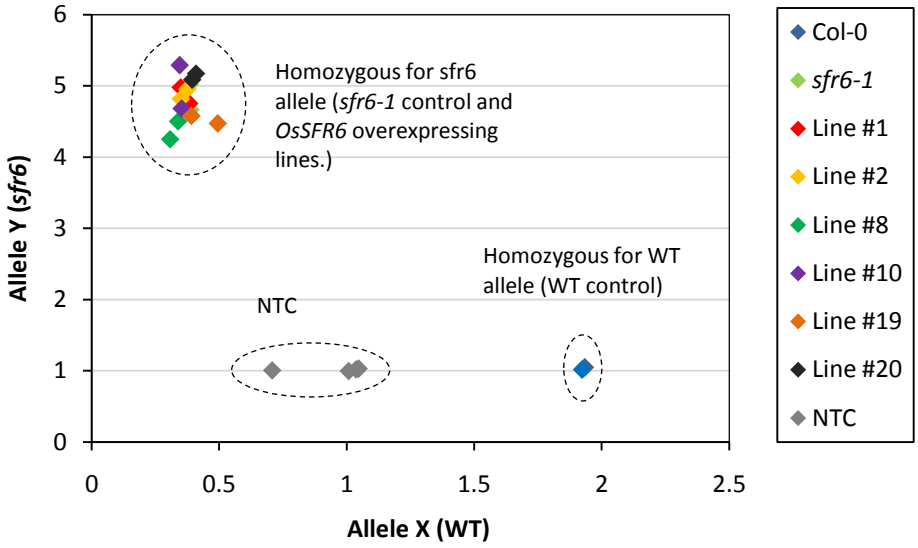
L1, L2, R1 and R2 are gateway sites. *pB7WG2* harbours *35SCaMV* (p35S) promoter and antibiotic resistivity is Bastar (Bar). *Not drown to scale*.

An allelic discrimination assay (Materials and methods section 2.15) was performed to ensure the origin of transformants was the *sfr6-1* mutant. The same probe

described in section 3.2.2.1 was used for this assay. If selected transgenic lines of *35S*::*OsSFR6** originated from *sfr6-1* mutant the T2 generation should be homozygous for the *sfr6-1* allele. Six lines out of 10 tested detected the *sfr6-1* mutant gene (*i.e.* homozygous for mutant gene; Figure 6.5.). Transgenic plants of the T2 generation were used for further analysis. From this point onward transgenic *sfr6-1* mutant plants expressing *OsSFR6** are designated as "*35S::OsSFR6*+sfr6-1*".

Figure 6.5

Results of allelic discrimination assay of *sfr6-1* complemented lines with *35S*::*OsSFR6*.



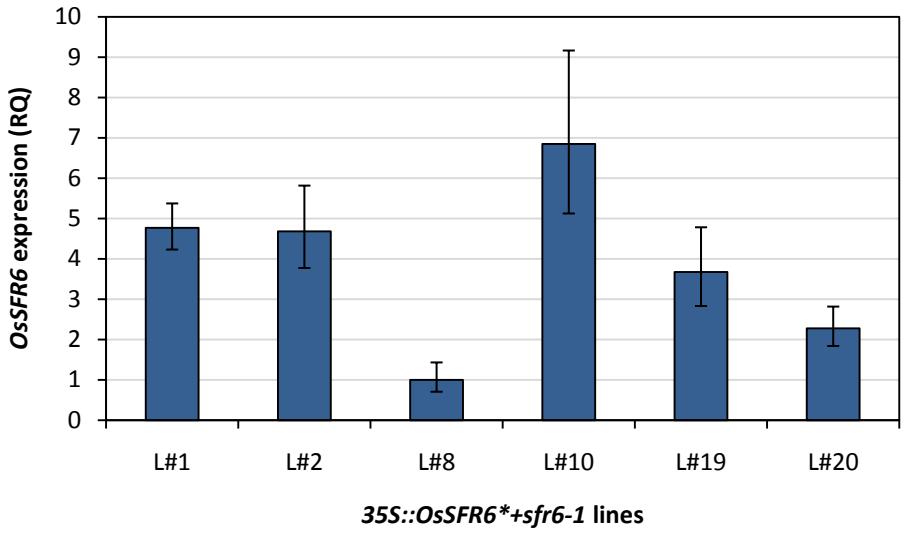
Genomic DNA was extracted from 7-day-old seedlings grown on 1×MS agar medium. Two technical replicates were performed for each sample. Each spot corresponds to a one technical sample. Water was used for non template control (NTC). Graph shows the assay data for *35S*::*OsSFR6** expressors in *sfr6-1* background. Graph presents data of only 6 lines identified (which were used for further studies) as homozygous to *sfr6* mutant allele along with *sfr6-1* and Col-0 homozygous lines as controls.

35S::OsSFR6+sfr6-1* lines were tested for *OsSFR6** expression using a TaqMan probe specially design for *OsSFR6* (Applied Biosystem) mRNA. Selected

35S::*OsSFR6*+sfr6-1* lines showed 2-7 fold higher levels compared to the lowest *OsSFR6* expresser of *35S*::*OsSFR6*+sfr6-1* line which is line #8 (Figure 6.6).

Figure 6.6

Analysis of *OsSFR6* expression levels due to *35S*::*OsSFR6** in *sfr6-1* mutant background.



Unstressed 7-day-old seedlings grown on 1×MS agar plates were used. Relative quantification of *OsSFR6* expression is presented relative to the level of *OsSFR6* expression in line #8 as this was the line which showed lowest *OsSFR6* expression level. *PEX4* was used as endogenous control. Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

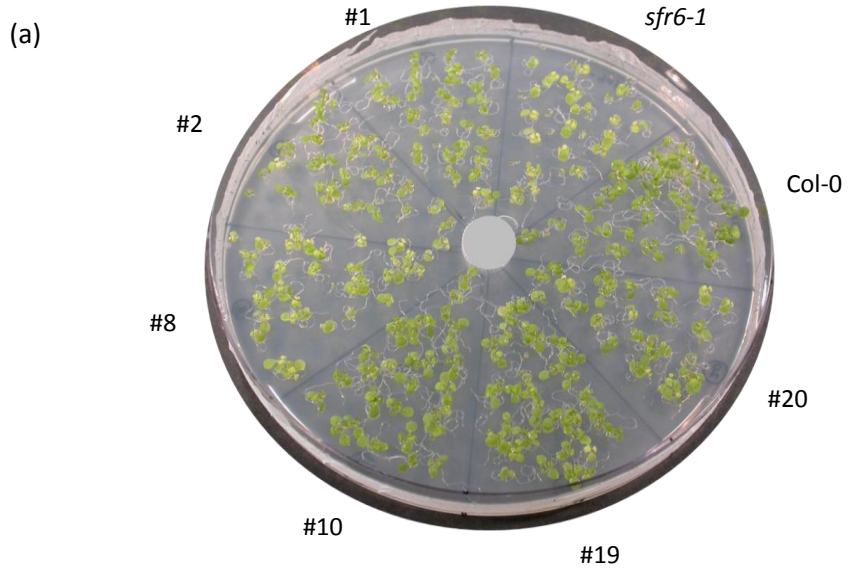
6.2.3.2 Phenotypic complementation

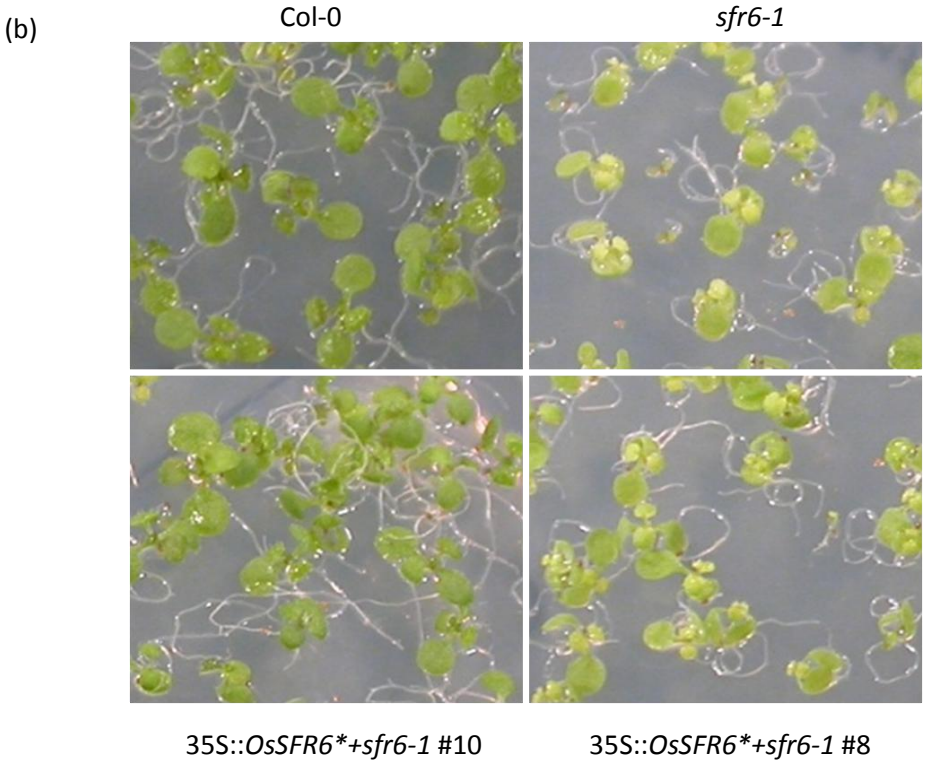
The original *sfr6-1* mutant displays a paler leaf colour compared to Col-0 (Knight *et al*., 2009). Therefore, the colour complementation of transgenic plants was tested. As shown in Figure 6.6. *35S*::*OsSFR6*+sfr6-1* plants showed restoration of leaf colour from pale green to dark green with the regaining of leaf colour proportional to the amount of transgene expression. The leaf colour of *35S*::*OsSFR6*+ sfr6-1* line #10 which had the highest expression was as dark green as Col-0 and line #8 with lowest

*OsSFR6** expression was not able to rescue leaf colour completely. Other lines tested displayed leaf colour in between these two lines. These results suggest that the level of expression of *OsSFR6** has an effect on leaf colour development in *Arabidopsis.* And also these results gave preliminary evidence that OsSFR6 might be a functional ortholog of AtSFR6.

Figure 6.6

Phenotypic complementation of *sfr6-1* by *OsSFR6** gene.





T2 progeny of *sfr6-1* plants containing transgene *OsSFR6** were grown on 1×MS agar medium and photographs were taken 10 days after germination. Col-0 and *sfr6-1* mutant were also grown for comparison. (a) Restoration of leaf colour of *sfr6-1* mutant by *OsSFR6** gene. (b) Close up pictures of transgenic plants expressing *OsSFR6**.

6.2.3.3 Low temperature-induced gene expression and freezing tolerance of *35S*::*OsSFR6*+sfr6-1* lines

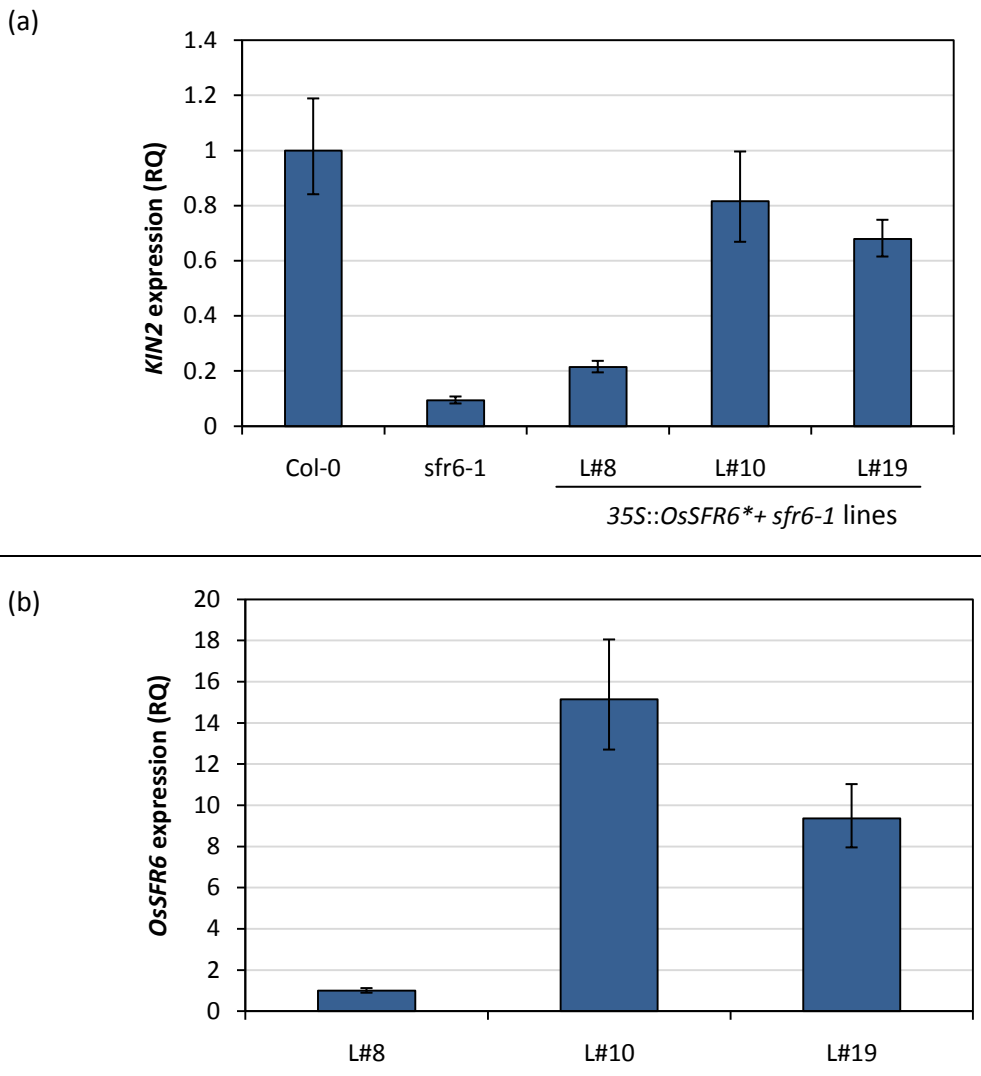
If the OsSFR6 protein of rice is a functional ortholog of AtSFR6 protein, expression of *OsSFR6* in *Arabidopsis* plants might also activate the *COR* gene expression and freezing tolerance of *Arabidopsis*. Therefore, to examine the low temperature induced gene expression and freezing tolerance of *35S*::*OsSFR6*+ sfr6-1* lines, *KIN2* and *COR78* levels were measured after plants were exposed to 4^{o}C for 6 h and plants were cold acclimated and analysed freezing tolerance by exposing plants to -6.5 and -7.5 ^{o}C.

6.2.3.3.1 KIN2 expression

Three *35S*::*OsSFR6*+ sfr6-1* lines (L#10, #19 and #8) with highest, medium and lowest expression of *OsSFR6** respectively were used for this study. Line #10 exhibited a higher level of *KIN2* transcript accumulation than *sfr6-1* but less than Col-0 and line #19 also exhibited higher level of *KIN2* accumulation than *sfr6-1*but less than line #10. Line #8 exhibited the lowest level but slightly higher than the *sfr6-1* mutant (Figure 6.7). These results suggest the level of *KIN2* expression may be roughly proportional to the level of *OsSFR6** expression in the lines (Figure 6.7).

Figure 6.7

KIN2 expression in *35S*::*OsSFR6**+ *sfr6-1* lines in response to low temperature treatment.



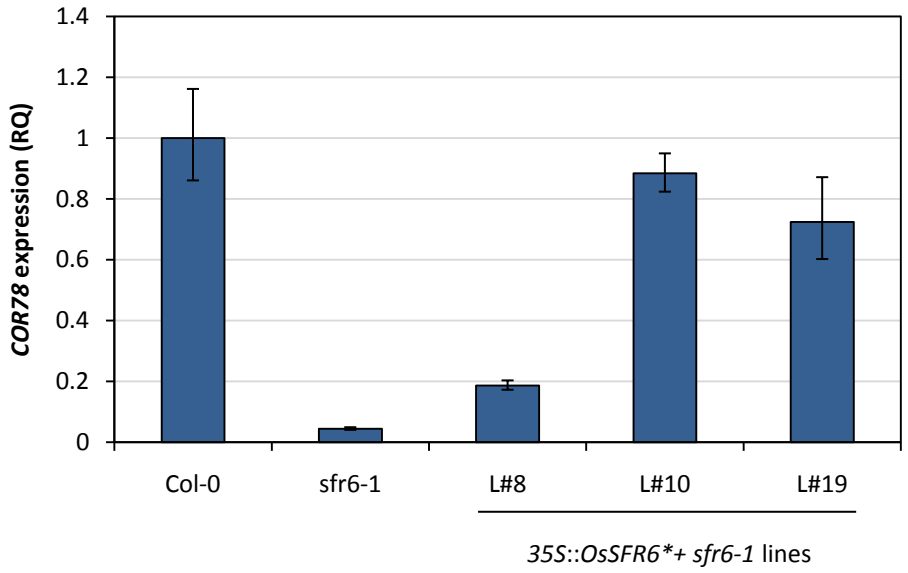
Seven-day-old seedlings grown on 1×MS agar plates were subjected to cold treatments at 4^{o}C for 6 h and samples were collected for RNA extraction. Data represented here are relative quantification (RQ) values of gene expression relative to cold treated Col-0. *PEX4* was used as endogenous control to normalize expression values. The graph shows the average of three biological replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to Student's *t* test. (a) *KIN2* mRNA levels of *35S*::*OsSFR6**+ *sfr6-1* lines. (b) *OsSFR6* expression of *35S*::*OsSFR6**+ *sfr6-1* in lines used to examine *KIN2* expression.

6.2.3.3.2 COR78 expression

COR78 expression in *35S*::*OsSFR6*+ sfr6-1* lines was also examined. The same samples taken to examine *KIN2* expression were used for this assay. As seen for *KIN2* expression, *COR78* expression was restored by the *OsSFR6** transgene and the expression levels were proportional to *OsSFR6** expression in the selected lines (Figure 6.8). These results further suggest *OsSFR6* is a functional orthologue of *AtSFR6* gene.

Figure 6.8

Restoration of *COR78* expression in the *sfr6-1* mutant by complementation with *OsSFR6**.



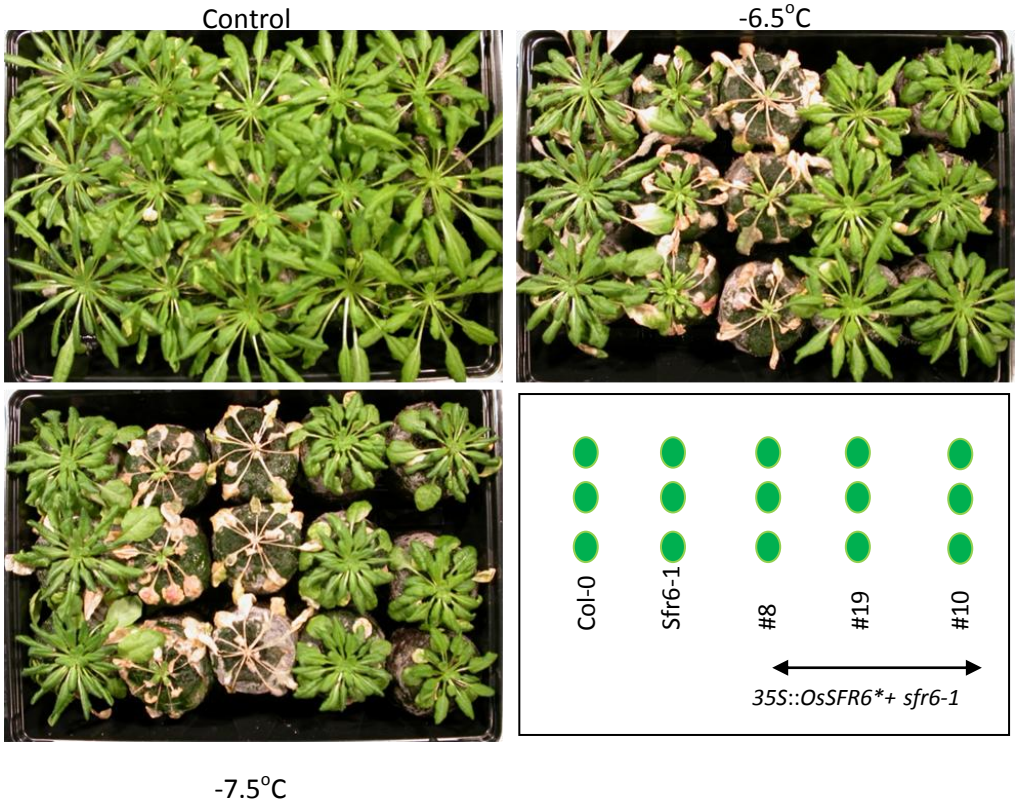
Seven-day-old seedlings grown on 1×MS agar plates were subjected to cold treatments at 4^{o}C for 6 h and samples were collected for RNA extraction. Data presented here are relative quantification (RQ) values of *COR78* expression relative to the *COR78* expression level of non transgenic but cold treated Col-0 plants. *PEX4* was used as an endogenous control to normalize expression value. Graphs shows average of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

6.2.3.3.3 Freezing tolerance

Three complemented lines of *35S*::*OsSFR6**+ *sfr6-1* (line #8, #10 and #19), Col-0 and *sfr6-1* mutant plants were grown on peat plugs under short day (8 h light and 16 h dark) conditions at 20^{o}C temperature and 150 µmol m^{-2} s^{-1} light level in a Sanyo growth chamber (model MLR 351; www.sanyo-biomedical.co.uk). After 5 weeks plants were cold acclimated for 11 days at 4^{o}C in a Sanyo growth chamber (model MLR 351) and then plants were exposed to freezing temperatures (-6.5 and -7.5 ^{o}C) for 24 h in the dark in a Sanyo incubator (model MIR 254; www.sanyo-biomedical.co.uk). Then plants were returned to a 20^{o}C short day Sanyo cabinet and the development of freeze thaw-induced damage was monitored. At both freezing temperatures used for this experiment *sfr6-1* and *35S*::*OsSFR6**+ *sfr6-1* #8 transgenic plants which express lowest level of *OsSFR6** showed the most severe damage after freezing and thawing. Ten days after returning to 20^{o}C after -7.5^{o}C freezing treatments all *sfr6-1* plants and *35S*::*OsSFR6**+ *sfr6-1* #8 plants had completely died (Figure 6.9). The other two *35S*::*OsSFR6**+ *sfr6-1* complemented lines (line #10 and #19), which expressed considerably higher levels of *OsSFR6**, and Col-0 plants grew relatively well (Figure 6.9). These results indicate that *OsSFR6** in *Arabidopsis* can complement the freezing sensitivity of the *sfr6-1* mutant. Further, these results suggest that OsSFR6 is a functional orthologue of AtSFR6 and SFR6 function may be conserved in other plant species. However, to better understand the function of OsSFR6 in rice needs further experiments (see Chapter 8).

Figure 6.9

Freezing tolerence of the *sfr6-1* plants expressing 35S::*OsSFR6**.



Five-week-old plants were cold acclimated at 4°C for 11 days then treated at freezing temperatures (to -6.5 and -7.5°C) for 24 h then returned to 20°C. *35S*::*OsSFR6** + *sfr6-1* lines (L#8, #10 and #19) were used along with Col-0 and the *sfr6-1* mutant. Pictures were taken 10 days after returning to 20°C. Six plants from each line per treatment were used.

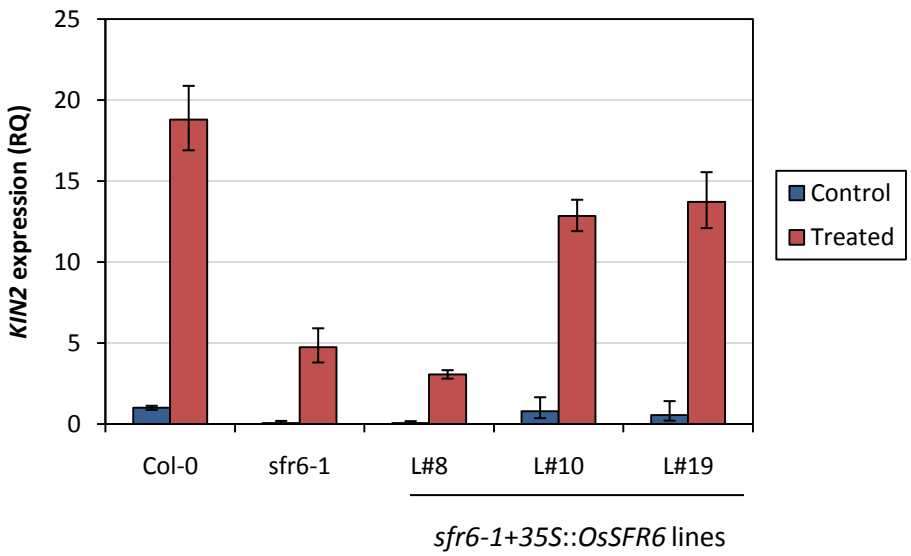
6.2.3.4 KIN2 expression of *35S*::*OsSFR6**+ *sfr6-1* lines following osmotic stress

sfr6-1 mutants display reduced expression of *KIN2* following osmotic stress (Boyce *et al*., 2003). Therefore, the *KIN2* expression of *35S*::*OsSFR6**+ *sfr6-1* lines after osmotic stress was also examined. Seven-day-old plants grown on 1×MS were transferred to 5 ml of water for 3 h and then concentrated mannitol solution was added to make a final concentration of 350 mM. Then seedlings were collected after 6 h for RNA extraction. Osmotic treatment increased *KIN2* expression of all lines

tested compared to water control plants. Line #10 and #19 showed the highest level of *KIN2* expression but slightly less than the Col-0 plants. Line #8 showed the lowest value as usual and the expression was slightly lower than the *sfr6-1* mutant.

Figure 6.10

KIN2 expression after osmotic stress in *35S*::*OsSFR6*+ sfr6-1* lines.



Seven-day-old seedlings grown on 1×MS agar plates were immersed in 5 ml of 350 mM mannitol solution for 6 h. Control plants were immersed in 5 ml of water for 6 h. Graph shows the relative quantification (RQ) value of *KIN2* expression relative to the *KIN2* levels of non-transgenic Col-0 water control plants. Data presented here are averages of three technical replicates. *PEX4* was used as endogenous control. Error bars indicate RQ_{MIN} and RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

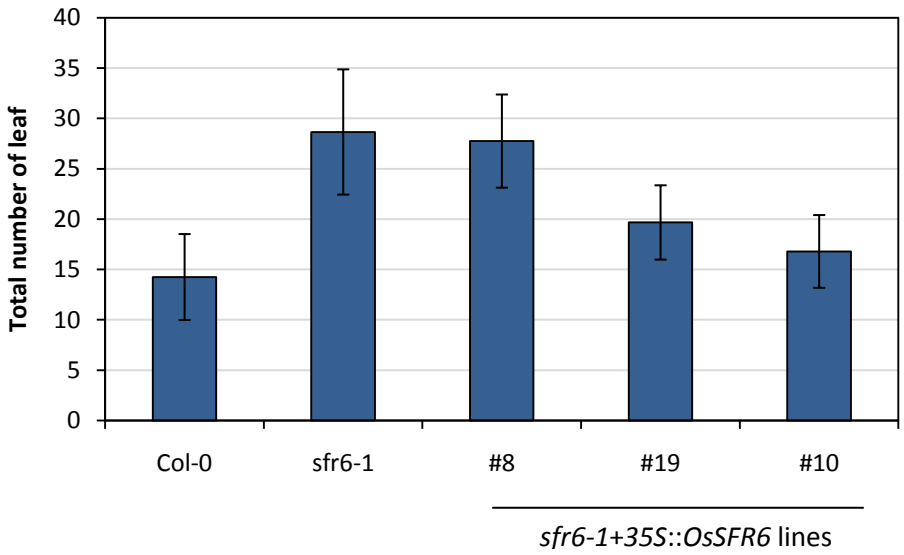
6.2.3.5 Complementation of the late flowering phenotype of *sfr6-1* mutant by *OsSFR6**

The *sfr6-1* mutant flowers later than Col-0 (Knight *et al*., 2008). Therefore, the possible complementation of this late flowering phenotype in *sfr6-1* mutant plants transformed with *OsSFR6** was analysed. Three complemented lines (line #8, #10 and #19) along with Col-0 and the *sfr6-1* mutant were used. Seven-day-old plants grown on 1×MS were transferred to peat plugs. Then plants were grown in 22^{o}C,

long day (16 h light and 8 h dark) photoperiod conditions (light level 150 µmol m^{-2} s^{-1}) until they bolted. The number of rosette and cauline leaves was counted when flower shoots became approximately 1 cm long. It was observed that the three complemented lines bolted in a dose-dependent manner relative to *OsSFR6** expression. *sfr6-1* and complemented line #8 which showed lowest *OsSFR6** expression bolted later having more than 25 leaves on average. Line #19 and #10 bolted earlier than *sfr6-1* and line #8 but little later than Col-0 (Figure 6.11). However there is no significant difference of number of leaves at bolting in between line #10 and 19 compare to Col-0.

Figure 6.11

Restoration of late flowering phenotype of *sfr6-1* mutant by *35S*::*OsSFR6**.



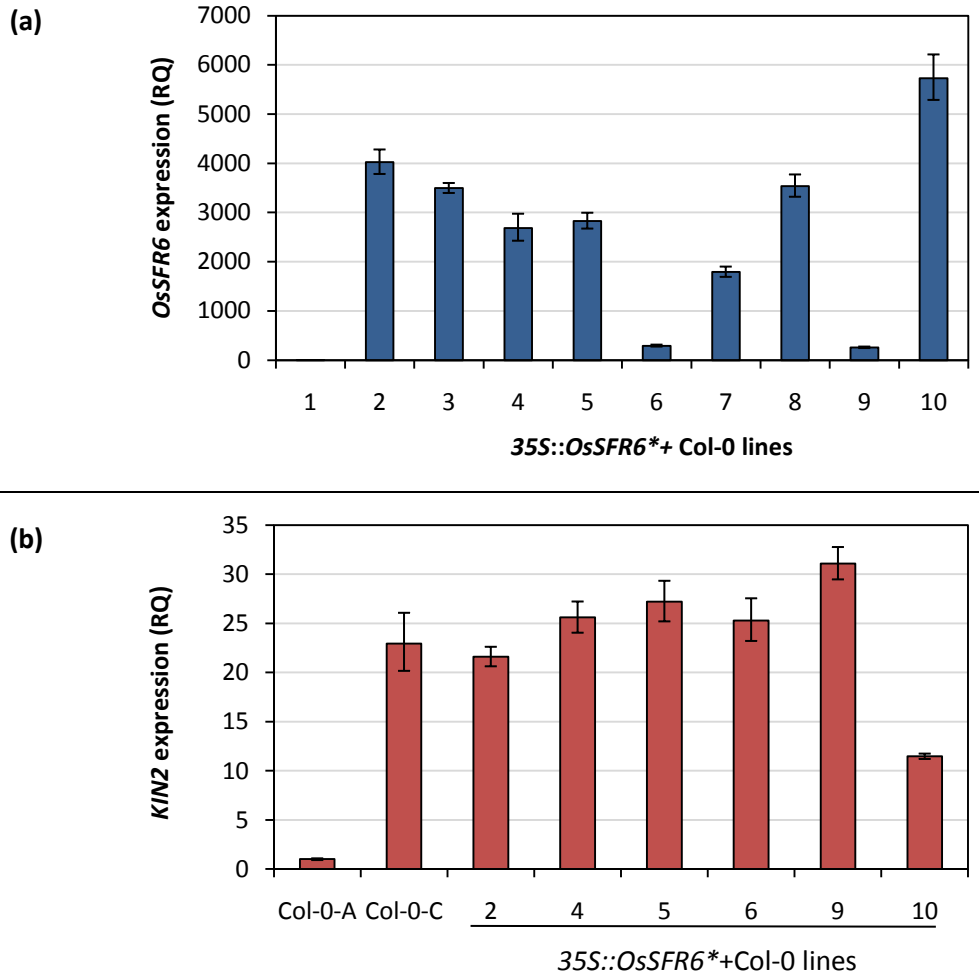
35S::Os*SFR6** + *sfr6-1* lines were grown under long day photoperiod (16 h light and 8 h dark). Col-0 and *sfr6-1* were also grown as controls. Number of leaves were counted when the flower shoot became approximately 1 cm long. Error bars are ±SD (n=25).

6.2.3.6 *KIN2* expression of Col-0 expressing *35S::OsSFR6**

Wild type *Arabidopsis* expressing *35S::OsSFR6** were produced to analyse the effect of *35S::OsSFR6** on *KIN2* expression in Col-0 plants following low temperature stress. The plasmid construct containing *pB7WG2+OsSFR6** (Figure 6.4) was introduced into the Col-0 *Arabidopsis* wild type plants by the *Agrobacterium* dipping method. Approximately 20 transgenic plants named *35S::OsSFR6**+Col-0 were selected. No phenotypic alterations were observed in *35S::OsSFR6**+Col-0 transgenic lines. Ten lines were tested for *OsSFR6** expression after low temperature exposure at 4^{o}C for 12 h and 6 lines out of these 10 lines showed more than 2000 fold high levels than the *35S::OsSFR6**+Col-0 line expressing the lowest level of *OsSFR6** (Figure 6.13a). Then *KIN2* expression of the 6 *35S::OsSFR6**+Col-0 lines with highest, medium and lowest level of *OsSFR6** expression was compared. RNA samples were isolated from seven-day-old plants grown on 1×MS medium treated at low temperature (5^{o}C) for 24 h. There was no significant difference in *KIN2* expression between the *35S::OsSFR6**+Col-0 lines and the non-transgenic cold treated Col-0 control plants, except the highest *OsSFR6** expresser of *35S::OsSFR6**+Col-0 lines (Line #10) showed significantly reduced level of *KIN2* expression (Figure 6.13b). This should be clarified by further study.

Figure 6.13

Analysis of *KIN2* expression of Col-0 expressing *35S::OsSFR6**.



Seven-day-old *35S::OsSFR6**+Col-0 seedlings grown on 1×MS agar plates were subjected to cold treatments at 4^{o}C for 12 h and samples were collected for RNA extraction. *PEX4* was used as endogenous control to normalize expression value. Graph shows average of three biological replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} constitute the acceptable error for a 95% confidence limit according to Student's *t* test. (a) *OsSFR6** expression levels of selected *35S::OsSFR6**+Col-0 lines. Data represented here are relative quantification (RQ) values of gene expression relative to the *35S::OsSFR6**+Col-0 line with lowest OsSFR6* expression. (b) *KIN2* expression of *35S::OsSFR6**+Col-0 lines are presented in this graph. *KIN2* values are relative quantification (RQ) value of gene expression relative to *KIN2* expression of unstressed, non-transgenic Col-0. Col-0-A and Col-0-C represent ambient and cold treated Col-0 samples respectively.

6.2.4 Section 2: Generation of OsSFR6 knockdown rice plants

Knocking down of *OsSFR6* in rice was performed to examine the function of OsSFR6 in rice (see materials and methods section 2.11.2). However, due to time constraints it was not possible to finish. Therefore, this section describes the generation of plasmid constructs for transformation, callus induction from rice seeds and *Agrobacterium*-mediated callus transformation.

6.2.4.1 Preparation of plasmids for rice transformation

Nowadays artificial microRNAs (amiRNA) and other small RNAs (sRNA) are widely used for gene silencing (Ossowski *et al*., 2008; Warthmann *et al*., 2008). amiRNAs have also been successfully used for gene silencing in rice (Warthmann *et al*., 2008). Endogenous microRNAs (miRNAs) are single stranded small RNAs which negatively regulate gene expression in plant and animals. amiRNAs are also single-stranded RNAs but not normally found in plants and which are processed from endogenous miRNA precursors (www.http://wmd3.weigelworld.org). The sequence of amiRNA is designed according to the target gene of miRNAs and therefore a amiRNA specifically targets the chosen gene (www.http://wmd3.weigelworld.org). Ossowski *et al*. (2008) have developed a Web MicroRNA Designer (WMD) platform which can easily use to design amiRNAs for gene silencing of >30 species. Therefore, WMD3-Web MicroRNA Designer tool was used to design amiRNAs for *OsSFR6* (refer to Material and Methods section 2.9). The amiRNA sequence and 4 oligos designed for selected amiRNA sequence by WMD3 web tool for *Os10g35560.1* (*OsSFR6*) and the sequences of 2 universal primers used for PCRs are presented in Table 6.1. The PCR amplification was done to replace MIR528 precursor in pNW55 vector by the amiRNA sequence (refer to Materials and methods section 2.9 for PCR amplification).

Table 6.1

Sequences of amiRNA and oligos used for PCR to produce amiRNA constructs from *pNW55* vector.

amiRNA sequence - TAAACTATGATACCATACCGG

Sequence of 4 oligos designed for amiRNA sequence		
Primer name	Orientation	Sequence
Primer I (miR-s)	Sense	agTGTATTTATGCTAATAGGCACcaggagattcagtttga
Primer II (miR-a)	Antisense	tgGTGCCTATTAGCATAAATACActgctgctgctacagcc
Primer III (miR*s)	Sense	ctGTGCCAATTTGCATAAATACAttcctgctgctaggctg
Primer IV (miR*a)	Antisense	aaTGTATTTATGCAAATTGGCACagagaggcaaaagtgaa
Sequence of 2 universal primers		
G-4368	Sense	CTG CAA GGC GAT TAA GTT GGG TAA C
G-4369	Antisense	GCG GAT AAC AAT TTC ACA CAG GAA ACA G

A PCR fragment of 554 bp was cloned into *pENTR1A* gateway entry vector using *BamH1* and *EcoRV* sites and it was confirmed that the construct had no mutations by sequencing (DBS, Durham University). Subsequently the amiRNA sequence in *pENTR1A* was cloned into *pIPKb002* and *pIPKb003* over-expression vectors (Kumlehn, 2008) (refer to appendix B.6 for vector maps) by LR reaction to generate a binary vector construct. These vectors contain maize *ubiquitin 1* and rice *actin 1* promoters respectively. Finally these binary vectors were introduced into *A. tumefaciens* EHA105 (Hood *et al*., 1993; Materials and methods section 2.11.2) and used to transform rice callus derived from seeds (Materials and methods section 2.11.2.1).

6.2.4.2 Introduction of the OsmiRNAi construct into rice

Callus generation from rice seeds and infection of callus with *Agrobacterium* were performed according to the protocol developed by Nishimura *et al*. (2006) (refer to Material and Methods section 2.11.2 for more details). The OsmiRNAi constructs with maize *ubi* promoter and rice *actin* promoter were introduced into rice by co-

cultivating calli with *Agrobacterium* harbouring *pIPKb002+OsmiRNAi* or *pIPKb003+OsmiRNAi*. Lemont and Nipponbare genotypes were used for this experiment. Active callus induction and growth was observed for both of these genotypes throughout the experimental period (Figure 6.14), however, after co-cultivation with *Agrobacterium* the selected putative transformed calli did not grow. As well as this *Agrobacterium* cells re-grow occasionally on plates even though frequent rewashing and re-transferring was performed.

Figure 6.14

Lemont and Nipponbare seeds cultured on callus induction medium

Nipponbare Lemont

Callus generated from scutella, 7 days after mature seeds were cultured on callus induction medium.

6.2.5 Section 3: CRT/DRE element regulation by OsSFR6

AtSFR6 specifically affects low temperature induction of genes dependent on the CRT/DRE sequence motif (Knight *et al*., 1999). Therefore, in order to investigate the effect of OsSFR6 on the expression of genes regulated by CRT/DRE motif, I used two constructs containing CRT/DRE motifs in their promoter region fused to the reporter gene LUC+. These plasmids and plasmids with OsmiRNAi (see section 6.2.4.1) were co-bombarded into rice seedlings (refer to section 2.11.3). The descriptions of constructs and vector combinations (I-VI) used for particle bombardment are presented in Table 6.1.

Table 6.1

Plasmid combinations used to examine the effect of OsSFR6 on the expression of gene regulated by *CRT/DRE* motif.

	[DRE/CRT]$_4$::LUC+ [a]	*KIN2 promoter::LUC +* [b]
pIPKb002 (Kumlehn, 2008).	I	II
pIPKb002+OsmiRNAi (refer to section 6.2.4.1)	III	IV
pIPKb003+OsmiRNAi (refer to section 6.2.4.1)	V	VI

[a] *[DRE/CRT]$_4$::LUC+* plasmid was produced by cloning 4×repeated *cis* element sequence with minimal (-70bp) *CaMV* promoter into *pDHLC+2* which contains the *LUC+* coding region in *pDH51* without the 35S promoter (PhD thesis, Helen J Rushton, University of Oxford, 2009).

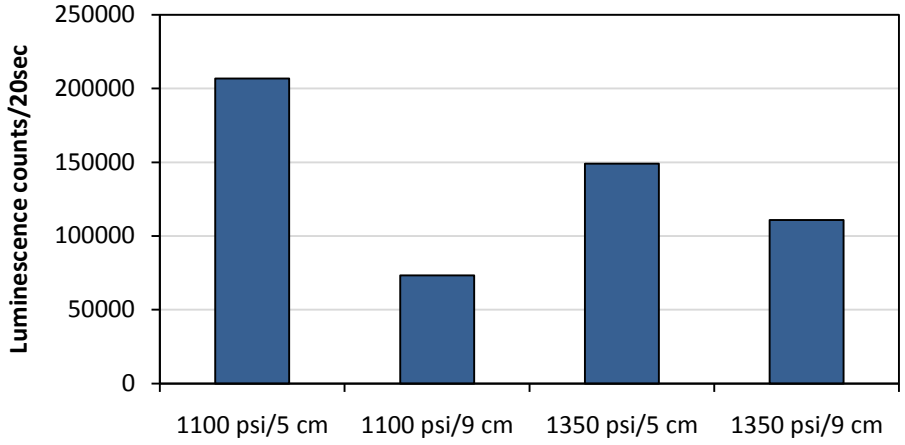
[b] *KIN2 promoter::LUC* plasmid was produced by cloning 750 bp upstream of *KIN2* promoter into *pDHLC+2* (personal communication with Dr. Heather Knight).

Optimization of the conditions for bombardment was performed by using the vector *pDHLC+1* (PhD thesis, Helen J Rushton, University of Oxford, 2009) which was made by inserting *LUC+* as *SmaI* and *XbaI* fragment into *pDH51*and LUC+ is driven by 35S promoter. Two levels of helium pressure (1100 and 1350 psi) and two distances (5 and 9 cm) from the microcarrier to the target tissue was tested (Figure 6.15). It was observed that a helium pressure of 1100 psi and 5 cm distance from the

microcarrier to the target tissue gave the highest transient *LUC* gene expression. Therefore, these conditions were used for the subsequent experiment.

Figure 6.15

Expression of pDHLC+1 in rice seedlings 48 h after ballistic bombardment.



pDHLC+1 was introduced into two-week old rice seedlings (see Materials and methods section 2.11.3.1) by particle bombardment. After bombardment seedlings were incubated 48 h before assaying for the transient expression of LUC+.

Three replicates were performed for each vector combination (Table 6.1). After bombardment plants were incubated 48 h in a growth chamber (seed Materials and methods section 2.11.3.1) and transferred to 4^{o}C for overnight before the collection of tissues for LUC assay. Whilst some luciferase activity was detected, values were close to background to be able to determine whether there was a significant difference between treatments. Due to time constraints it was not possible to repeat the experiment. However, it would be worthwhile in the future to repeat this experiment to understand not only the effect of OsSFR6 on CRT/DRE element but also the suitability of the miRNAi constructs for silencing OsSFR6 in a transient assay system.

6.3 Discussion

6.3.1 OsSFR6 is an ortholog of SFR6/MED16

The ultimate objective of studying the function of stress response genes is manipulating them in crop plants to achieve a better stress tolerance trait. Therefore, in this study the homologue of AtSFR6 in rice was identified and characterized and its orthology tested by functional complementation. This demonstrated that the functional complementation of phenotypes of *sfr6-1* mutant by *35S::OsSFR6* was correlated to the level of *OsSFR6* expression. Moreover, similar to the over-expression of *35S::AtSFR6* in *Arabidopsis*, the expression of *35S::OsSFR6* in wild type *Arabidopsis* did not enhance *COR* gene expression following low temperature stress. Therefore, taken together, these results provide preliminary evidence that OsSFR6 might be an ortholog of SFR6/MED16. Rice is a freezing-intolerant species and in other freezing intolerant species CBF/DREB1 transcription factors have been shown to control chilling, rather than freezing, tolerance (Choi *et al*., 2002; Hsieh *et al*., 2002). The presence of a functional SFR6 ortholog in rice capable of restoring *Arabidopsis* CBF function suggests that OsSFR6 may play a role in chilling tolerance in rice.

6.3.2 CBF cold response pathway in chilling-sensitive species

The CBF pathway is a well-studied genetic system involved in freezing tolerance in *Arabidopsis*. Over-expression of CBF transcription factors in *Arabidopsis* results in large increases of expression of *COR* genes and freezing tolerance even at ambient temperatures (Jaglo-Ottosen *et al*., 1998). These observations were a step towards increasing crop plant freezing tolerance effectively just by over-expressing a single transcription factor gene. Therefore, CBF orthologues were identified in many important crop plants, both freezing tolerant and intolerant. As examples CBF homologues were identified in crops such as tomato (Jaglo *et al*., 2001) and rice (Dubouzet *et al*., 2003) which are freezing intolerant species and crops such as rye (Jaglo *et al*., 2001), barley (Choi *et al*., 2002; Skinner *et al*., 2005) and wheat (Jaglo

et al., 2001; Kume *et al*., 2005) which cold acclimate and are freezing tolerant species. Induction of CBF genes following exposure to low temperature were also observed in these species. Over-expression of CBF genes of chilling sensitive species like tomato and rice in *Arabidopsis* induce the CBF regulon and low temperature tolerance (Dubouzet *et al*., 2003; Zhang *et al*., 2004) indicating these plants have functional CBF cold response pathway. The possible identification of a functional CBF cold response pathway in freezing intolerant species raises the question as to why these species are unable to cold acclimate to become freezing tolerant. The analysis of the CBF regulon and CBF regulators of these species might help to answer this question. The studies on the CBF regulon of CBF1 over-expressing tomato plants revealed lack of expression of known COR homologues suggesting this might be associated with freezing sensitivity of tomato (Hsieh *et al*., 2002) and emphasises the importance of studying CBF regulon of chilling sensitive species to clarify potential CBF activity in these plants. Moreover the studies on wheat and barley CBF genes revealed that cereal CBF genetic pathway is much more complex than *Arabidopsis* as the genome of cereals contains large numbers of CBF genes compared to *Arabidopsis* (currently 4 CBF genes have been identified) (Skinner *et al*., 2005; Miller *et al*., 2006). As an example 20 CBF genes have been identified in barley (Skinner *et al*., 2005) and 25 CBFs in wheat (Badawi *et al*., 2007). Therefore, the function of CBF regulators in cereals might be more complex than the CBF regulators in *Arabidopsis*. SFR6 plays a central role in regulating the expression of the CBF regulon upon low temperature stress in *Arabidopsis* (Knight *et al*., 1999; Knight *et al*., 2009). Therefore, identification of *SFR6*-like genes and characterization of their functions will help clarify the potential role of CBF activity of these species and it might also help to improve low temperature tolerance trait of chilling-sensitive agriculturally important crop plants.

6.3.3 CBF cold response pathway in rice

In 2003 Dubouzet *et al*. reported the identification of *OsDREB* genes in rice. They identified 5 cDNAs of DREB homologs: OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D and OsDREB2A. Over-expression of OsDREB1A in *Arabidopsis*

induces the over-expression of target stress inducible genes of DREB1A and resulted in increased tolerance to freezing, drought and high-salt stresses (Dubouzet *et al*., 2003). The over-expression of *OsDREB1A*, *OsDREB1B*, *DREB1A*, *DREB1B* and *DREB1C* in rice also produced increased tolerance to drought, salt and cold with growth retardation similar to over-expression of *DREBs* in *Arabidopsis* (Ito *et al*., 2006). Later another DREB1 homolog designated as OsDREBD1F was identified in rice (Wang *et al*., 2008). Over-expression of this gene increased salt, drought and low temperature tolerance in both rice and *Arabidopsis.* OsDREB1F specifically binds to the DRE/CRT element but not to ABRE, however, over-expression of *OsDREB1F* in *Arabidopsis* increased ABA-dependent gene expression suggesting that *OsDREB1F* may be involved in the ABA-dependent pathway as well (Wang *et al*., 2008). ICE1 is known to be a transcription factor regulating *COR* gene expression following low temperature stimuli via CBF3 (Chinnusamy *et al*., 2003). The over-expression of *ICE1* in rice leads to enhanced cold tolerance and increased accumulation of proline content (Dian-jun *et al*., 2008). All these observations indicate the cold tolerance signalling pathway is conserved in rice and as rice is a chilling-sensitive species the CBF/DREB1 genes of rice might be involved in regulation of chilling response gene expression.

However, differences between the cold response signalling pathways of rice and *Arabidopsis* have also been observed. As an example Dubouzet *et al*. (2003) observed the expression level of target genes in *35S::OsDREB1A* plants was not as high as *35S::AtDREB1A*. Moreover, some stress induced target genes of DREB1A that have only ACCGAC as a DRE were not over-expressed in the *OsDREB1A* transgenic *Arabidopsis* (Dubouzet *et al*., 2003). DREB1A binds to both ACCGAC and GCCGAC DRE-related core motif (Dubouzet *et al*., 2003). Although, over-expression of either *OsDREB1* or *DREB1* could increase stress tolerance of *Arabidopsis*, the microarray analysis of downstream gene expression revealed that the up-regulated genes of OsDREB1A in rice are less than those of DREB1A in *Arabidopsis* (Ito *et al*., 2006). Furthermore, over-expression of *CBF1/DREB1b* of *Arabidopsis* in rice did not increase cold tolerance but some cold responsive genes such as *LIP5*, *LIP9* and *OSDHN1* were up-regulated in transgenic plants (Lee *et al*., 2004). The one possibility for these differences might be differential function of CBF

regulators such as OsSFR6. In this study restoration of *KIN2* and *COR78* gene expression and increased freezing tolerance of *sfr6-1* mutant by *35S::OsSFR6* was observed indicating that the biological function of OsSFR6 and SFR6 are very similar. However, the restoration of gene expression of other cold induced genes which show defective expression in the *sfr6-1* mutant was not analysed. Microarray analysis of gene expression in *35S::OsSFR6* in *sfr6-1* background will help to understand whether OsSFR6 and SFR6 have a differential effect on expression of cold genes in *Arabidopsis*.

Chapter 7

Identification of positive and negative regulators of SFR6 activity

7.1 Introduction

An enhancer/suppressor mutant screen was performed to identify positive and negative regulators of SFR6 activity. Enhancer/suppressor screening is a common type of genetic screen used to uncover more information on known genes or mutants by identifying genetic interactors which might be in the same pathway. The screen begins with a mutant allele of a gene which causes either a weak or strong phenotype in the biological process of interest. Further mutagenesis of the original mutant, selection of plants showing a stronger or weaker phenotype and subsequent mapping can identify the secondary mutation site and the gene involved in suppression or enhancement of the original phenotype. The results of the enhancer/suppressor mutant screen performed in this study are discussed in this chapter.

The specific aim of the work described in this chapter was;

- Identification of mutations in genes encoding other proteins interacting with SFR6.

7.2 Results

7.2.1 Isolation of enhancer/suppressor mutants

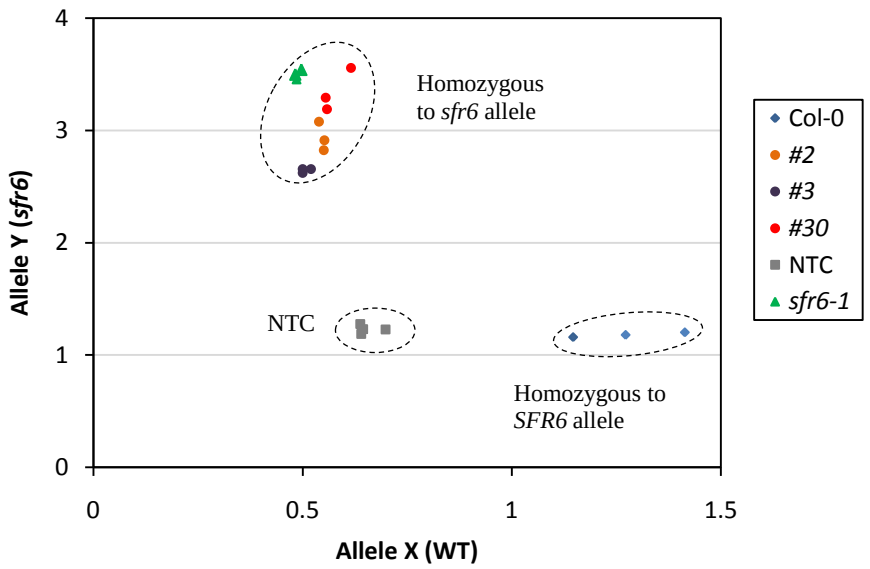
M2 seeds from ethyl methanesulfonate (EMS) mutagenesised *sfr6-1* mutant plants (Lehle Seeds, Round Rock, Texas, USA) were used for screening. These represented a total of about 20500 original M1 families across 18 pools of M2 seeds. The enhancer/suppressor screen was conducted on 10-day-old seedlings grown on 1×MS agar medium as described in Materials and methods section 2.12. Green coloured seedlings resembling the wild type phenotype were selected as potential suppressor mutants and the seedlings with both paler leaves and/or larger cotyledons were selected as enhancer mutants. Two plates of Col-0 and *sfr6-1* mutant were also grown simultaneously each time to identify mutant phenotypes easily and properly. Putative mutants identified were transferred to peat plugs and grown to maturity at 22^{o}C and 16 h photoperiod (see Material and method section 2.2.4). Approximately, 200,000 seedlings (half of the target) were screened and a total of 52 putative mutants were identified. A secondary screen was performed by growing these 52 mutant lines again on 1×MS agar medium along with Col-0 and *sfr6-1* controls to test the repeatability of their phenotypes. Many of the mutants showed a phenotype similar to *sfr6-1*. However, 13 mutant lines showed consistent enhancer/suppressor phenotypes and they were selected for further characterizations.

Since the aim was to isolate mutations interacting with the activity of SFR6 protein it was important to confirm that the selected mutant lines contained the original *sfr6-1* mutation in their genome. Therefore, an allelic discrimination assay was conducted using specifically designed TaqMan® MGB probe (Applied Biosystems) which can discriminate between wild type *SFR6* and mutant *sfr6-1* gene variants (probe is similar to the probe described in section 3.2.2.1 and 6.2.3.1) by real time qPCR. If selected mutant lines originated from *sfr6-1* mutant plants they should be homozygous for the original *sfr6-1* mutant gene. Among the 13 mutant lines tested by allelic discrimination assay 3 mutant lines (lines *#2*, *#3* and *#30*) were homozygous for the *sfr6-1* mutant allele and these three lines were selected for further investigations. All of the other lines showed wild type SFR6 alleles indicating contamination of the mutagenized seeds with wild type seeds. Therefore, all these

lines were eliminated from further consideration. Allelic discrimination assay was repeated to confirm further the origin of mutant lines *#2*, *#3* and *#30* (Figure 7.1).

Figure 7.1

Results of allelic discrimination assay of enhancer suppressor mutants.



Genomic DNA was extracted from 7-day-old seedlings grown on 1×MS agar medium. Three technical replicates were performed for each sample. Each spot corresponds to one technical replicate. Water was used as non template control (NTC). Graph presents data of only three lines identified as homozygous to the *sfr6-1* mutant allele. *sfr6-1* and Col-0 homozygous lines were also used as controls.

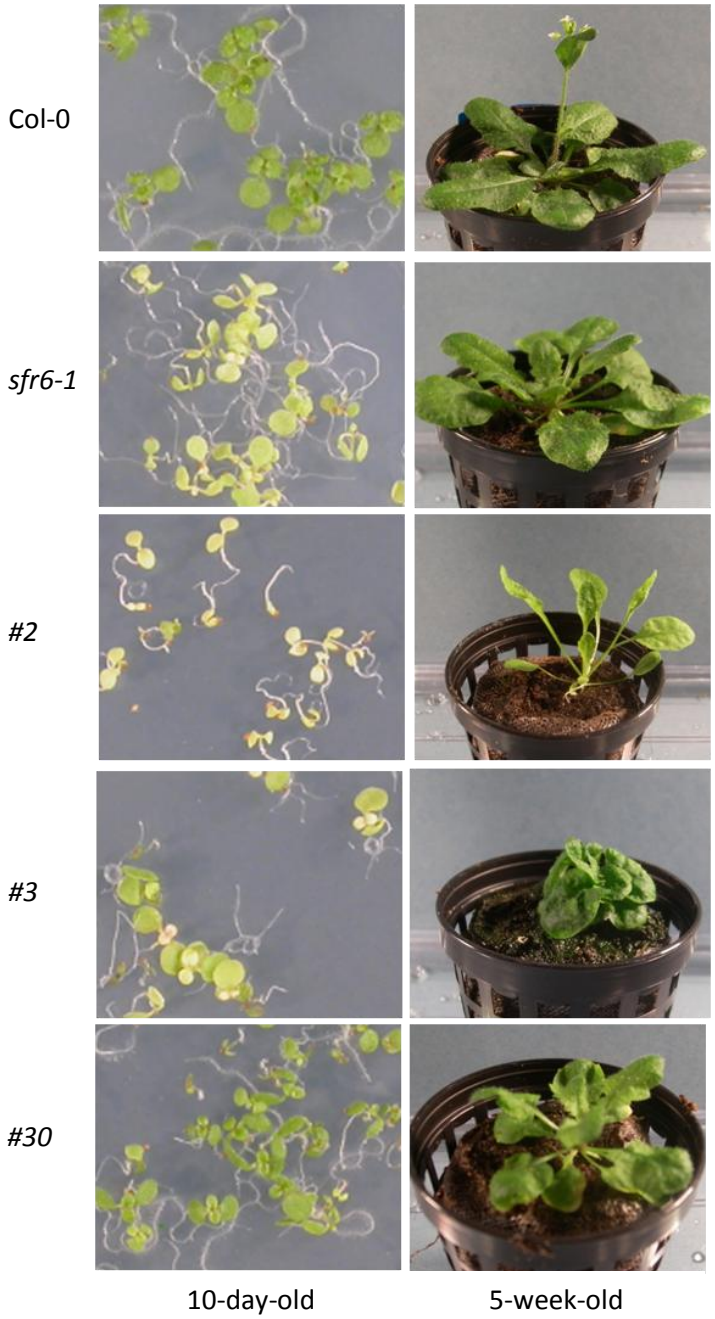
7.2.2 Phenotypes of the selected enhancer/suppressor mutants

The visual phenotypic differences of three selected mutant lines (from this point onwards *#2*, *#3* and *#30* mutant lines were designated as "*ems2/sfr6-1*, *ems3/sfr6-1* and *ems30/sfr6-1*" respectively) are presented in Figure 7.2. *ems2/sfr6-1* was selected as enhancer mutant. This mutant showed paler coloured leaves than the *sfr6-1* mutant. Apart from that, this mutant was weaker than *sfr6-1* producing fewer numbers of leaves, reproductive stems, branches and flowers and subsequently less numbers of seed set. *ems3/sfr6-1* was also selected as an enhancer mutant with

bigger cotyledons than *sfr6-1* and showed a pale colour phenotype similar to *sfr6-1*. However, when this line was grown to maturity leaves became dark green and showed a densely packed leaf arrangement. Very late flowering was also observed or sometimes plants did not flower at all during their life cycle. These plants produced very low number of seeds per plant and seeds were bigger than normal WT *Arabidopsis* seeds. *ems30/sfr6-1* selected as a putative suppressor mutant with darker green leaves than *sfr6-1* but this phenotype was not very strong when plants were grown to maturity on peat plugs. At maturity it displayed phenotype more or less similar to the *sfr6-1* mutant phenotype. The visible phenotypes of these three mutants at day-10 on agar medium and 5-week on peat plugs are presented in Figure 7.2.

Figure 7.2

Phenotype of 3 enhancer/suppressor mutants homozygous for the *sfr6-1* mutant allele.



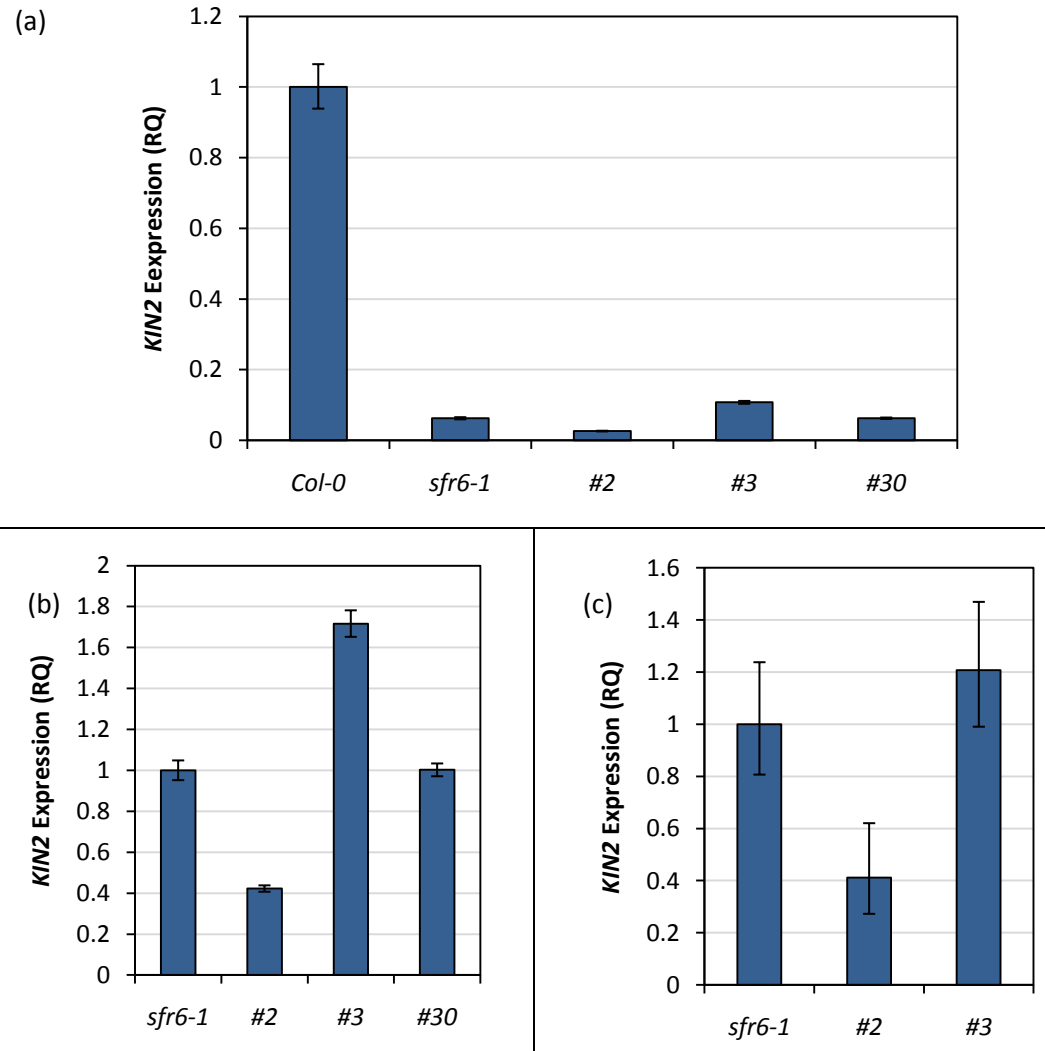
Appearances of enhancer/suppressor mutants homozygous for the *sfr6-1* mutant allele are presented here. Photographs were taken after 10 days on agar plates and 5 weeks after transferring plants to the growth room. *sfr6-1* and Col-0 plants are also shown as controls.

7.2.3 *KIN2* expression upon low temperature stress

sfr6 mutants are defective in expressing groups of cold induced genes (Knight *et al*., 1999 and Knight *et al*., 2009). Therefore, if a second site mutation is affecting the pathway that SFR6 is already affecting, the cold induced gene expression of *ems/sfr6-1* mutant lines should be altered. Therefore, in order to examine whether new mutations had altered levels of *COR* gene expression, *KIN2* expression upon low temperature exposure was analysed. Seven-day-old seedlings grown on 1×MS medium were subjected to 4^{o}C for 6 h. Then samples were collected for RNA extraction. *KIN2* expression was measured using real time qPCR as described in earlier chapters.

Figure 7.3

KIN2 expression of three selected enhancer/suppressor mutants of *sfr6-1*.



Seven-day-old seedlings grown on 1×MS agar plates were subjected to cold treatments at 4^{o}C for 6 h and then samples were collected for RNA extraction. Data represented in graph (a) are relative quantification (RQ) values for *KIN2* expression relative to the *KIN2* levels of cold treated Col-0. Data presented in graphs (b) and (c) are RQ values for *KIN2* expression relative to the *KIN2* levels of cold treated *sfr6-1*. Graph c data represent data of repeat experiment only with *sfr6-1*/*ems2* and *sfr6-1*/*ems3* ß-*TUBULIN4* was used as endogenous control to normalize expression values. Graphs show average of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to a Student's *t* test.

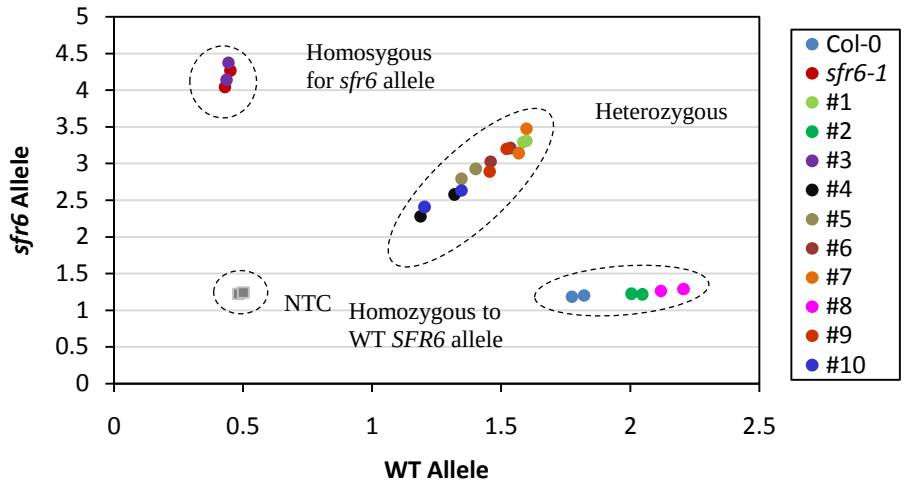
After 6 h cold treatment all *ems/sfr6-1* mutants showed a significantly reduced level of *KIN2* expression compared to Col-0 indicating that none of the new mutations were strong suppressors of the *sfr6-1* mutant (Figure 6.3a) even in the *ems30/sfr6-1* suppressor mutant. However, when comparing *KIN2* expression levels of selected mutant lines relative to the *KIN2* expression levels of cold treated *sfr6-1* mutant, *ems2/sfr6-1* lines showed significantly reduced *KIN2* expression and *ems3/sfr6-1* mutant showed significantly higher expression than *sfr6-1* original mutant. Therefore, I repeated the experiment only with *ems2/sfr6-1* and *ems3/sfr6-1* to examine reproducibility of previous results. *ems2/sfr6-1* again showed significantly reduced levels of *KIN2* expression after 6 h cold treatment compare to *sfr6-1* (Figure 6.3c). However, *ems3/sfr6-1* did not show a higher level of *KIN2* expression as previously. Therefore, only *ems2/sfr6-1* was used for further experiments.

7.2.4 Segregation of the *ems2* mutation from the *sfr6-1* mutation

To isolate the *ems2* mutation form the *sfr6-1* mutation, *ems2/sfr6-1* double mutant plants were crossed with Col-0 wild type plants and all F1 plants exhibited a wild type phenotype indicating that the new mutation (*ems2*) is recessive. Then F1 progeny were allowed to self fertilize and F2 seeds were screened to observe segregation of the *sfr6-1* and *ems2* mutant phenotype. All seedlings showing a phenotype other than Col-0 were selected and grown to maturity. Mutants, most likely to be *ems2* single mutants were healthier looking than the *ems2/sfr6-1* double mutant. However, the difference was not enough to clearly discriminate. Therefore, mainly two type of seedlings were selected *i.e. sfr6-1*-like and *ems2/sfr6-1* double mutant like. Seedlings of both these groups were tested for the presence of *sfr6-1* original mutation by allelic discrimination assay. All *sfr6-1*-like mutant lines had the *sfr6-1* mutant allele and 9 *ems2*-like lines out of 10 lines had the wild type SFR6 allele and only one line was homozygous for the mutant *sfr6-1*allele. Of these 9 lines 2 lines were homozygous for the wild type SFR6 allele and other 7 lines were heterozygous (Figure 7.4).The 2 lines homozygous for the WT SFR6 allele were used for further experiments.

Figure 7.4

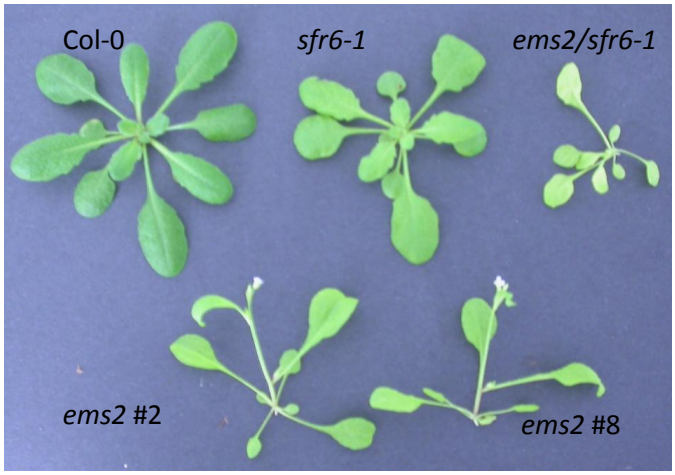
Results of allelic discrimination assay of *ems2*-like seedlings from the F2 generation of Col-0 and *ems2/sfr6-1* mutant crosses.



Genomic DNA was extracted from leaves of 2-week-old seedlings grown on peat plugs. Two technical replicates were performed for each sample. Water was used for non-template control (NTC). Graph presents data of only four lines identified as heterozygous to *SFR6* along with *sfr6-1* and Col-0 homozygous lines.

Figure 7.5

Comparison of phenotypic differences of new mutant lines.



The phenotypes of eighteen-day old seedlings of Col-0, *sfr6-1*, *ems2/sfr6-1* and two lines of *ems2* mutant grown on peat plugs under short day conditions (8h light/16h dark) are shown in this photograph.

New mutant lines (*ems2*) were early flowering and the colour of the foliage was in between the foliage colours of *sfr6-1* original mutant and *ems2/sfr6-1* double mutant indicating the effect of both mutations on leaf colour.

7.2.5 Cloning and sequencing the *sfr6* gene from *ems2/sfr6-1* double mutant lines

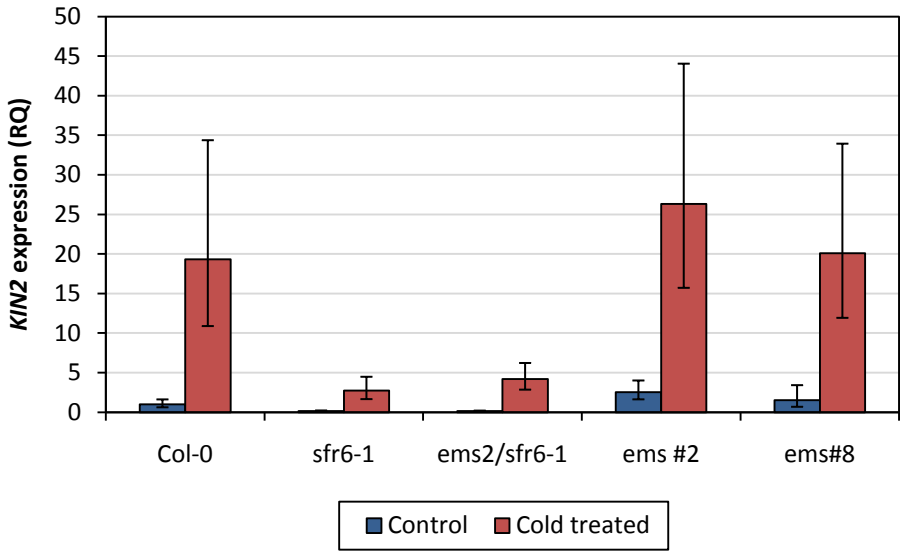
The enhanced *sfr6-1* phenotype in *ems2/sfr6-1* double mutant could be due to another mutation in the *SFR6* gene. Therefore, the *sfr6* gene from the *ems2/sfr6-1* double mutant was cloned and sequenced. No new mutations in the *sfr6* gene of *ems2/sfr6-1* double mutant lines were identified suggesting this enhanced phenotype of *sfr6-1* mutant is due to a mutation in a gene other than *sfr6*.

7.2.6 *KIN2* expression of *ems2* lines

The reduced level of *KIN2* expression in *ems2/sfr6-1* double mutant compared to *sfr6-1* mutant following cold treatments was observed previously (see section 7.2.3). Therefore, I investigated whether this reduced level of *KIN2* expression was due to the *ems2* mutation. *KIN2* expression of new mutant lines (line *#2* and #8) following cold treatment of 4^{o}C for 24 h was analysed.

Figure 7.6

KIN2 expression of *ems2* mutant lines.



Seven-day-old seedlings grown on 1×MS agar plates were subjected to cold treatments at 4^{o}C for 24 h and then samples were collected for RNA extraction. Data represented in graph are relative quantification (RQ) values for *KIN2* expression relative to the *KIN2* levels of cold treated Col-0. *PEX4* was used as endogenous control to normalize expression values. Graphs show average of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX} and constitute the acceptable error for a 95% confidence limit according to a Student's *t* test.

A similar level of *KIN2* expression was observed in the new mutant lines and Col-0 control lines. *ems2/sfr6-1* double mutant lines showed significantly reduced levels of *KIN2* similar to the *sfr6-1* original mutant. These results suggest that the lower expression of *KIN2* in double mutant is due to *sfr6-1* mutation.

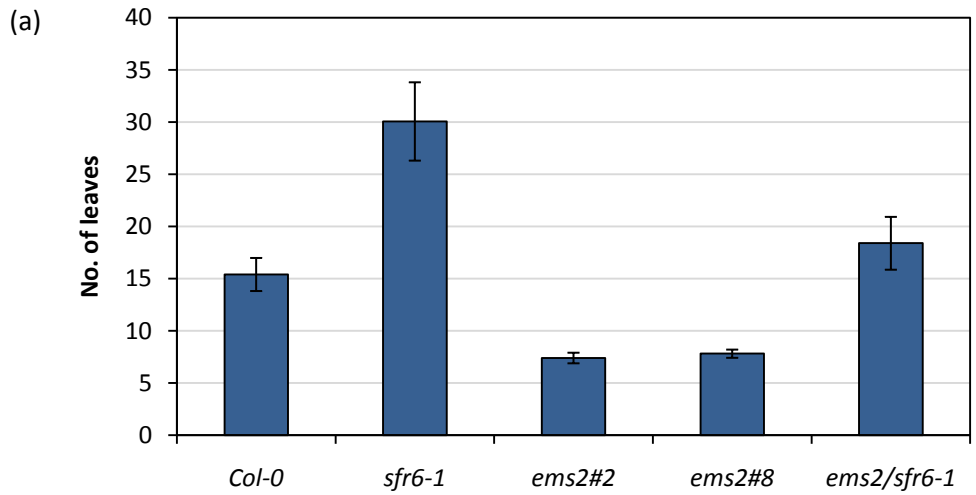
7.2.7 Early flowering phenotype of the *ems2* mutant

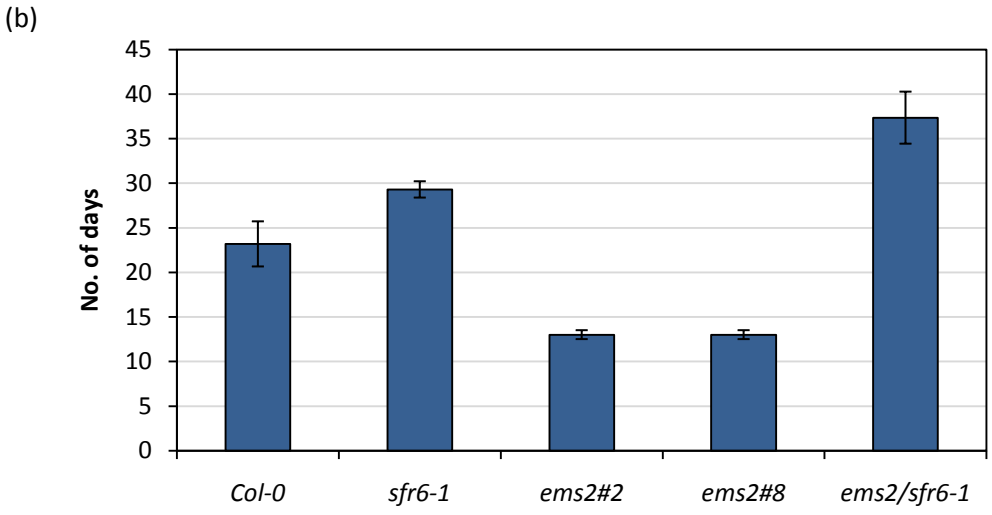
The *ems2* mutant was early flowering and as the phase transition from vegetative to reproduction was observed at 6-7 leaf stage (16 h light and 8 h dark under 22^{o}C) (Figure 7.7a). The leaf number of the *ems2/sfr6-1* double mutant at flowering was similar to Col-0 (Figure 7.7a). However, the number of days to flowering of

ems2/*sfr6-1* was higher than all other lines (Figure 7.7b). *sfr6-1* is already known to be a late flowering mutant (Knight *et al*., 2008). These results indicate *sfr6-1* is epistatic to the *ems2* mutation. The early flowering phenotype and other visible phenotypes of *ems2* lines are similar to the *phyB* mutant of *Arabidopsis* (Michael *et al*., 2000).

Figure 7.7

Flowering time and leaf number of *ems2* mutant.





(a) Col-0, *sfr6-1*, *ems2/sfr6-1* double mutant and 2 lines of *ems2* were grown under short day photoperiod (8 h light and 16 h dark). Number of leaves were counted when flower shoot became approximately 1 cm long. (b) No. of days taken for flowering are presented in this graph. Error bars are ±SD (n=25).

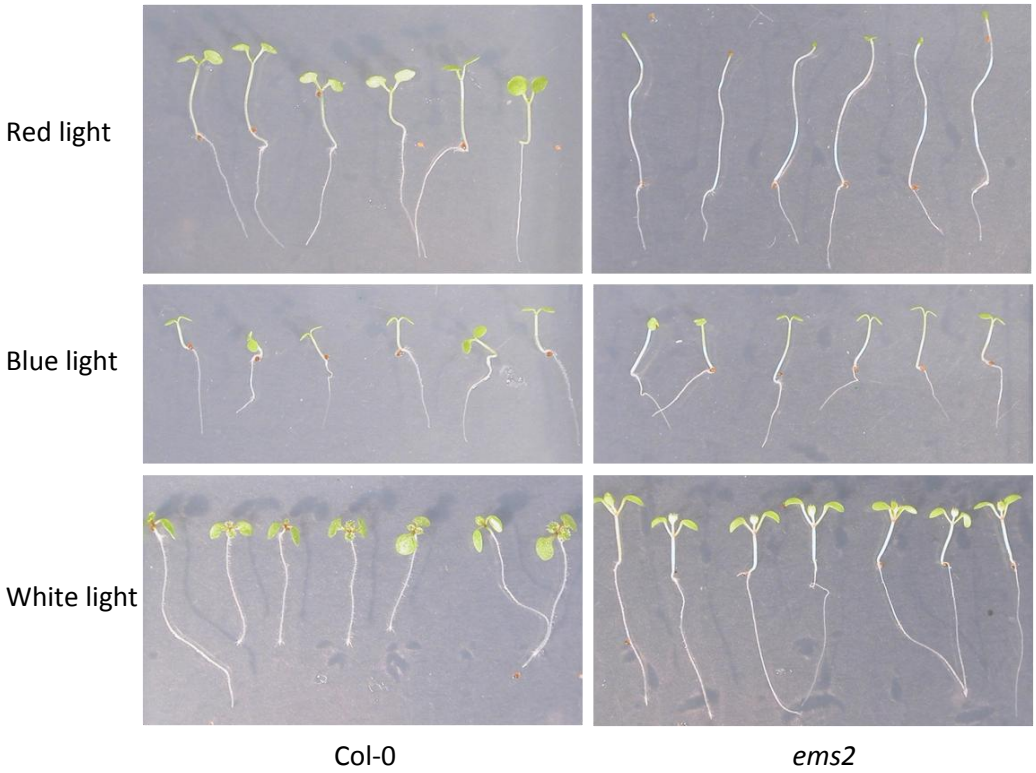
7.2.8 Hypocotyl elongation of *ems2* mutant under different light conditions

Phytochromes act as red and far-red light phoreceptors of plants and mediate shade avoidance characteristics. Tracking the wavelength of red and far-red light is important for several developmental processes of plant. The *Arabidopsis* genome contains 5 phytochrome photoreceptors (phyA-E) (Clack *et al*., 1994).

Therefore, in this study, hypocotyl elongation of the *ems2* mutant was analysed under different light conditions to further confirm whether the *ems2* mutation might be a phytochrome mutant. Seeds were sown on 1×MS medium and stratified as described in Materials and Methods section 2.2.4. Then plates were subjected to 2 h white light to induce germination. Plates were then wrapped either with red or blue filters (HT019-Fire and HT363-Special medium blue) and placed vertically under constant light conditions for 7 days (see section 2.14; Figure 7.8). Hypocotyl elongation was analysed and the *ems2* mutant receiving red light showed considerably longer hypocotyls (Figure 7.8). However, *ems2* plants receiving blue light did not display considerably longer hypocotyls compared to Col-0. According to these results and the visible phenotype it is proposed that the *ems2* mutant is a phytochrome mutant and most probably a mutation of phyB as *phyB* mutants plants show as similar phenotype as *ems2* mutant.

Figure 7.8

Hypocotyl elongation of *ems2* mutant under different light conditions.



Seeds were sown on 1×MS agar plates and stratified 3 days at 4^{o}C. Then plates were wrapped either with red or blue filter papers and placed in a growth chamber vertically under constant light. Unwrapped plates were used as control plants. All plates were subjected to 2 h white light before wrapping to induce germination. Photographs were taken 7 days after treatment.

7.3 Discussion

To clarify SFR6 activity on *COR* gene expression upon low temperature stimuli a mutant screen was performed with the purpose of identifying positive and negative regulators of SFR6 activity. The main objective was identification of revertants of the *sfr6-1* mutant. However, as described in this Chapter no suppressor mutants were observed and the mutants identified as enhancers of *sfr6-1* showed only enhancement of visual phenotypes. These mutations did not affect cold gene expression upon low temperature stimulus (Figure 7.6). However, with the subsequent identification of SFR6 as a subunit of the mediator complex of *Arabidopsis* (Backstrom *et al*., 2007) this explained the results: suppression of mediator activity by another gene is not likely possible as *Arabidopsis* has ~15,000 transcription factor genes (Riechmann *et al*., 2000) but it has 27 mediator subunits (Backstrom *et al*., 2007). Therefore, one subunit of mediator likely regulates gene expression of several signalling pathway. Thus, loss of function of more than one mediator subunit probably is lethal or much weaker than the original mutant.

As it was observed that the *ems2* mutant has a *phyB* like phenotype (Figure 7.8) the sequencing of the phyB locus from *ems2* is proposed as future work.

Chapter 8
General discussion and future work

8.1 Implication to the current work

Developing stress tolerant crops to cope with the rapid environmental degradation that is occurring is an absolute requirement in order to provide enough food for growing population. One of the basic genetic engineering approaches currently being used to improve crop stress tolerance is generation of transgenic plants by introducing novel genes into the genome of agriculturally important crops or altering the expression of existing genes. Understanding stress response signalling pathways is the prime requirement to manipulate stress tolerance of crop plants by this approach. The aim of this thesis was to understand SFR6 function in stress tolerance in *Arabidopsis* and other crop plants, as a possible route to crop improvement in the future.

The *sfr6* mutant of *Arabidopsis* was isolated on the basis of its failure to cold acclimate (Warren *et al*., 1996). Further studies showed the freezing sensitivity of the *sfr6* mutant is due to greatly reduced levels of the expression of *COR* genes controlled by CBF/DREB1 transcription factors in response to low temperature (Knight *et al*., 1999; Knight *et al*., 2009). However, CBF/DREB1 gene expression itself was not mis-regulated at the transcriptional and translational level in the *sfr6-1* mutant indicating that SFR6 operates downstream of CBF translation (Knight *et al*., 1999; Knight *et al*., 2009). In addition *sfr6* is compromised in its ability to express genes in response to drought and salinity (Knight *et al*., 1999; Boyce *et al*., 2003) and in other important developmental processes such as flowering (Knight *et al*., 2008). Mapping and cloning of *SFR6* identified *At4g04920* in chromosome 4 near the centromere as the locus for *SFR6* and as a 135kDa protein with unknown function (Knight *et al*., 2009). The work discussed in this thesis was started from this point with the idea to engineer SFR6 to improve crop tolerance to stress without prior knowledge of its identity as a plant mediator subunit.

The aim of this Chapter is to make conclusion on the most important findings and suggest further experiments.

8.2 Important findings of the current work

1. Over-expression of *SFR6* alone cannot be used as a molecular tool to improve crop tolerance to stress.

In this study overexpression of *SFR6* (a subunit of plant mediator complex) in wild type *Arabidopsis* did not affect *KIN2* and *CBF1* gene expression. However, the complementation of *sfr6-1* mutant by a *35S*::*SFR6* construct revealed that SFR6 is essential but alone not sufficient for *KIN2* gene expression. Consistent with previous observations it was suggested that mediator subunits combine in a complex in equal molar ratios (Guglielmi *et al*., 2004). Therefore, over-expression of one subunit of the mediator complex might not affect the function of the complex as a whole. Moreover, mediator subunits are linked to each other and these links are essential to get maximum output of mediator function on regulation of transcription (Jiang and Stillman, 1992; Kidd *et al*., 2009). Therefore, over-expression of SFR6 cannot be used as a basic molecular tool to improve crop tolerance to stress. However, protein sequences could be mutated to enhance the activity and thereby it might possible to use SFR6 to improve plant tolerance to stress. Before this step the proper understanding of the function of SFR6 on regulation of stress induced gene expression is important.

2. SFR6/MED16 is a possible convergence point between freezing, UV irradiance and pathogen induced signalling pathways

The results reported in Chapter 4 showed a possible link between cold, UV irradiance and pathogen induced signalling pathways via SFR6. Previous studies on the *sfr6-1* mutant also showed a role for SFR6 in drought response gene expression (Knight *et al*., 1999; Boyce *et al*., 2003; Knight *et al*., 2009) and genes involved in

flowering and circadian clock function (Knight *et al*., 2008). Altogether, the results of this study and previous studies indicate a wider role of SFR6/MED16 in shaping the plant stress transcriptome profile.

3. SFR6/MED16 regulates expression of defence genes via SA- and JA-mediated signalling pathways as well as the metacaspase induced programmed cell death pathway

Similar to the freezing sensitivity of *sfr6* mutants which is due to defective expression of a group of *COR* gene (Knight *et al*., 1999; Knight *et al*., 2009), current work revealed the hyper-sensitivity of *sfr6* mutants to UV irradiance and pathogen infection is most likely due to reduced expression of plant defence genes (Chapter 5). This effect extended to not only SA- and JA-mediated defence genes but also the metacaspase induced programme cell death pathway indicating possible interactions of SFR6/MED16 with transcription factors responsible for defence gene expression via all these signalling pathways. The data presented in this thesis also showed that the expression of *NPR1* transcripts was not altered in *sfr6* mutants either upon UV irradiance and pathogen infection or exogenous application of sodium salicylate. This indicates that SFR6/MED16 might act after *NPR1* transcription indicating similarities with SFR6 action on the CBF cold-response pathway (Knight *et al*., 1999; Knight *et al*., 2009). Taken together these results indicate that SFR6 is a possible convergence point for the activation of SA- and JA-mediated defence genes as well as the metacapase induced programmed cell death pathway. However, further investigations of defence gene expression in *sfr6* mutants is required to determine whether SFR6/MED16 acts as a convergence point of these three plant defence mechanisms.

4. *AtSFR6* function is conserved in rice

Finally, the demonstration of functional complementation of the *sfr6-1* mutant phenotype by *OsSFR6* revealed that the SFR6 function might be conserved in rice.

Rice is a chilling-sensitive monocot plant. The function of OsSFR6 might be in mediating responsive to chilling-tolerance in rice. Therefore, further characterization of OsSFR6 function in rice will advance our understanding of the conservation and the evolutional divergence of SFR6 function in freezing tolerant and intolerant species.

8.3 Future work

Identification of proteins interacting with SFR6

Present (Chapter 5) and previous (Knight *et al*., 2009) analysis of SFR6 function on stress response gene expression showed a possibility that SFR6 binds transcription factors directly. Therefore, identification of proteins interacting with SFR6 will help to understand the specific roles of its transcriptional regulation of gene expression in plants. Similarly, identification of other mediator subunits interacting with SFR6 will also be important in advancing our knowledge on the role of mediator on transcriptional regulation because, as shown by Jiang and Stillman (1992), yeast MED16 physically binds with other tail subunits. Yeast -2-hybrid screening and large scale purification of SFR6 interacting proteins by using TAP or STREP tagged SFR6 under various induction conditions such as UV, freezing, mannitol *etc.* can be used to identify which proteins interact with SFR6. Screening T-DNA insertion lines for mutations in other mediator subunits will also be important to identify other mediator subunits interact with SFR6. Once identified T-DNA lines mutated in other tail subunits can be tested for their response to cold, UV irradiance, pathogen infection and alteration of flowering time. These results will help us to understand whether SFR6 acts as a linking subunit of other tail subunits to middle part of the mediator.

Microarray analysis of UV- and pathogen-induced gene expression

Conducting microarray analysis by comparing samples from UV treated and *P. syringae* infiltrated *sfr6-1* mutant and wild type plants, will help to examine the extent of the effect of SFR6 upon UV irradiance and pathogen infections induce defence gene expression. Similar to the identification of effects of SFR6 on genes with CRT/DRE motif by microarray analysis (Boyce *et al*., 2003) this will lead to identification of transcription factors involved in UV- and pathogen-responsive defence pathways through SFR6. Microarray results will also provide important insights into SFR6 activity on other plant defence mechanisms which are not investigated in this work such as the flavonoid biosynthesis pathway.

Further characterization of effect of SFR6 upon NPR1-dependent *PR* gene expression

Previous research showed SFR6 operates downstream of CBF protein (Knight *et al*., 2009). This work demonstrates unaltered levels of *NPR1* expression during significant reduction of UV irradiance and pathogen-induced *PR1* gene expression (Chapter 5). However, NPR1 does not directly bind to the promoter of pathogenesis related genes (Zhang *et al*., 1999; Despres *et al*., 2000). As described in Chapter 5 section 5.1.3 NPR1 induces TGA transcription factors to bind with the promoter of the *PR1* gene (Zhang *et al*., 1999; Despres *et al*., 2000). Current work did not analyse expression of TGAs in *sfr6* mutants. Therefore, analysing expression of TGAs in *sfr6-1* mutant will be important to suggest whether the effect of SFR6 is upstream or downstream of TGAs. Parallel with this work, producing genetic crosses of *sfr6* mutants with *35S::NPR1* and *35S::TGAs* and measuring the protein levels of NPR1 and TGAs in *sfr6* mutant and wild type plants responding to UV irradiance and pathogen infection will also be important to reveal the role of SFR6 on NPR1-dependent defence gene expression and more importantly these results will allow us to elucidate the defence mechanism involving SFR6. These results will also allow us to assess the similarities and differences of SFR6 action in freezing and defence gene expression

Further characterization of the function of OsSFR6 in rice

Current work revealed functional orthology of *OsSFR6* by a complementation assay involving the *Arabidopsis sfr6-1* mutant and the *OsSFR6* gene. However, generation of *OsSFR6* knockout rice and examining the phenotypic differences are a must for proper understanding of the function of this gene. It is also important to mention that according to the rice genome database the rice knock out of mediator subunit has not yet been reported. Current work identified an AtSFR6 orthologue from rice (Chapter 6) and a miRNA construct has been produced for *OsSFR6* knockdown analysis (Chapter 6). Therefore, *OsSFR6* knockout rice can be generated either by using RNAi or miRNAi constructs for further investigations. Once *OsSFR6* knockdown rice plants have been generated, microarray analysis can be performed to identify the effect on the expression of stress induced genes of rice.

In addition, bioluminescence assays can be used to examine specific effect of *OsSFR6* gene on CRT/DRE motif. This can be performed using the promoters of cold induced rice genes with CRT/DRE element in their promoter such as *jacalin1* (*Jac1*) and *lipoxygenase* (*LOX*). Oh *et al.* (2005) showed that *Jac1* (*AK066682* or *Os12g0247700*) is a highly cold induced rice gene with CRT/DRE motif in its promoter and *LOX* (*AJ270938* or *Os12t05592000*) gene is not induced as high as *Jac1* but has three copies of CRT/DRE element in its promoter. The constructs of the promoters of these two genes fused to the LUC+ gene can bombarded into rice seedlings. Then the expression of LUC can be measured using a luminometer. These results will allow us to understand the specificity of OsSFR6 for genes with CRT/DRE motif.

8.4 Conclusion

In conclusion the results reported in this thesis demonstrate the requirement of SFR6/MED16 for the activation of many but not all stress response gene expression, and indicated conserved *AtSFR6* function in rice. However, the mechanism of regulation of stress induced gene expression via SFR6/MED16 remains to be further

investigated. The future research on specific roles of individual subunits and of the whole complex will widen our knowledge of the transcriptional regulation of gene expression in plant and will create new routes to improve crop tolerance to environmental stress.

Bibliography

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell **15:** 63-78

Agarwal M, Hao YJ, Kapoor A, Dong CH, Fujii H, Zheng XW, Zhu JK (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. Journal of Biological Chemistry **281:** 37636-37645

Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology **55:** 373-399

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin CT, Thomashow MF (1996) Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. Proceedings of the National Academy of Sciences of the United States of America **93:** 13404-13409

Asturias FJ, Jiang YW, Myers LC, Gustafsson CM, Kornberg RD (1999) Conserved structures of mediator and RNA polymerase II holoenzyme. Science **283:** 985-987

Atici O, Nalbantoglu B (2003) Antifreeze proteins in higher plants. Phytochemistry **64:** 1187-1196

Autran D, Jonak C, Belcram K, Beemster GTS, Kronenberger J, Grandjean O, Inze D, Traas J (2002) Cell numbers and leaf development in *Arabidopsis*: a functional analysis of the STRUWWELPETER gene. Embo Journal **21:** 6036-6049

Backstrom S, Elfving N, Nilsson R, Wingsle G, Bjorklund S (2007) Purification of a plant mediator from *Arabidopsis thaliana* identifies PFT1 as the Med25 subunit. Molecular Cell **26:** 717-729

Badawi M, Danyluk J, Boucho B, Houde M, Sarhan F (2007) The CBF gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal CBFs. Molecular Genetics and Genomics **277:** 533-554

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008) Structure and functional analysis of wheat ICE (inducer of CBF expression) genes. Plant and Cell Physiology **49:** 1237-1249

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5'-region of *Arabidopsis*-thaliana cor15a has cis-acting elements that confer cold-regulated, drought-regulated and aba-regulated gene-expression. Plant Molecular Biology **24:** 701-713

Bjorklund S, Gustafsson CM (2005) The yeast Mediator complex and its regulation. Trends in Biochemical Sciences **30:** 240-244

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals of Botany **91:** 179-194

Bourbon HM (2008) Comparative genomics supports a deep evolutionary origin for the large, four-module transcriptional mediator complex. Nucleic Acids Research **36:** 3993-4008

Bourbon HM, Aguilera A, Ansari AZ, Asturias FJ, Berk AJ, Bjorklund S, Blackwell TK, Borggrefe T, Carey M, Carlson M, Conaway JW, Conaway RC, Emmons SW, Fondell JD, Freedman LP, Fukasawa T, Gustafsson CM, Han M, He X, Herman PK, Hinnebusch AG, Holmberg S, Holstege FC, Jaehning JA, Kim YJ, Kuras L, Leutz A, Lis JT, Meisterernest M, Naar AM, Nasmyth K, Parvin JD, Ptashne M, Reinberg D, Ronne H, Sadowski I, Sakurai H, Sipiczki M, Sternberg PW, Stillman DJ, Strich R, Struhl K, Svejstrup JQ, Tuck S, Winston F, Roeder RG, Kornberg RD (2004) A unified nomenclature for protein subunits of Mediator complexes linking transcriptional regulators to RNA polymerase II. Molecular Cell **14:** 553-557

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong XI (1994) A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired-resistance. Plant Cell **6:** 1845-1857

Boyce JM, Knight H, Deyholos M, Openshaw MR, Galbraith DW, Warren G, Knight MR (2003) The sfr6 mutant of *Arabidopsis* is defective in transcriptional activation via CBF/DREB1 and DREB2 and shows sensitivity to osmotic stress. Plant Journal **34:** 395-406

Bryant GO, Ptashne M (2003) Independent recruitment in vivo by Gal4 of two complexes required for transcription. Molecular Cell **11:** 1301-1309

Campbell SA, Close TJ (1997) Dehydrins: genes, proteins, and associations with phenotypic traits. *New Phytologist*, **137**, 61–74

Cao H, Bowling SA, Gordon AS, Dong XN (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired-resistance. Plant Cell **6:** 1583-1592

Cao H, Glazebrook J, Clarke JD, Volko S, Dong XN (1997) The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88:** 57-63

Cerdan PD, Chory J (2003) Regulation of flowering time by light quality. Nature **423:** 881-885

Chadick JZ, Asturias FJ (2005) Structure of eukaryotic Mediator complexes. Trends in Biochemical Sciences **30:** 264-271

Chen WQ, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou GZ, Whitham SA, Budworth PR, Tao Y, Xie ZY, Chen X, Lam

S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X, Zhu T (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell **14:** 559-574

Chen WQ, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou GZ, Whitham SA, Budworth PR, Tao Y, Xie ZY, Chen X, Lam S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X, Zhu T (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell **14:** 559-574

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong XH, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes & Development **17:** 1043-1054

Chinnusamy V, Schumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. Journal of Experimental Botany **55:** 225-236

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends in Plant Science **12:** 444-451

Choi DW, Rodriguez EM, Close TJ (2002) Barley Cbf3 gene identification, expression pattern, and map location. Plant Physiology **129:** 1781-1787

Choi HI, Hong JH, Ha JO, Kang JY, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. Journal of Biological Chemistry **275:** 1723-1730

Clack T, Mathews S, Sharrock RA (1994) The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: the sequences and expression of *PHYD* and *PHYE*. *Plant Mol. Biol.* 25: 413-427

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant Journal **16:** 735-743

Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F (1998) Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell*, **10**, 623–638

Dean JV, Mohammed LA, Fitzpatrick T (2005) The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures. Planta **221:** 287-296

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gutrella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic-acid in plant-disease resistance. Science **266:** 1247-1250

Dempsey DA, Shah J, Klessig DF (1999) Salicylic acid and disease resistance in plants. Critical Reviews in Plant Sciences **18:** 547-575

Despres C, DeLong C, Glaze S, Liu E, Fobert PR (2000) The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12:** 279-290

Desveaux D, Subramaniam R, Despres C, Mess JN, Levesque C, Fobert PR, Dangl JL, Brisson N (2004) A "whirly" transcription factor is required for salicylic acid-dependent disease resistance in *Arabidopsis*. Developmental Cell **6:** 229-240

Dhawan R, Luo HL, Foerster AM, AbuQamar S, Du HN, Briggs SD, Scheid OM, Mengiste T (2009) HISTONE MONOUBIQUITINATION1 Interacts with a Subunit of the Mediator Complex and Regulates Defense against Necrotrophic Fungal Pathogens in *Arabidopsis*. Plant Cell **21:** 1000-1019

Dian-jun M, Xiang-yang H, Yu Z, Kui-de Y (2008) Over-expression of ICE1 gene in transgenic rice improves cold tolerence. Rice Science **15(3):** 173-178

Dong CH, Agarwal M, Zhang YY, Xie Q, Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proceedings of the National Academy of Sciences of the United States of America **103:** 8281-8286

Dong XN (2001) Genetic dissection of systemic acquired resistance. Current Opinion in Plant Biology **4:** 309-314

Dong XN (2004) NPR1, all things considered. Current Opinion in Plant Biology **7:** 547-552

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant Journal **33:** 751-763

Durrant WE, Dong X (2004) Systemic acquired resistance. Annual Review of Phytopathology **42:** 185-209

Eulgem T (2005) Regulation of the *Arabidopsis* defense transcriptome. Trends in Plant Science **10:** 71-78

Falk A, Feys BJ, Frost LN, Jones JDG, Daniels MJ, Parker JE (1999) EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proceedings of the National Academy of Sciences of the United States of America **96:** 3292-3297

Fan J, Crooks C, Lamb C (2008) High-throughput quantitative luminescence assay of the growth in planta of Pseudomonas syringae chromosomally tagged with Photorhabdus luminescens luxCDABE. Plant Journal 53: 393-399

Flanagan PM, Kelleher, R.J., lll, Sayre, M.H., Tschochner, H., and Kornberg, R.D. (1991) A mediator required for activation of RNA polymerase ll transcription in vitro. Nature **350:** 436-438

Fourrier N, Bedard J, Lopez-Juez E, Barbrook A, Bowyer J, Jarvis P, Warren G, Thorlby G (2008) A role for SENSITIVE TO FREEZING2 in protecting chloroplasts against freeze-induced damage in *Arabidopsis*. Plant Journal **55:** 734-745

Fowler S, Thomashow MF (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell **14:** 1675-1690

Franklin KA, Whitelam GC (2005) Phytochromes and shade-avoidance responses in plants. *Ann Bot (Lond)* 96: 169-175.

Friedrich L, Lawton K, Dietrich R, Willits M, Cade R, Ryals J (2001) NIM1 overexpression in *Arabidopsis* potentiates plant disease resistance and results in enhanced effectiveness of fungicides. Molecular Plant-Microbe Interactions **14:** 1114-1124

Fujibe T, Saji H, Arakawa K, Yabe N, Takeuchi Y, Yamamoto KT (2004) A methyl viologen-resistant mutant of *Arabidopsis*, which is allelic to ozone-sensitive rcd1, is tolerant to supplemental ultraviolet-B irradiation. Plant Physiology **134:** 275-285

Fujikawa S, Jitsuyama Y, Kuroda K (1999) Determination of the role of cold acclimation-induced diverse changes in plant cells from the viewpoint of avoidance of freezing injury. Journal of Plant Research **112:** 237-244

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Current Opinion in Plant Biology **9:** 436-442

Fursova OV, Pogorelko GV, Tarasov VA (2009) Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana*. Gene **429:** 98-103

Gaudet DA, Laroche A, Frick M, Davoren J, Puchalski B, Ergon A (2000) Expression of plant defence-related (PR-protein) transcripts during hardening and dehardening of winter wheat. Physiological and Molecular Plant Pathology **57:** 15-24

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology **124:** 1854-1865

Gilmour SJ, Thomashow MF (1991) Cold-acclimation and cold-regulated gene-expression in aba mutants of *Arabidopsis*-thaliana. Plant Molecular Biology **17:** 1233-1240

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant Journal **16:** 433-442

Griffith M, Yaish MWF (2004) Antifreeze proteins in overwintering plants: a tale of two activities. Trends in Plant Science **9:** 399-405

Guglielmi B, van Berkum NL, Klapholz B, Bijma T, Boube M, Boschiero C, Bourbon HM, Holstege FCP, Werner M (2004) A high resolution protein interaction map of the yeast Mediator complex. Nucleic Acids Research **32:** 5379-5391

Guo Y, Xiong L, Ishitani M, Zhu JK (2002) An *Arabidopsis* mutation in translation elongation factor 2 causes superinduction of CBF/DREB1 transcription factor genes but blocks the induction of their downstream targets under low temperatures. Proceedings of the National Academy of Sciences of the United States of America **99:** 7786-7791

Guy CL, Niemi KJ, Brambl R (1985) Altered gene-expression during cold-acclimation of spinach. Proceedings of the National Academy of Sciences of the United States of America **82:** 3673-3677

Haake V, Cook D, Riechmann JL, Pineda O, Thomashow MF, Zhang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. Plant Physiology **130:** 639-648

Hahn S (2004) Structure and mechanism of the RNA polymerase II transcription machinery. Nature Structural & Molecular Biology **11:** 394-403

He R, Drury GE, Rotari VI, Gordon A, Willer M, Farzaneh T, Woltering EJ, Gallois P (2008) Metacaspase-8 modulates programmed cell death induced by ultraviolet light and H2O2 in *Arabidopsis*. Journal of Biological Chemistry **283:** 774-783

He SY (1998) Type III protein secretion systems in plant and animal pathogenic bacteria. Annual Review of Phytopathology **36:** 363-392

Heath MC (2000) Hypersensitive response-related death. Plant Molecular Biology **44:** 321-334

Heino P, Sandman G, Lang V, Nordin K, Palva ET (1990) Abscisic-acid deficiency prevents development of freezing tolerance in *Arabidopsis thaliana* (l) heynh. Theoretical and Applied Genetics **79:** 801-806

Helen J Rushton (2009) PhD thesis: Investigating calcium regulated gene expression in Arabidopsis thaliana. University of Oxford.

Hematy K, Cherk C, Somerville S (2009) Host-pathogen warfare at the plant cell wall. Current Opinion in Plant Biology **12:** 406-413

Hipkins MF and Baker NR (1986) Spectroscopy. In Photosynthesis-Energy Transduction: A practical approach. *Edited by* Hipkins and Barker N.R. IRL press, Oxford.

Hoeberichts FA, ten Have A, Woltering EJ (2003) A tomato metacaspase gene is upregulated during programmed cell death in Botrytis cinerea-infected leaves. Planta **217:** 517-522

Holsters M, Silva B, Vanvliet F, Hernalsteens JP, Genetello C, Vanmontagu M, Schell J (1978) Invivo transfer of ti-plasmid of Agrobacterium tumefaciens to Escherichia coli. Molecular & General Genetics **163:** 335-338

Hon WC, Griffith M, Mlynarz A, Kwok YC, Yang DSC (1995) Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiology **109:** 879-889

Hood EE, Gelvin SB, Melchers S, Hoekema A (1993) New Agrobacterium helper plasmids for gene transfer to plants (EHA105). *Trans Res* 2:208-218

Hsieh TH, Lee JT, Yang PT, Chiu LH, Charng YY, Wang YC, Chan MT (2002) Heterology expression of the *Arabidopsis* C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. Plant Physiology **129:** 1086-1094

Imai R, Chang L, Ohta A, Bray EA, Takagi M (1996) A lea-class gene of tomato confers salt and freezing tolerance when expressed in Saccharomyces cerevisiae. Gene **170:** 243-248

Ishitani M, Xiong LM, Lee HJ, Stevenson B, Zhu JK (1998) HOS1, a genetic locus involved in cold-responsive gene expression in *Arabidopsis*. Plant Cell **10:** 1151-1161

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant and Cell Physiology **47:** 141-153

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in Brassica napus and other plant species. Plant Physiology **127:** 910-917

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. Science **280:** 104-106

Jiang YW, Dohrmann PR, Stillman DJ (1995) Genetic and physical interactions between yeast rgr1 and sin4 in chromatin organization and transcriptional regulation. Genetics **140:** 47-54

Jiang YW, Stillman DJ (1992) Involvement of the sin4 global transcriptional regulator in the chromatin structure of saccharomyces-cerevisiae. Molecular and Cellular Biology **12:** 4503-4514

Jin HL, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000) Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. Embo Journal **19:** 6150-6161

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J (1999) *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proceedings of the National Academy of Sciences of the United States of America **96:** 13583-13588

Jones JDG, Dangl JL (2006) The plant immune system. Nature **444:** 323-329

Kang JY, Choi HI, Im MY, Kim SY (2002) *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343-357

Karimi M, Inze D, Depicker A (2002) GATEWAY((TM)) vectors for Agrobacterium-mediated plant transformation. Trends in Plant Science **7:** 193-195

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology **17:** 287-291

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004) A combination of the *Arabidopsis* DREB1A gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant and Cell Physiology **45:** 346-350

Katagiri F, Thilmony R, He SY (2002) The *Arabidopsis thaliana* –Pseudomonase syringae interaction. In: The *Arabidopsis* Book. Eddited by Somerville, C.R. and Meyerowitz EM

Kidd BN, Edgar CI, Kumar KK, Aitken EA, Schenk PM, Manners JM, Kazan K (2009) The Mediator Complex Subunit PFT1 Is a Key Regulator of Jasmonate-Dependent Defense in *Arabidopsis*. Plant Cell **21:** 2237-2252

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K (2007) The AtGenExpress global stress

expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. Plant Journal **50:** 347-363

Kim YJ, Bjorklund S, Li Y, Sayre MH, Kornberg RD (1994) A multiprotein mediator of transcriptional activation and its interaction with the c-terminal repeat domain of rna-polymerase-II. Cell **77:** 599-608

King EO, Ward MK and Raney DE (1954) Two simple media for the demonstration of phycocyanin and fluorescin. J Lab Clin Med 44, 301–307

Klement Z, Bozso Z, Kecskes ML, Besenyei E, Arnold C, Ott PG (2003) Local early induced resistance of plants as the first line of defence against bacteria. Pest Management Science **59:** 465-474

Knight H, Knight MR (2001) Abiotic stress signalling pathways: specificity and cross-talk. Trends in Plant Science **6:** 262-267

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8:** 489-503

Knight H, Mugford SG, Ulker B, Gao DH, Thorlby G, Knight MR (2009) Identification of SFR6, a key component in cold acclimation acting post-translationally on CBF function. Plant Journal **58:** 97-108

Knight H, Thomson AJW, McWatters HG (2008) Sensitive to freezing6 integrates cellular and environmental inputs to the plant circadian clock. Plant Physiology **148:** 293-303

Knight H, Veale EL, Warren GJ, Knight MR (1999) The sfr6 mutation in *Arabidopsis* suppresses low-temperature induction of genes dependent on the CRT DRE sequence motif. Plant Cell **11:** 875-886

Knight H, Zarka DG, Okamoto H, Thomashow ME, Knight MR (2004) Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. Plant Physiology **135:** 1710-1717

Kreps J, Budworth P, Goff S, Wang RL (2003) Identification of putative plant cold responsive regulatory elements by gene expression profiling and a pattern enumeration algorithm. Plant Biotechnology Journal **1:** 345-352

Kreps JA, Wu YJ, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiology **130:** 2129-2141

Kume S, Kobayashi F, Ishibashi M, Ohno R, Nakamura C, Takumi S (2005) Differential and coordinated expression of Cbf and Cor/Lea genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. Genes & Genetic Systems **80:** 185-197

Kumlehn J (2008) The IPKb vector set: Molecular binary plasmids for cereal transformation. ISB News Report. April

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Current Opinion in Plant Biology **5:** 325-331

Kuras L, Borggrefe T, Kornberg RD (2003) Association of the mediator complex with enhancers of active genes. Proceedings of the National Academy of Sciences of the United States of America **100:** 13887-13891

Lafitte HR IA, Bennett J (2004) Abiotic stress tolerance in rice for Asia: Progress and the future. International Rice Research Institute, www.irri.cgiar.org

Lee H, Guo Y, Ohta M, Xiong LM, Stevenson B, Zhu JK (2002) LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase. Embo Journal **21:** 2692-2702

Lee HI, Leon J, Raskin I (1995) Biosynthesis and metabolism of salicylic-acid. Proceedings of the National Academy of Sciences of the United States of America **92:** 4076-4079

Lee HJ, Xiong LM, Gong ZZ, Ishitani M, Stevenson B, Zhu JK (2001) The *Arabidopsis* HOS1 gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. Genes & Development **15:** 912-924

Lee SC, Huh KW, An K, An G, Kim SR (2004) Ectopic expression of a cold-inducible transcription factor, CBF1/DREB1b, in transgenic rice (Oryza sativa L.). Molecules and Cells **18:** 107-114

Li X, Zhang YL, Clarke JD, Li Y, Dong XN (1999) Identification and cloning of a negative regulator of systemic acquired resistance, SNlI1, through a screen for suppressors of npr1-1. Cell **98:** 329-339

Li Y, Bjorklund S, Jiang YW, Kim YJ, Lane WS, Stillman DJ, Kornberg RD (1995) Yeast global transcriptional regulators SIN4 and RGR1 are components of mediator complex rna-polymerase-II holoenzyme. Proceedings of the National Academy of Sciences of the United States of America **92:** 10864-10868

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell **10:** 1391-1406

Lorenzo O, Solano R (2005) Molecular players regulating the jasmonate signalling network. Current Opinion in Plant Biology **8:** 532-540

Mackerness SAH, Surplus SL, Blake P, John CF, Buchanan-Wollaston V, Jordan BR, Thomas B (1999) Ultraviolet-B-induced stress and changes in gene expression in *Arabidopsis thaliana*: role of signalling pathways controlled by jasmonic acid, ethylene and reactive oxygen species. Plant Cell and Environment **22:** 1413-1423

Mahalingam R, Fedoroff N (2003) Stress response, cell death and signalling: the many faces of reactive oxygen species. Physiologia Plantarum **119:** 56-68

Martinez C, Pons E, Prats G, Leon J (2004) Salicylic acid regulates flowering time and links defence responses and reproductive development. Plant Journal **37:** 209-217

Mayda E, Mauch-Mani B, Vera P (2000) *Arabidopsis* dth9 mutation identifies a gene involved in regulating disease susceptibility without affecting salicylic acid-dependent responses. Plant Cell **12:** 2119-2128

McKnow R. KGaWG (1996) Cold responses of *Arabidopsis* mutants impaired in freezing tolerence. Journal of Experimental Botany **47:** 1919-1925

Michael MN, Fankhauser C, Chory J (2000) Light: an indicator of time and place. Genes & Development 14:257–271

Mikkelsen MD, Thomashow MF (2009) A role for circadian evening elements in cold-regulated gene expression in *Arabidopsis*. Plant Journal **60:** 328-339

Miller AK, Galiba G, Dubcovsky J (2006) A cluster of 11 CBF transcription factors is located at the frost tolerance locus Fr-A(m)2 in Triticum monococcum. Molecular Genetics and Genomics **275:** 193-203

Mou Z, Fan WH, Dong XN (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113:** 935-944

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15(3): 473-497

Myers LC, Gustafsson CM, Bushnell DA, Lui M, Erdjument-Bromage H, Tempst P, Kornberg RD (1998) The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain. Genes & Development **12:** 45-54

Myers LC, Gustafsson CM, Hayashibara KC, Brown PO, Kornberg RD (1999) Mediator protein mutations that selectively abolish activated transcription. Proceedings of the National Academy of Sciences of the United States of America **96:** 67-72

Myers LC, Kornberg RD (2000) Mediator of transcriptional regulation. Annual Review of Biochemistry **69:** 729-749

Nakashima K, Shinwari ZK, Sakuma Y, Seki M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2000) Organization and expression of two *Arabidopsis* DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. Plant Molecular Biology **42:** 657-665

Nawrath C, Heck S, Parinthawong N, Metraux JP (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. Plant Cell **14:** 275-286

Nawrath C, Metraux JP (1999) Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11:** 1393-1404

Nishimura A, Aichi I, Matsuoka M (2006) A protocol for Agrobacterium-mediated transformation in rice. Nature Protocols **1:** 2796-2802

Nobuta K, Okrent RA, Stoutemyer M, Rodibaugh N, Kempema L, Wildermuth MC, Innes RW (2007) The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent Defense responses in *Arabidopsis*. Plant Physiology **144:** 1144-1156

Novillo F, Alonso JM, Ecker JR, Salinas J (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in, stress tolerance in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **101:** 3985-3990

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH, Kim JK (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiology **138:** 341-351

Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell **13:** 1959-1968

Oono Y, Seki M, Nanjo T, Narusaka M, Fujita M, Satoh R, Satou M, Sakurai T, Ishida J, Akiyama K, Iida K, Maruyama K, Satoh S, Yamaguchi-Shinozaki K, Shinozaki K (2003) Monitoring expression profiles of *Arabidopsis* gene expression during rehydration process after dehydration using ca. 7000 full-length cDNA microarray. Plant Journal **34:** 868-887

Ossowski S, Schwab R, Weigel D (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. Plant Journal **53:** 674-690

Park SY, Noh KJ, Yoo JH, Yu JW, Lee BW, Kim JG, Seo HS, Paek NC (2006) Rapid upregulation of Dehyrin3 and Dehydrin4 in response to dehydration is a characteristic of drought tolerant genotypes in barley. Journal of Plant Biology **49:** 455-462

Parker JE, Holub EB, Frost LN, Falk A, Gunn ND, Daniels MJ (1996) Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to Peronospora parasitica specified by several different RPP genes. Plant Cell **8:** 2033-2046

Pearce RS (1999) Molecular analysis of acclimation to cold. Plant Growth Regulation **29:** 47-76

Penninckx I, Eggermont K, Terras FRG, Thomma B, DeSamblanx GW, Buchala A, Metraux JP, Manners JM, Broekaert WF (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell **8:** 2309-2323

Pieterse CMJ, van Loon LC (1999) Salicylic acid-independent plant defence pathways. Trends in Plant Science **4:** 52-58

Puhakainen T, Hess MW, Makela P, Svensson J, Heino P, Palva ET (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis*. Plant Molecular Biology **54:** 743-753

Qiang Liu MK, Yoh Sakuma, Hiroshi Abe, Setsuko Miura, Kazuko Yamaguchi-Shinozaki, and Kazuo Shinozaki (1998) Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low-Temperature-Responsive Gene Expression, Respectively, in *Arabidopsis*. Plant Cell. **Vol. 10:** 1391-1406

Rate DN, Cuenca JV, Bowman GR, Guttman DS, Greenberg JT (1999) The gain-of-function *Arabidopsis* acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell **11:** 1695-1708

Riechmann JL, Heard J, Martin G, Reuber L, Jiang CZ, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu CL (2000) *Arabidopsis* transcription factors: Genome-wide comparative analysis among eukaryotes. Science **290:** 2105-2110

Rotari VI, He R, Gallois P (2005) Death by proteases in plants: whodunit. Physiologia Plantarum **123:** 376-385

Rushton PJ, Somssich IE (1998) Transcriptional control of plant genes responsive to pathogens. Current Opinion in Plant Biology **1:** 311-315

Rymen B, Fiorani F, Kartal F, Vandepoele K, Inze D, Beemster GTS (2007) Cold nights impair leaf growth and cell cycle progression in maize through transcriptional changes of cell cycle genes. Plant Physiology **143:** 1429-1438

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell **18:** 1292-1309

Samuelsen CO, Baraznenok V, Khorosjutina O, Spahr H, Kieselbach T, Holmberg S, Gustafsson CM (2003) TRAP230/ARC240 and TRAP240/ARC250 Mediator subunits are functionally conserved through evolution. Proceedings of the National Academy of Sciences of the United States of America **100:** 6422-6427

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM (2000) Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proceedings of the National Academy of Sciences of the United States of America **97:** 11655-11660

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K (2001) Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell **13:** 61-72

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant Journal **31:** 279-292

Shah J, Tsui F, Klessig DF (1997) Characterization of a salicylic acid-insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. Molecular Plant-Microbe Interactions **10:** 69-78

Shinozaki K, YamaguchiShinozaki K (1997) Gene expression and signal transduction in water-stress response. Plant Physiology **115:** 327-334

Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Current Opinion in Plant Biology **3:** 217-223

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Plant Biology **6:** 410-417

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochemical and Biophysical Research Communications **250:** 161-170

Singh KB, Foley RC, Onate-Sanchez L (2002) Transcription factors in plant defense and stress responses. Current Opinion in Plant Biology **5:** 430-436

Skinner JS, von Zitzewitz J, Szucs P, Marquez-Cedillo L, Filichkin T, Amundsen K, Stockinger EJ, Thomashow MF, Chen THH, Hayes PM (2005)

Structural, functional, and phylogenetic characterization of a large CBF gene family in barley. Plant Molecular Biology **59:** 533-551

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Metraux JP, Brown R, Kazan K, Van Loon LC, Dong XN, Pieterse CMJ (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15:** 760-770

Steponkus PL (1984) Role of the plasma-membrane in freezing-injury and cold-acclimation. Annual Review of Plant Physiology and Plant Molecular Biology **35:** 543-584

Steponkus PL, Lynch DV, Uemura M (1990) The influence of cold-acclimation on the lipid-composition and cryobehavior of the plasma-membrane of isolated rye protoplasts. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **326:** 571-583

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proceedings of the National Academy of Sciences of the United States of America **94:** 1035-1040

Tantau H, Dorffling K (1991) TantauIn vitro-selection of hydroxyproline-resistant cell lines of wheat *(Jfiticum aestivum):* accumulation of proline, decrease in osmotic potential, and increase in frost tolerance. Physiologia plantarum 82: 243-248.

Thomas MC, Chiang CM (2006) The general transcription machinery and general cofactors. Critical Reviews in Biochemistry and Molecular Biology **41:** 105-178

Thomashow MF (1994) *Arabidopsis thaliana* as a model for studying mechanisms of plant cold tolerance. *In* E Meyerowitz, C Somerville, eds, *Arabidopsis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 807–834

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiology **118:** 1-7

Thomashow MF (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annual Review of Plant Physiology and Plant Molecular Biology **50:** 571-599

Thomashow MF (2001) So what's new in the field of plant cold acclimation? Lots! Plant Physiology **125:** 89-93

Thomashow MF, Stockinger EJ, Jaglo-Ottosen KR, Gilmour SJ, Zarka DG (1997) Function and regulation of *Arabidopsis thaliana* COR (cold-regulated) genes. Acta Physiologiae Plantarum **19:** 497-504

Thordal-Christensen H (2003) Fresh insights into processes of nonhost resistance. Current Opinion in Plant Biology **6:** 351-357

Thorlby G, Fourrier N, Warren G (2004) The sensitive to freezing2 gene, required for freezing tolerance in *Arabidopsis thaliana*, encodes a beta-Glucosidase. Plant Cell **16:** 2192-2203

Tsutsui T, Asada Y, Tamaoki M, Ikeda A, Yamaguchi J (2008) *Arabidopsis* CAD1 negatively controls plant immunity mediated by both salicylic acid-dependent and -independent signaling pathways. Plant Science **175:** 604-611

Tsutsui T, Kato W, Asada Y, Sako K, Sato T, Sonoda Y, Kidokoro S, Yamaguchi-Shinozaki K, Tamaoki M, Arakawa K, Ichikawa T, Nakazawa M, Seki M, Shinozaki K, Matsui M, Ikeda A, Yamaguchi J (2009) DEAR1, a transcriptional repressor of DREB protein that mediates plant defense and freezing stress responses in *Arabidopsis*. Journal of Plant Research **122:** 633-643

Uemura M, Joseph RA, Steponkus PL (1995) Cold-acclimation of *Arabidopsis*-thaliana - effect on plasma-membrane lipid-composition and freeze-induced lesions. Plant Physiology **109:** 15-30

Uemura M, Warren G, Steponkus PL (2003) Freezing sensitivity in the sfr4 mutant of *Arabidopsis* is due to low sugar content and is manifested by loss of osmotic responsiveness. Plant Physiology **131:** 1800-1807

Uren AG, O'Rourke K, Aravind L, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM (2000) Identification of paracaspases and metacaspases: Two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Molecular Cell **6:** 961-967

Vasyukova NI, Ozeretskovskaya OL (2009) Jasmonate-Dependent Defense Signaling in Plant Tissues. Russian Journal of Plant Physiology **56:** 581-590

Vlachonasios KE, Thomashow MF, Triezenberg SJ (2003) Disruption mutations of ADA2b and GCN5 transcriptional adaptor genes dramatically affect *Arabidopsis* growth, development, and gene expression. Plant Cell **15:** 626-638

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG, Thomashow MF (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. Plant Journal **41:** 195-211

Wang QY, Guan YC, Wu YR, Chen HL, Chen F, Chu CC (2008) Overexpression of a rice OsDREB1F gene increases salt, drought, and low temperature tolerance in both *Arabidopsis* and rice. Plant Molecular Biology **67:** 589-602

Warren G, McKown R, Marin A, Teutonico R (1996) Isolation of mutations affecting the development of freezing tolerance in *Arabidopsis thaliana* (L) Heynh. Plant Physiology **111:** 1011-1019

Warthmann N, Chen H, Ossowski S, Weigel D, Herve P (2008) Highly Specific Gene Silencing by Artificial miRNAs in Rice. Plos One **3**

Webb MS, Uemura M, Steponkus PL (1994) A Comparison of Freezing Injury in Oat and Rye: Two Cereals at the Extremes of Freezing Tolerance. Plant Physiol. 104(2): 467–478

Weigel D (1995) The APETALA2 DOMAIN is related to a novel type of DNA-binding domain. Plant Cell **7:** 388-389

Whalen MC, Innes RW, Bent AF, Staskawicz BJ (1991) Identification of Pseudomonas-syringae pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell **3:** 49-59

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562-565

Wilson IW, Kennedy GC, Peacock JW, Dennis ES (2005) Microarray analysis reveals vegetative molecular phenotypes of *Arabidopsis* flowering-time mutants. Plant and Cell Physiology **46:** 1190-1201

Wolkers WF, Balasubramanian SK, Ongstad EL, Zec HC, Bischof JC (2007) Effects of freezing on membranes and proteins in LNCaP prostate tumor cells. Biochimica Et Biophysica Acta-Biomembranes **1768:** 728-736

Xin Z, Browse J (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. Plant Cell and Environment **23:** 893-902

Xin ZG, Browse J (1998) eskimo1 mutants of *Arabidopsis* are constitutively freezing-tolerant. Proceedings of the National Academy of Sciences of the United States of America **95:** 7799-7804

Xiong LM, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell **14:** S165-S183

Xu DP, Duan XL, Wang BY, Hong BM, Ho THD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiology **110:** 249-257

Yalpani N, Enyedi AJ, Leon J, Raskin I (1994) Ultraviolet-light and ozone stimulate accumulation of salicylic-acid, pathogenesis-related proteins and virus-resistance in tobacco. Planta **193:** 372-376

Yamaguchishinozaki K, Shinozaki K (1994) A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251-264

Yasuda M, Ishikawa A, Jikumaru Y, Seki M, Umezawa T, Asami T, Maruyama-Nakashita A, Kudo T, Shinozaki K, Yoshida S, Nakashita H (2008)

Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. Plant Cell **20:** 1678-1692

Yoshida M, Abe J, Moriyama M, Kuwabara T (1998) Carbohydrate levels among winter wheat cultivars varying in freezing tolerance and snow mold resistance during autumn and winter. Physiologia Plantarum **103:** 8-16

Yu DQ, Chen CH, Chen ZX (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell **13:** 1527-1539

Zhang X, Fowler SG, Cheng HM, Lou YG, Rhee SY, Stockinger EJ, Thomashow MF (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. Plant Journal **39:** 905-919

Zhang YL, Fan WH, Kinkema M, Li X, Dong XN (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proceedings of the National Academy of Sciences of the United States of America **96:** 6523-6528

Zhang YL, Tessaro MJ, Lassner M, Li X (2003) Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15:** 2647-2653

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J (1998) PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. Plant Cell **10:** 1021-1030

Zhu JH, Dong CH, Zhu JK (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Current Opinion in Plant Biology **10:** 290-295

Zhu JH, Shi HZ, Lee BH, Damsz B, Cheng S, Stirm V, Zhu JK, Hasegawa PM, Bressan RA (2004) An *Arabidopsis* homeodomain transcription factor gene, HOS9, mediates cold tolerance through a CBF-independent pathway. Proceedings of the National Academy of Sciences of the United States of America **101:** 9873-9878

Zhu JH, Verslues PE, Zheng XW, Lee B, Zhan XQ, Manabe Y, Sokolchik I, Zhu YM, Dong CH, Zhu JK, Hasegawa PM, Bressan RA (2005) HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. Proceedings of the National Academy of Sciences of the United States of America **102:** 9966-9971

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. Plant Physiology **136:** 2621-2632

Appendix A

Solution and media recipes

A.1 KB medium

10 g Glycerol

10 g Tryptone

10 g Peptone

1.5 g MgSO4.7H2O

1.5 g K2HPO4

15 g agar

No need to pH the media

A.2 STET buffer

4.0 g Sucrose

2.5ml of Triton X-100

200 µl of 0.25 M EDTA

2.5 ml of 1 M Tris-HCl

50 µl of 20 % Sodium Azide

Dissolved in 50 ml of water

A.3 1 × TE buffer

Add the following to 990 ml distilled water

400 µl 0.25 M EDTA

10 ml 1M Tris-HCl (pH 8.0)

A.4 TER buffer

Add RNAse A to 1×TE to a concentration of 10 µg/ml

A.5 Edwards' extraction buffer

1 ml 1M Tris-HCl (pH 7.5)

0.25 ml 0.5 M EDTA (pH 8.0)

1.5 ml 1 M NaCl

0.25 ml 10 % SDS

Add 2.25 ml to make up to 5 ml

A.6 10 × TBE buffer

9.3 g EDTA

55 g Boric acid

108 g Tris base

Dissolved in 1 L of distilled water

A.7 50 × TAE buffer

242 g Tris base

57.1 ml Glacial Acetic Acid

18.6 g EDTA

Dissolved in 1 L of distilled water

A.8 10 × Blue DNA sample loading buffer

0.025 g Xilene cyanol

0.025 g Bromophenol blue

1.25 ml 10 % SDS

1.25 ml Glycerol

Dissolve in 6.25 ml of distilled water

A.9 10 × Orange G DNA sample loading buffer

20 g Sucrose

100 mg Orange G

Dissolve in 50 ml of distilled water

A.10 N6D medium

3.98 g CHU(N6) basal salt mixture – (Sigma Aldrich cat. No. 1416)

10 ml of 100×N6-Vitamin (see A.11 for recipe)

0.1 g myo inositol- (Sigma Aldrich, cat. No.15125)

0.3 g casamino acid- (Melford laboratories cat. No. 1301)

2.878 g L-proline - (Melford laboratories, cat. No. P0717)

10 ml of 100× 2,4-D- (Melford laboratories, cat. No. D0911) (see A.12 for recipe)

30 g sucrose

Mix all these in 800 ml distilled water. Adjust the pH to 5.8 and add 3g gellan gum- (Sigma Aldrich, cat. No. P8169). Adjust the volume to 1 L with distilled water and autoclave. Cool the medium to 50^{o}C and pour in to plates.

A.11 100×N6-Vitamin

100 mg glycine- (Melford laboratories, cat. No. G0709)

25 mg nicotinic acid- (Melford laboratories, cat. No.)

25 mg pyridoxine hydrochloride-(Melford laboratories, cat. No. P3207)

50 mg thiamine hydrochloride- (Melford laboratories cat. No.T0614)

Dissolve 100 mg 2,4-D in 1 ml of 1 N sodium hydroxide solution and adjust the volume to 500 ml with distilled water. Store at 4^{o}C

A.12 100× 2,4-D

Dissolve 100 mg 2,4-D in 1 ml of 1 N sodium hydroxide solution and adjust the volume to 500 ml with distilled water. Store at 4^{o}C

A.13 AB medium

5 g glucose- (Melford laboratories cat. No. G1400)

15 g agar - (Melford laboratories, cat. No. M1002)

Dissolve in 800ml distilled water and adjust the volume to 900 ml with distilled water and then autoclave it and add

50 ml of 20 × AB buffer (see section A.14 for recipe)

50 ml of 20 × AB salt (see section A.15 for recipe)

Appropriate antibiotics

Then pour in to Petri plates.

A.14 20 × AB buffer

60 g dipotassium hydrogenphosphate (BDH cat. No. 103494G)

20 g Sodium phosphate monobasic dihydrate (Sigma Aldrich, cat. No 71500)

Dissolve in 800 ml distilled water, adjust the pH to 7.2 and adjust the volume to 1 ml and autoclave. Store at room temperature.

A.15 20 × AB salt

20 g Ammonium chloride (Sigma Aldrich, cat. No.A0171)

6 g magnesium sulphate heptahydrate (BDH, cat. No. 101514Y)

3 g potassium chloride (BDH, cat. No. 101985M)

240mg calcium chloride dehydrate (BDH, cat. No. 100704Y)

50 mg iron(II) sulphate heptahydrate (Fisher scientific, cat. No. 20139-2500)

Dissolve in 800 ml distilled water, adjust the volume to 1 L and autoclave and store at room temperature.

A.16 AAM medium

1 ml of 1,000×AA-1 (see section A.17 for recipe)

1 ml of 1,000×AA-2 (see section A.18 for recipe)

1 ml of 1,000×AA-3 (see section A.19 for recipe)

1 ml of 1,000×AA-4 (see section A.20 for recipe)

1 ml of 1,000×AA-5 (see section A.21 for recipe)

5 ml of 200×AA-6 (see section A.22 for recipe)

10 ml of 100×AA-Sol (see section A.23 for recipe)

0.5 g casamino acid

68.5 g sucrose

36 g glucose

0.9 g L-glutamine

0.3 g L-aspartic acid

3 g potassium chloride

Dissolve in 800 ml of water and adjust the pH to 5.2 and the volume to 1 L with distilled water and autoclave

A.17 1,000×AA-1

0.7g manganese (II) sulphate monohydrate (Melford laboratories cat. No.M17899)

300 mg Boric acid (MERCK chemicals cat. No.203667)

200mg Zinc sulphate heptahydrate (Melford laboratories cat. No.Z0526)

2.5 g copper (II) sulphate pentahydrate (Sigma Aldric, cat. No.C3036)

25 mg disodium molybdate dehydrate (Fisher scientific, cat. No. 20637-1000)

2.5 mg cobalt (II) chloride hexahydrate (Fisher scientific, cat. No. 19209-1000)

75 mg potassium iodide (Sigma Aldrich, cat. No.30315)
Dissolve in 90 ml of distilled water, adjust the volume to 100 ml and store at 4^{o}C

A.18 1,000×AA-2

Dissolve 15 g calcium chloride dehydrate (BDH cat. No. 4122) in 90 ml distilled water, adjust the volume to 100 ml. Store at 4^{o}C

A.19 1,000×AA-3

Dissolve 25 g magnesium sulphate heptahydrate 90 ml distilled water; adjust the volume to 100 ml. Store at 4^{o}C

A.20 1,000×AA-4

Dissolve 4 g EDTA ferric sodium salt (Melford laboratories cat. No.E0509)
in 90 ml distilled water, adjust the volume to 100 ml. Store at 4^{o}C

A.21 1,000×AA-5

Dissolve 15 g sodium dihydrogenphosphate (Sigma Aldrich cat. No. 71505) in 90 ml distilled water, adjust the volume to 100 ml. Store at 4^{o}C

A.22 200×AA-6

Dissolve 20 mg nicotinic acid,
20 mg pyridoxine hydrochloride
200 mg thiamine hydrochloride
2 g mayo-inositol in 90 ml distilled water, adjust the volume to 100 ml. Store at 4^{o}C

A.23 100×AA-Sol

Dissolve 5.3 g L-arginine (Melford laboratories cat. No.A0704)
225 mg glycine in 250 ml distilled water, adjust the volume to 300 ml. Store at 4^{o}C

A.24 1,000×As

Dissolve acetosyringone (Sigma Aldrich, cat. No.D134406) at a concentration of 10 mg ml^{-1} in DMSO, filter sterilize. Store at -20^{o}C

A. 25 2N6-AS medium

3.98g CHU (N6) basal salt mixture

10 ml of 100×N6 vitamins

0.1 g myo-inositol

0.3 g casamino acid

10 ml f 100×2,4D

30 g sucrose

10 g glucose mix and dissolve in 800 ml distilled water. Adjust the pH to 5.2 and add

3 g gellan gum adjusts the volume in 1 L autoclave. Cool the medium to 50^{o}C and add 1 ml of 1,000×AS (See section A.24) and pour the medium to petri plates.

A.26 1,000×Car

Dissolve carbencillin (Melford laboratories) at a concentration of 250 mg ml-1 in distilled water, filter sterilise. Store at -20^{o}C.

A.27 N6D-S medium

Prepare a 1 L of N6D medium and add

2 ml of 1,000×Car

Appropriate antibiotics

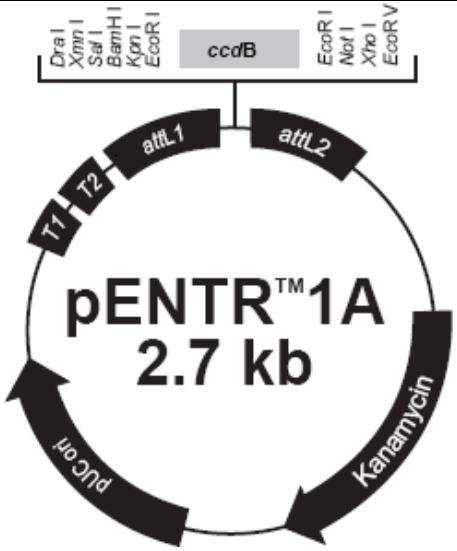
Appendix B

Plasmid vectors

B.1 *pENTR1A*

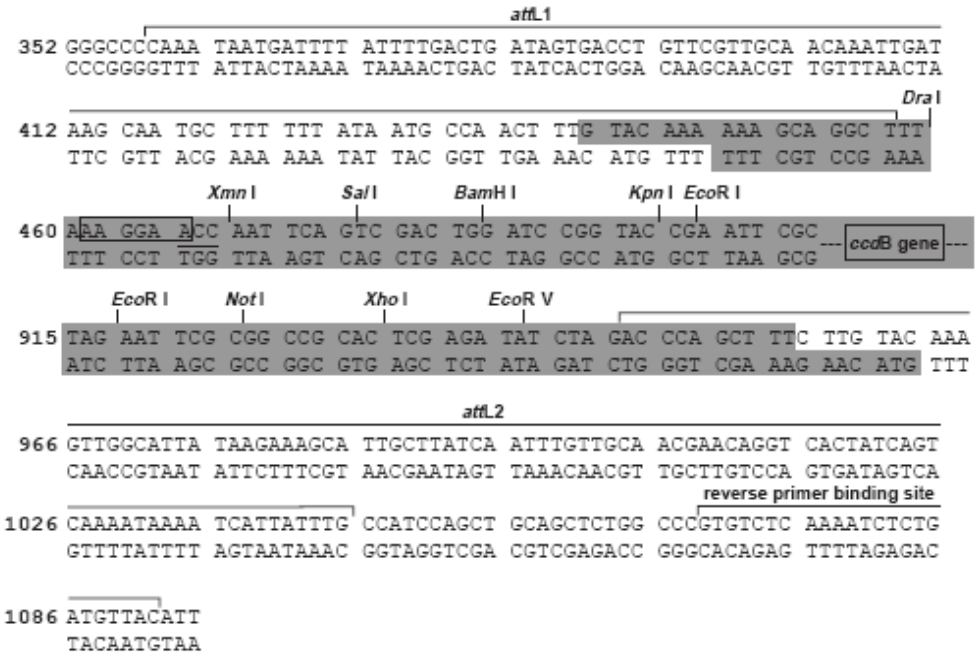
Figure B.1

pENTR1A vector map as shown at http//www.invitrogen.com



Comments for pENTR™1A
2717 nucleotides

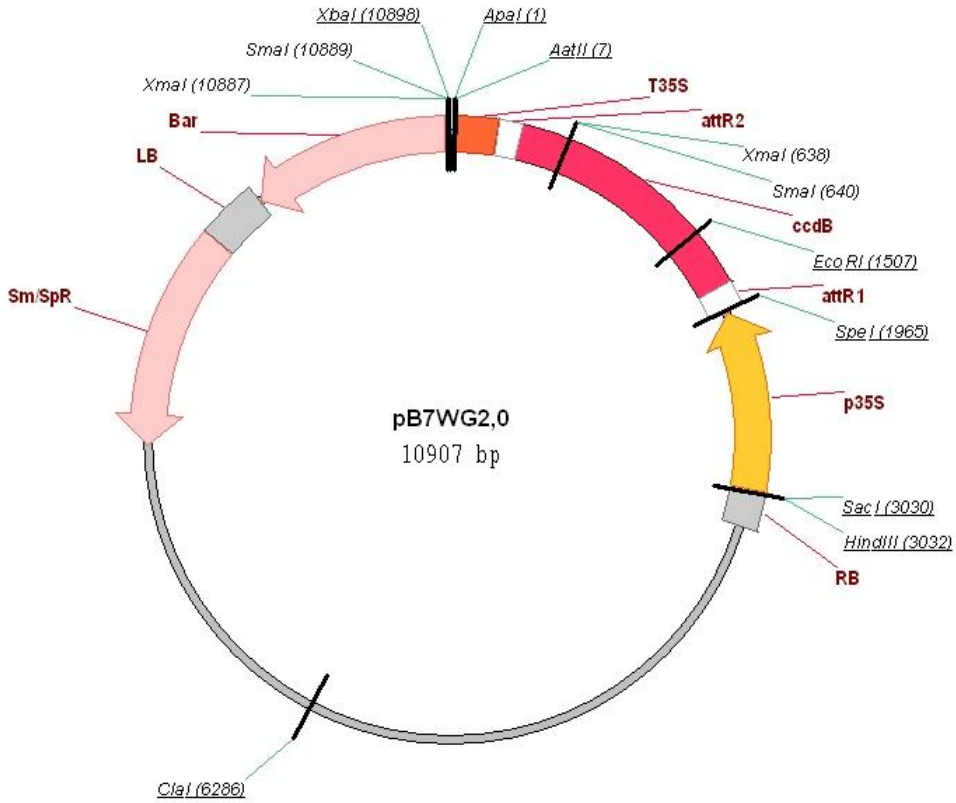
*rrn*B T1 transcription termination sequence: bases 106-149
*rrn*B T2 transcription termination sequence: bases 281-308
*attL*1: bases 358-457 (complementary strand)
*ccd*B gene: bases 612-917
*attL*2: bases 946-1045
Kanamycin resistance gene: bases 1168-1977
pUC origin: bases 2041-2714



B.2 *pB7WG2*

Figure B.2

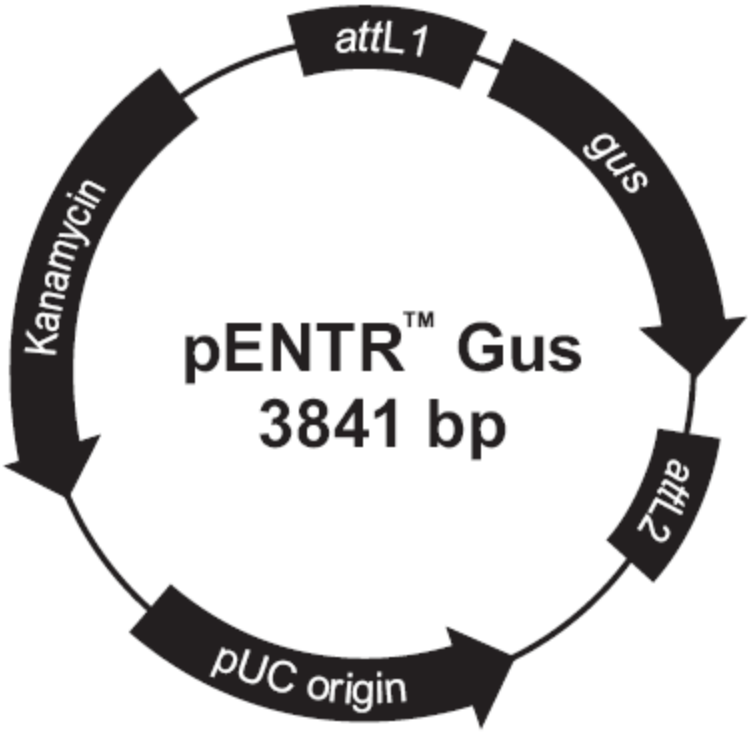
pB7WG2 Gateway destination vector map as shown at http//www.psb.rug.ac.be/gateway



B.3 *pENTR-Gus*

Figure B.3

pENTR-Gus vector map as shown at http//www.invitrogen.com



Comments for pENTR™ Gus
3841 nucleotides

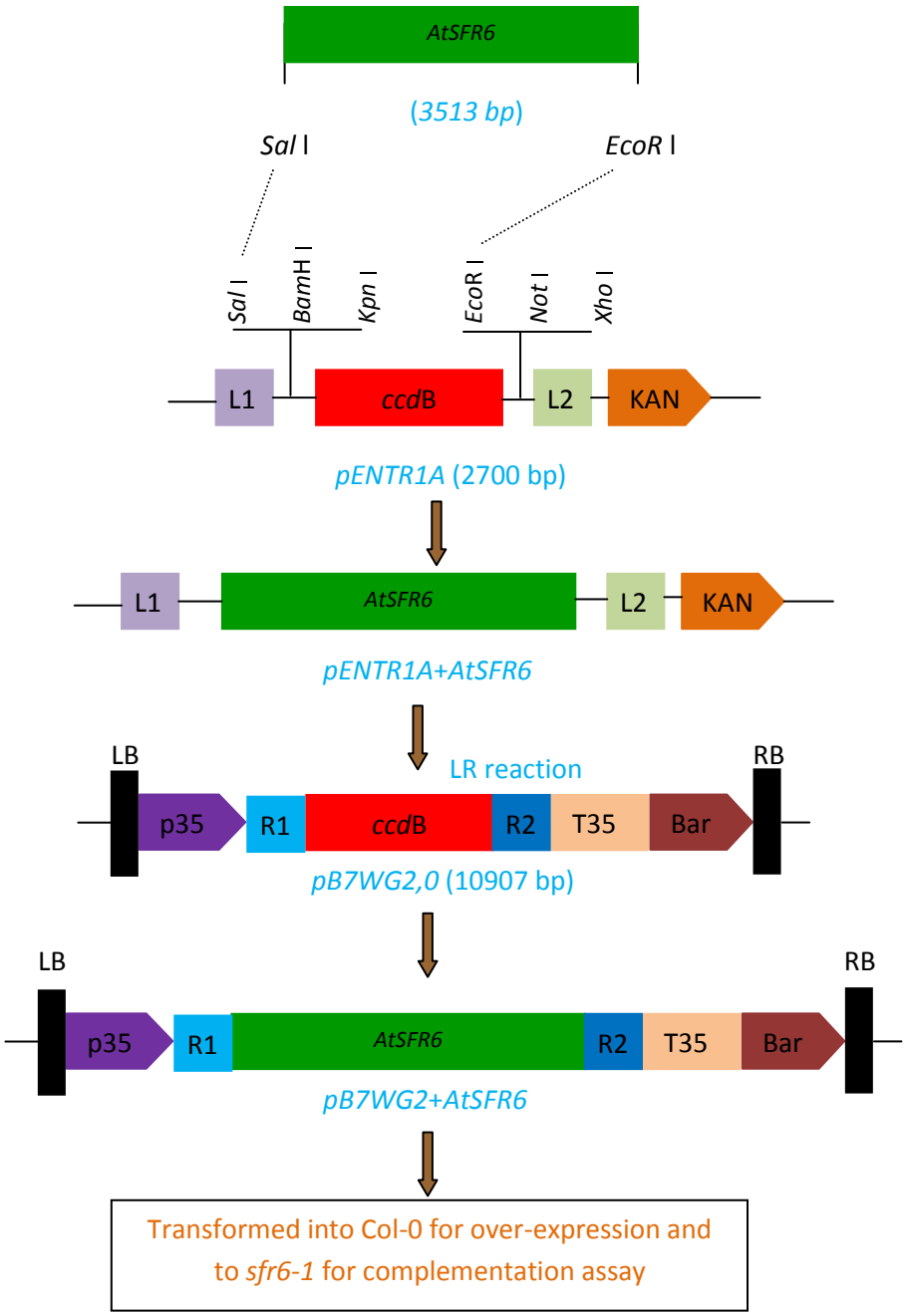
*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

B.4 Cloning *AtSFR6* into *pB7WG2*

Figure B.4

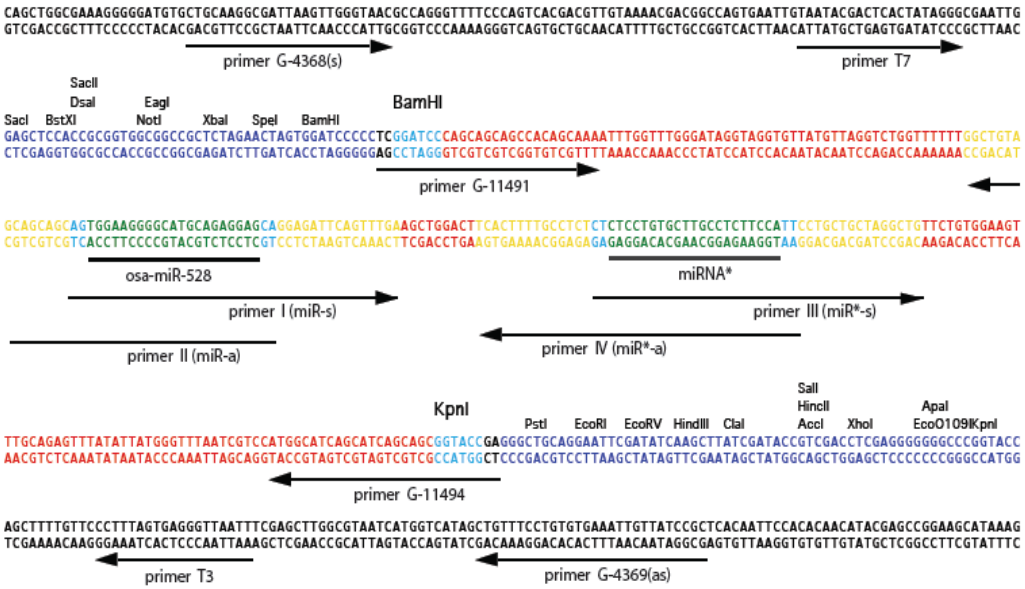
Schematic representation of cloning AtSFR6 into pB7WG2



B.5 *pNW55*

Figure B.5

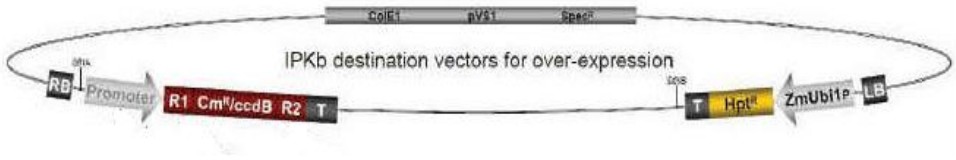
pNW55- OsaMIR528 stem-loop in pBluescript KS downloaded from http://wmd2.weigelworld.org



B.6 *IPKb* over-expression vector

Figure B.6

IPKb over-expression binarry plasmid as shown in Kumlehn, 2008.



Appendix C

Probes and Primers

All the Applied Biosystems TaqMan probes used for gene expression and genotyping assays are listed with their gene code and Applied Biosystems probe identifier. All syber primers used for gene expression assay were also listed with their gene code and forward and reverse primers. All primers used for sequencing are also listed with their sequences and directions.

C.1 Probe for *sfr6-1* genotyping assay

Table C.1

Descriptions of probe used for single nucleotide polymorphism (SNP) genotyping assay of *sfr6-1*

Applied Biosystems probe identifier	SNP492-SNP1
Forward primer name	SNP492-SNP1F
Forward primer sequence	CGTATGATCCAGATGAAGGTCCTT
Reverse primer name	SNP492-SNP1R
Reverse primer sequence	GCAGTACAACAGGTTGAACACTTGA
Reporter 1 Name	SNP492-SNP1V1
Reporter 1 Sequence	CACAGGCTGGAGAGTA
Reporter 2 Name	SNP492-SNP1M1
Reporter 2 Sequence	CACAGGCTGAAGAGTA

C.2 Probes for gene expression assays

Table C.2

Descriptions of Applied Biosystems TaqMan probe used for gene expression assays

Gene	Gene code	Applied Biosystems probe identifier
PDF1.1	*AT1G75830*	*At02359800_s1*
KIN2	*AT5G15970*	*At02354775_s1*
ß-TUBULIN4	*AT5G44340*	*At02337699_g1*
CBF1	*AT4G25490*	*At02238420_sH*
PEX4	*AT4G25760*	*At02304594_g1*
SFR6	*AT4G04920*	*At02209654_g1*
ATMC8 (metacaspase8)	*AT1G16420*	*At02156537_g1*
EDS5	*AT4G39030*	*At02170432_g1*
ICS1	*AT1G74710*	*At02286260_g1*
PR1	*AT2G14610*	*At02170748_s1*
LTI78	*AT5G52310*	*At02320470_g1*
AT4G26410	*AT4G26410*	*At02239002_g1*
OsSFR6	*OS10g35560*	

Table C.3

Descriptions of Syber primers used for gene expression assays

Gene	Gene code	Forward primer	Reverse primer
NPR1	*AT1G64280*	GCCGCCGAACAAGTACTCA	GCTGTTGGAGAGCAATTGCA

C.3 Primers for sequencing *OsSFR6*

Figure C.1

Primers used for sequencing of *OsSFR6* coding region.

atgacctcttcctccgccccaaaccctagccccctcgtccccacccccacccccctctccccccaccgc
caacgccgccgccgccgccgccggagccgtgcccgtctcctcgccgacgccgccgcccaaggatcagc
agcaggagggccaggggggtggaggaggagtgggggatgggggtggagtggaggaggtgggcgtaggg
ggaggcgaggccatggaggtggatggtggcgcgggaggtggtggtgggggggttggggatgtggaggg
gggtgggggtggtggtggtgcgggggga
ggaggaggtggaggtggaggtgggcagcaggcgtcgccggcgaccgtgttccggatccggctcaagca
gccgccctcgagcctccgccacaag

ATGCGCGTGCCCGAGCTCTGCAGGAACTT→CAGTGCAGTTGCTTGGTGCGGGAAGCTCAATGCAATT
GCATGCGCATCAGAGACTTGTGCACGCATACCAAGCTCTAATTCAAGCCCACCATTTTGGATTCCCAT
ACACATTCTAAATCCAGAGAGACCAACAGAATGTTCTGTTTTCAATGTGAAAGCAGATTCTCCACGCG
ACTTTGTTCAATTCATTGAATGGTCTCCTCGATCATGCCCTCGTGCATTACTGGTGGCAAATTTTCAT
GGAAGGATTACTATATGGACACAGCCAACTAAGGGTCCTACTAATCTTGTACGTGATGCCAGTTCCTG
GCAATGTGAACACGAATGGCGTCAAGATCTTTCGGTGGTGACTAAGTGGTTGTCAGGAATTTCTCCGT
ATAGATGGCTTCCTGCAAACTCTAGTACTTCATCAAACTTGAAAACCTTTGAGGAAAAGTTCCTTACC
CAGCAGCCTCAAAGTTCGGCTGGGTGGCCAAACATTCTATGTGTCTGTTCAGTTTTTTCATCGGGTTC
TGTTCAGCTTCATTGGTCACAATGGCCTTCTCAAAACTCAGCACAACCTAGATGGTTTTCTACTAGCA
AAGGGCTTTTAGGAGCAGGGCCAAGCGGCATAATGGCTGCTGATGCTATTATTACTGAAACTGGAGCA
TTACATGTTGCTGGTGTTCCCCTTGTTAATCCATCTACTGTAGTGGTTTGGGAGGTGATGCCAGGCCT
TGGCAATGGTATTCAGGCAACTGCAAAGATAAATGCAACAAGCTCTCTTCCTCCATCACTAAATCCCC
CACTCTGGGCTGGTTTTGCTCCACTTGCATCTTACCTCTTCTCTTTGCAAGACTACCTTGTTTCCGAG
GGCGCACAGACAAAAAAACAGGCACAGGTAGATAATGAGACCACTGAGGTAGCATCGATCCATTGTTG
TCCAGTTTCCAACTTTTCAGCTTACGTCAGTCCTGAAGCTGCTGCCCAGTCAGCCACTACCACAACAT
GGGGATCT→GGGGTTACCTCAGTTGCTTTTGATCCCACTCGAGGGGGATCAGTTATTACAGTTGTAA
TAGTTGAAGGGCAGTACATGTCTCCTTATGATCCTGATGAAGGACCTTCCATCACTGGATGGAGAGTC
CAGTGCTGGGAATCTTCAGTCCAACCTGTTGTTCTTCATCCAATATTTGGAAGCCCTGCAAACTTTGG
TGGACAGCCACCTACACAGACTGTTTGGTCCACAAGAGTTAACAAAAGCATCCCACCATCTGAGGACC
TTAAGAACCCTCAATCATATGTTCCAATGCCAACAACTTCAGATGAGCGGAGTTCTTCTGAGTGCAGT
GTTGACAGGGCGAACCGACTTAGCTTTGACCCTTATGATCTTCCAAATGATGTCAGACAATTGGCCCA
AATAGTTTATTCTGCTCATGGTGGTGAGGTTGCAGTTGCATTCCTGCGTGGAGGTGTG→CACATTTT
TTCAGGTCCAAACTTTGAACAGGTTGATAGCTATCATGTCAATGTTGGCTCAGCAATTGCTCCACCAG
CCTTCTCCTCCAGTGGTTGTTGCTTGGCATCAGTATGGCATGACACACTCAAAGATCGAACCATACTA

(Figure continues to the following page)

Figure C.1 *(Continued from the previous page)*

AAGATAATACGTGTGCTTCCTCCTGCAATTCTTAATGCTCAGACAAAGGTTAGCTCAGCTGTTTGGGA
ACGAGCAATAGCAGATAGATTTTGGTGGAGTCTATTGGCTGGTGTGGATTGGTGGGATGCTGTTGGCT
GCACACAAAGTGCTGCTGAAGATGGTATTGTCTCACTGAACAGTGTGATAGCTTTGCTGGACGCGGAC
TTCCATTGTCTTCCAACTATACAACAGAGGCAACAACACTGTCCTAATCTTGATAGGATAAAGTGTAG
ATTGTTGGAAGGAACAAATGCTCAAGATGTCAGAGCACTTGTGTTGGACATGCAAGCAAGATTGCTTC
TGGATATGCTTGGCAAGGGAATTGAGTCTGCCCT→GATAAATCCATCAACTCTGCTACCTGAACCGT
GGCAAGCTTCCAGTGACATGTTATCTAGCATTGGGCCTGACAAAATGACTGTTGACCCAGCTCTACTT
TTAAGCATCCAGGGGTACGTTGATGCTGTTCTAGATTTAGCGTCACATTTTATCACACGCTTGCGACG
CTATGCGAGCTTCTGCCGAACTTTGGCTAGCCATGCAGTTGGAGCATCTTCTGGTTCAGGCAATTCTA
GGAATATGGTTACAAGTCCAACCAACAGTTCTCCTTCACCTTCAACTAACCAAGGTAATCAAGGTGGA
GTAGCGTCTACAACAGGGAGCTCACAAATGCAAGAGTGGGTCCAAGGTGCCATTGCTAAGATTAGTAA
CAATACTGATGGTGCTGCAAATGCTGCACCAAATCCAATTAGCGGGAGGTCATCATTCATGCCTATTA
GCATAAATACGGGAACATTCCCTGGCACACCAGCTG→TTAGACTTATTGGGGACTGCCATTTCCTTC
ATAGATTATGTCAGCTGTTGCTATTTTGTTTGCTTTTTCGGAGAAGGCAATCTCCAAGGATACCTGCA
AATGCACAAAAAAGTTCTGATTCTAGCATGCAGAAACAACACTTGATGAACAGTAAGACAGAGGATAA
TACTTTGGCAGTCAGATCTGGTCTAGGTGCTGCCAAATTGGAAGATGGCACAACTTCACGTGGACAGA
TGGTTGGAGCAAAGGGTGCTGAAGAAAATCCAGTGGGCAACAAATCTGCTAGGATAGGTTCTGGCAAT
GCTGGCCAAGGTTATACTTCAGACGAGGTGAAAGTCCTTTTTCTCATATTAGTTGACCTATGTAAACG
GACTGCAACCTTGCAACATCCGTTGCCTTCTTCTCAGGTTGGTTCGAGCAATATTATTATAAGGCTGC
ATTACATCGATGGCAATTACACTGTGCTCCCTGAGGTAGTGGAAGCATCTCTTGGCCCTCATATGCAG
AATATGCCTCGTCCACGTGGAGCTGATGCTGCTGGCCTT→CTACTTCGAGAATTAGAACTGCAGCCC
CCTGCTGAAGAATGGCATAGACGCAACATGTTTGGTGGGCCATGGTCAGAACCAGATGATCTTGGTCC
ATTGGATAATACGCGACAGCTAAAAATCAATGGCTCTACCAATCGCCACTTATCGGACATGGAAGAGG
ATGGCGACAGCTCCTTTGGGATTCAAAATCTTTGGCCAAGAAAGCGCCGGTTGTCTGAAAGAGATGCA
GCATTTGGTCTGAAAACATCCGTGGGGCTGGGATCTTTTCTAGGTGTGATGGGTTCTCGGAGAGATGT
TATTACAGCTGTGTGGAAAACAGGCCTCGAAGGTGAATGGTACAAGTGCATACGATGTTTGAGGCAAA
CCTGTGCATTTGCTCAGCCTGGTGCTCTAGCTCCGAACACGTCGAATGAGCTTGAGGCATGGTGGATC
AGCCGATGGACCCATGCTTGCCCAATGTGCGGTGGGACATGG←GTGAAAGTCGTTTGAATTTGACG

Lowercase represent OsSFR6 sequence which was not included to the OsSFR6* fragment (Chapter 6) used for this thesis. Blue areas denote primers used for sequencing. Arrows indicate the primer orientation.

C.4 Primers for sequencing *AtSFR6*

Figure C.2

Primers used for sequencing of *AtSFR6* coding region.

ATGAATCAGCAAAACCCAGAAGAAGAAGTTTCTTTGGTTAATAATAGCGGTGGTGGAGGAATCATCGA
AGCTCCAGCTATAGTGGAGGAGAAAGAGGAAGAAGGATTACAGCAGAAGCAGGAAGAGACTATTGAGT
CTACCGATCCGATTCTCGTCGTCGTTGAGGAGAAATTGTTAGAGAAATCAGTAGACGGAGAGAAAGAA
GACGATAATAGTAGTAGTAGTAACATGGAGATTGATCCTGTGAGTCCGGCTACAGTTTTCTGTGTTAA
GCTTAAGCAGCCCAATTCCAATTTGCTTCATAAGATGAGTGTTCCTGAATTGTGCCGTAACTTCAGTG
CTGTTGCGTGGTGTGGCAAATTGAATGCTATTGCTTGTGCTTCCGAGACCTGTGCCAGGATTCCAAGC
TCCAAGGCAAATACACCTTTTTGGATACCAATACATATCTTGATACCTGAGCGCCCTACTGAGTGTGC
GGTGTTTAATGTTGTGGCAGACTCTCCTCGTGATTCTGTCCAATTTATCGAATGGTCTCCCACTTCTT
GTCCTCGTGCGTTACTCATTGCTAATTTTCATGGACGTATAACTATCTGGACGCAGCCTACTCAGGGT
TCGGCTAATTTAGTGCACGACGCTACCTCCTGGCAGTGTGAG→CATGAATGGCGTCAGGACATTGCT
GTTGTTACAAAGTGGCTGACAGGGGCTTCCCCATATAGGTGGTTGTCCTCCAAGCCAAGTTCTGGTAC
AAATGCAAAGTCAACTTTCGAGGAGAAATTTCTCTCGCAGAGCTCTGAAAGCTCAGCTCGGTGGCCCA
ACTTTCTCTGTGTATGCTCTGTTTTCTCATCCGGCTCTGTTCAAATTCATTGGTCCCAGTGGCCTTCT
AACCAGGGAAGCACTGCAC→CAAAGTGGTTTAGTACAAA←GAAAGGTCTTTTAGGTGCAGGGCCAAG
TGGAATTATGGCTGCTGATGCTATTATAACAGACAGTGGTGCCATGCATGTAGCAGGAGTTCCAATTG
TAAACCCTTCAACAATTGTAGTATGGGAGGTGACCCCTGGCCCTGGAAATGGACTCCAGGCGACTCCA
AAAATCTCTACAGGCAGTCGTGTGCCACCATCCCTTAGTTCTTCTTCTTGGACAGGTTTTGCTCCTTT
AGCTGCGTACTTGTTTAGCTGGCAAGAATACTTAATATCCGAGATAAAGCAAGGGAAGAAGCCCTCAG
ATCAAGATTCCAGTGATGCTATATCGCTAAGTTGCTCACCGGTTTCCAATTTTTCTGCTTATGTAAGT
CCAGAGGCTGCAGCTCAGTCTGCAGCAACCACAACATGGGGATCTGGTGTTACCGCTGTTGCTTTTGA
TCCAACTCGTGGTGGTTCAGTGATAGCAGTTGTTATAGTTGAAGGGCAGTACATGTCTCCGTATGATC
CAGATGAAGGTCCTTCAATCACAGGCTGGAGAGTACAG←CGCTGGGAATCAAGTGTTCAACCTGTTG
TACTGCATCAGATATTTGGAAACCCAACTTCAAATTTTGGAGGACAGGTCCCCACGCAAACTGTCTGG
GTATCCAGAGTGGATATGAGCATACCAC→CTACTAAAGATTTTAAGAATCATCAAGTAGCTGCAGCA
GGACCAAGTGTGGATGCACCAAAGGAGCCTGATTCTGGTGATGAGAAGGCTAACAAGGTTGTATTTGA
TCCTTTTGATTTGCCAAGTGATATTCGGACACTTGCACGGATTGTCTATTCTGCTCATGGTGGTGAAA
TTGCGATT→GCTTTTCTTCGTGGTGGAGTTCATATCTTTTCTGGTCCAACTTTTTCACCTGTTGAAA
ACTATCAAATAAATGTTGGATCTGCAATTGCTGCACCCGCATTTTCACCAACAAGCTGTTGTTCGGCT
TCTGTATGGCATGATGCTGCTAAGGACTGCGCAATGTTGAAAATCATCCGTGTTCTTCCTCCTGCTCT
TCCGCGTAACCAATCAAAGGTTGATCAATCAACATGGGAGCGGGCGATCGCTGAGAGATTCTGGTGGA

(Figure continues to the following page)

Figure C.2 *(Continued from the previous page)*

GTCTTTTGGTCGGAGTTGATTGGTGGGATGCAGTTGGCTGCACACAGAGTGCTGCAGAGGATGGGATA
GTTTCATTGAACAGCGTGATTGCTGTCATGGATGCTGATTTTCACTCCCTTCCTTCAACACAGCACAG
ACAACAATATGGCCCTAACCTAGATAGGATCAAATG←TCGGTTACTTGAAGGAACCAATGCTCAAGA
GGTTCGTGCCATGGTTTTAGATATGCAA←GCAAGGTTGTTGTTGGACATGCTTGGAAAAGGTATTGA
ATCAGCTCTTGTGAATCCTTCGGCGTTGGTTTTTGAGCCATGGCGAGTAGATGGGGAGACAATAACAG
GCATCAATCCGGAGGCAATGGCTGTTGATCCTGCTCTTGTTTCCAGTATTCAGG→CTTATGTGGATG
CTGTTCTTGATCTTGCTTCTCATTTCATCACACGTTTAAGGCGTTATGCGAGTTTTTGTCGGACTCTT
GCAAGCCATGCTGCTTCTGCTGGAACTGGCAGTAATCGCAACAATGTTACCAGTCCCACACAAAATGC
ATCATCTCCTGCAACACCTCAGGTTGGTCAACCTACTACTACTACTACTACTGCTACGACAAACT
CCAGCGGAAGCTCACATGTGCAAGCTTGGATGCAGGGGGCCATAGCGAAAATTAGTAGCTCGAATGAT
GGATCCAACTCTACTGCAAGCCCAATCAGTGGTTCACCTACCTTCATGCCAATAAGCATCAATACAGG
AACATTTCCAGGAACACCTGCTGTTCGGCTCATTGGGGATTGTCATTTCCTTCATCGGTTATGCCAGC
TGTTGCTCTTCTGTTTTCTCCAGCGGTCTTCACGATTTCCACAGCGAAATGCTGATGTTAGTTCACAA
AAACTTCAAACGGGGGCTACCAGCAAATTGGAAGAAGTCAACTCTGCTAAACCAACCCCTGCCTTGAA
CAGGATA→GAGGACGCCCAGGGATTCCGGGGTGCCCAGTTGGGTACTGGAGTGAAAGGGATTGATGA
AAATTCTGCTCGTACAACAAAGATGGGTTCTGGGAATGCCGGTCAAGGATATACTTATGAGGAGGTGA
GAGTTCTTTTCCATATACTAATGGATCTCTGCAAGCGAACATCTGGTCTTGCGCATCCCTTACCTGGC
TCTCAGGTAGGTAGTG→GAAACATTCAAGTTCGACTGCATTATATTGATGGAAATTACACTGTGTTA
CCCGAGGT←GGTAGAAGCGGCTCTTGGACCACATATGCAGAACATGCCTCGCCCAAGAG→GAGCTGA
TGCTGCTGGTCTTCTACTTCGGGAGTTAGAGCTTCATCCGCCTTCCGAAGAATGGCATAGAAGAAATT
TATTTGGTGGTCCCGGGTCAGAGCCTGAGGATATGATCTTGACAGACGATGTTTCCAAGCTGAGTAAT
TCCTTAGATCTGCCTGATACAAACTTTTCCGGAATATGTGATGGATACAACAGAGTCCATAGTCTTTG
GCCAAGAAAACGCAGGATGTCTGAAAGAGATGCAGCTTTTGGTTCAAATACTTCTGTGGGTTTGGGTG
CATATCTTGGGATCATGGGTTCTCGTAGGGATGTTGTGACCGCGACATGGAAAACTGGTCTTGAAGGA
GTTTGGTACAAGTGCATAAGATGCCTAAGGCAGACATCTGCATTTG→CTTCACCAGGTGCCACTAAG
CAGCCAAATC→CGAATGAACGAGAAACCTGGTGGACAAGTCGTTGGGTTTATTGCTGCCCCA→TGTG
TGGTGGAACGTGGGTCCGTGTTGTA**TAG**

Blue areas denote primers used for sequencing. Arrows indicate the primer orientation.

Acknowledgements

This work was carried out at the plant cell signalling lab in the School of Biological and Biomedical Sciences, Durham University, Durham, United Kingdom.

I am grateful to my supervisor Professor Marc R. Knight, who guided me so patiently during my studies. His guidance, ideas and encouragement over these three years have been invaluable. I am also indebted to Dr. Heather Knight, without her guidance finishing this thesis would have been much more difficult.

I am grateful for the support that I have received from all past and present members of Plant Cell Signalling Lab, Dr. Piers Hemsley, Dr. Margaret Pullen, Dr. Bekir Ulker, Dr. Rob Ingle, Dahai Gao, Alex Sargeant, Paul Loftus, Liam O'Hara, Hillary Wilkinson and Dr. Vanja Pekovic. It has been pleasure working with all of them. Special thanks are due to Dr. Steve Chivasa for assistance with pathogen work and Dr. Robert Baxter for assistance with freezing assays.

I am grateful for my examiners Dr. Roger Pearce, Newcastle University and Dr. Tony Fawcett, Durham University for their fruitful comments.

In addition, a thank you to Prof. Joy A. Palmer-Cooper, Prof. Keith Lindsey, Prof. Ranjith Senarathne and Prof. S. Subasinghe for giving me the opportunity to do my doctoral research at Durham University under the project Sri Lanka studentship. Special thanks go to Dr. Kanthi Senanayake, Kumari Fonseka and Sajinee Pathiraja for their friendship. I am particularly indebted to Lakshika Liyanage, Kanchana Madurapperuma and their son Bimsara for looking after and being good friends to my daughter.

Most of all, I owe a debt of thanks to my husband Janaka Manohara and my daughter Jayadhi Nuwandhana for their irresistible support, love and for sharing with me the happiness and sadness all the time.

Wathugala GD Lakmini