Protein S-Thiolation and oxidative stress in plants

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Protein *S*-Thiolation in Response to Oxidative Stress in Plants

Abstract

The tripeptides glutathione (GSH; γglutamyl-cysteinyl-glycine) and homoglutathione (hGSH; γglutamyl-cysteinyl-β-alanine) are abundant cytosolic tripeptides in legumes. The reactive cysteinyl sulphydryl group enables GSH or hGSH to act as the major cellular redox buffer through the formation of disulphides with other GSH/hGSH molecules. GSH can also form disulphides with cysteinyl groups within proteins, which is termed *S*-thiolation, a reversible modification, protecting proteins from irreversible inactivation of thiol residues, as well as being important in regulating protein activity. Following treatment with fungal cell wall elicitors, plant cells produce reactive oxygen species (ROS) which results in cellular oxidative stress. In animal cells ROS generation induces antioxidant defences which include the accumulation of glutathione (GSH) and the formation of mixed disulphides between proteins and GSH. It was hypothesised that following treatment with a fungal elicitor, plant cells also thiolate proteins. It was of interest to determine how protein thiolation changed in response to changes in thiol metabolism known to occur during elicitation, as well as identifying proteins which underwent this modification.

Using cell cultures of alfalfa (*Medicago sativa* L.), a leguminous plant containing both GSH and hGSH, changes in thiol content upon treatment with a fungal cell wall preparation elicitor were determined. By inhibiting protein synthesis and labelling the thiol pools with L-[^{35}S]cysteine, the degree and rate of protein mixed disulphide formation could be monitored *in-vivo*. To induce the elicitation response, alfalfa cell cultures were treated with a fungal cell-wall elicitor. Following elicitor treatment GSH, but not hGSH, was found to accumulate, with an associated increase in GSH, but not hGSH, forming mixed disulphide with protein. In order to use proteomic tools to identify thiolated proteins, the oxidative stress response in cell cultures of Arabidopsis, a GSH containing species, was then characterised. The level of protein-bound GSH was found to increase following treatment of cell cultures with the oxidant *tert*-butyl hydroperoxide and this was associated with changes in cellular thiols. When proteins *S*-thiolated either *in-vivo*, or *in-vitro*, with [^{35}S]-GSH were resolved by SDS-PAGE under non-reducing conditions, a large number of radiolabelled polypeptides were identified in oxidatively stressed preparations. Testing the hypothesis that GSH-dependent enzymes may undergo *S*-thiolation, proteins which bound GSH were isolated from Arabidopsis using GSH-affinity chromatography. A number of 30 kDa polypeptides were isolated and found to be *S*-thiolated under oxidative conditions *in-vitro*. Several of these were subsequently identified, notably members of the glutathione transferase (GST) superfamily. Representative recombinant GSTs from Arabidopsis, maize and soybean were expressed, thiolated *in-vitro* and the effect on activity determined. Several thiolatable GSTs were identified from Arabidopsis, notably the members of the family of dehydroascorbate reductases (DHAR I, II, III) and lambda GSTs. Further analysis by electrospray mass-spectroscopy confirmed the covalent binding of GSH to DHAR isoenzymes during *in-vitro* thiolation. It was concluded that *S*-thiolation of proteins is a commonly observed reversible modification of proteins in plants exposed to oxidative stress with potentially important consequences in cytoprotection and regulation.

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1.0 Introduction

1.1 Cellular Responses to Biotic and Abiotic Elicitation in Plants

In order for plants to successfully defend themselves against micro-organisms, many biochemical and physical mechanisms can be invoked. These include the production of the reactive oxygen species (ROS) H_2O_2, O_2^-, $OH^\bullet$ and singlet oxygen. ROS agents can act as signal molecules to initiate other defence mechanisms, or in the case of infection by microbes can inhibit pathogen invasion directly through a process of localised cell death known as the hypersensitive response (HR) (Hammond-Kossack and Jones, 1996). ROS are also continually produced during normal photosynthetic processes. For example, in the case of photosynthetic electron transport, oxygen uptake is associated with the photoreduction of oxygen to superoxide, the Mehler reaction (Alscher *et al*, 1997). Photoreduction of oxygen also occurs during conditions of limited CO_2 availability i.e. under conditions of high light intensity when oxygen is utilised as an alternative electron acceptor, resulting in the formation of H_2O_2, superoxide, hydroxyl radicals and singlet oxygen (Casano *et al*, 1994). As ROS agents are phytotoxic, plants require antioxidant mechanisms to deal quickly and efficiently to detoxify ROS to ensure long term survival.

In addition to the HR, plants also defend themselves against fungal pathogens by initiating the *de-novo* synthesis of low molecular weight anti-microbial compounds, termed phytoalexins. Other defence mechanisms include cell wall fortification whereby the cell wall is physically strengthened by the production of lignin thus resisting the penetration of fungal hyphae from entering. This process also produces ROS. The plant can also produce pathogenesis related (PR) proteins, a group of proteins which include chitinases and glucanases, which prevent hyphal growth through degradation of the fungal cell wall (Hammond-Kossack and Jones, 1996).

1.1.1 The Role of Reactive Oxygen Species in the Elicitation Process

In plants, the oxidative burst associated with plant defence interaction is generally defined as the rapid production of high levels of ROS in response to external stimuli (Jones and Dangl, 1996; Mehdy, 1994). The oxidative burst was first demonstrated in potato tuber discs following inoculation with the resistance (incompatible) response to a race of *Phytophthora infestans* (Doke, 1983). However, this oxidative burst was not observed during the susceptible (compatible) response. The oxidative burst has since been observed in many plants challenged with micro-organisms, fungal cell-wall elicitor preparations which mimic infection, or mechanical injury. The two major forms of ROS produced in the oxidative burst following pathogen elicitation are superoxide and hydrogen peroxide. The type of ROS produced is dependent on the plant and elicitation system in use. For example, when tomato cotyledons were challenged with *Cladosporium fulvum* race-specific elicitors, the major ROS produced was superoxide (O_2^-), while French bean challenged with a crude cell wall elicitor from the fungal pathogen *Colletotrichum lindemuthianum*, predominantly produced H_2O_2 (Bolwell *et al*, 1995; Wojtaszek *et al*, 1995).

The production of ROS is a rapid response to infection. Within 13 min of sensing infection, hydrogen peroxide can accumulate to localised concentrations of up to 1 mM in infected plants (Jacks and Davidonis, 1996). However, it is believed that with incompatible interactions, a second, prolonged oxidative burst is required. In the case of tomato cell suspension cultures challenged with an oligogalacturonic acid elicitor preparation, this response is mediated by *Pto* kinase, the product of a resistance (R) gene, which is a key element in initiating a signalling cascade which results in a second oxidative burst and an incompatible response (Chandra *et al*, 1996). Figure 1.1, taken from Wojtaszek (1997), shows a schematic representation of the possible components of ROS generation, and their effects following recognition of a pathogen. In addition to ROS being produced through

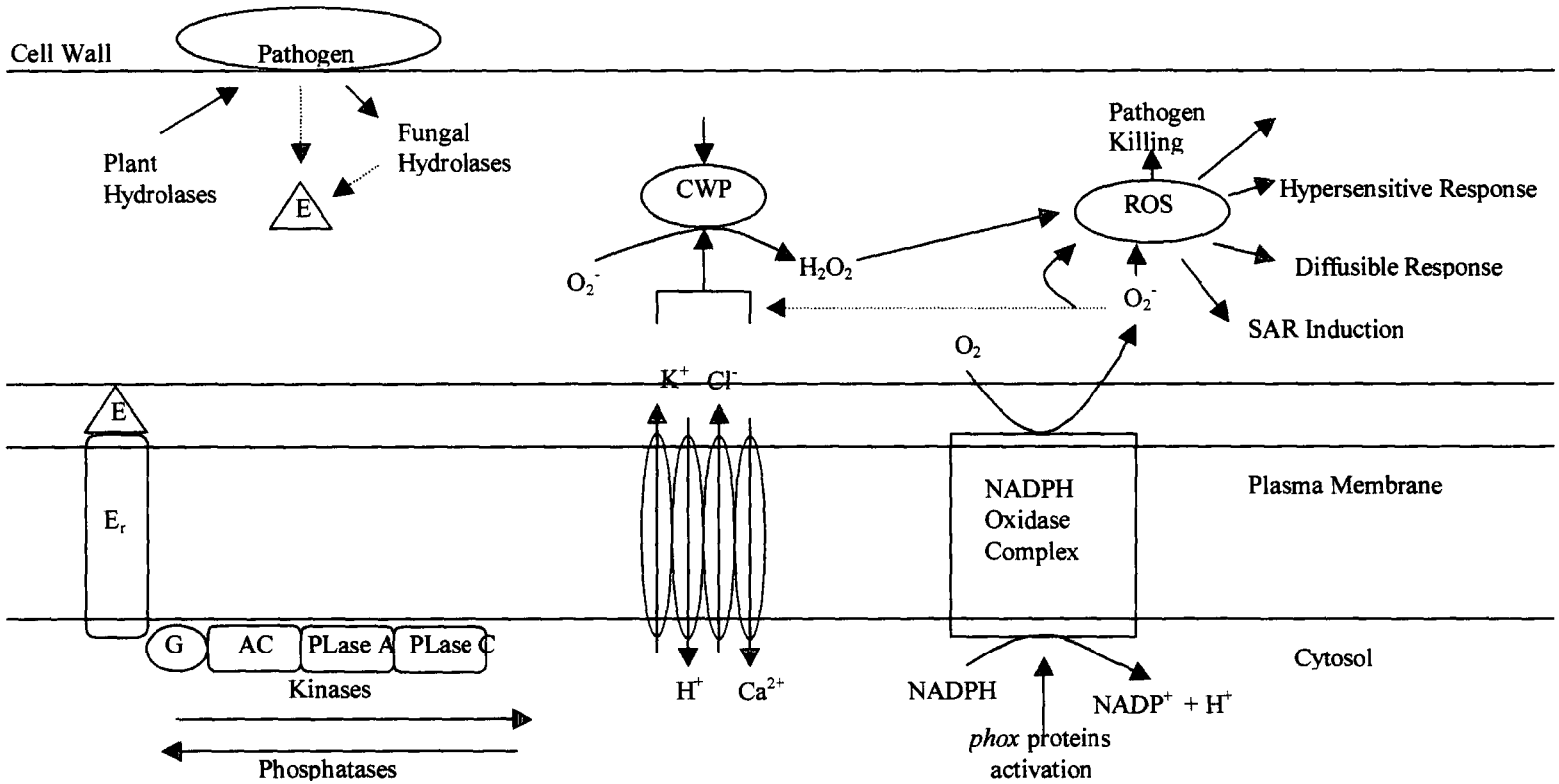

Figure 1.1. Schematic Representation of major sources of reactive oxygen species (ROS) in plants. After Wojtaszek 1997.
Following elicitation, plant and fungal cell wall hydrolases are released, initiating signalling response cascades in various aspects of the plant defence response. Abbreviations used: AC, adenylate cyclase; CWP, cell-wall bound peroxidase; E, elicitor; E_r, Elicitor receptor; G, GTP-binding protein; PLase A and C, phospholipase A and C; R, reductant; *phox*, phagocyte oxidase type proteins.

normal photosynthetic electron transport, the production of O_2^- is believed to be derived from the action of cell wall peroxidases and NAD(P)H oxidase (Wojtaszek, 1997). Both O_2^- and H_2O_2 are only moderately reactive but are readily converted to more reactive protonated species at low pH, yielding the hydroperoxy radical. In addition H_2O_2 also undergoes the Fenton reaction (figure 1.2) resulting in the formation of hydroxyl radicals ($OH^{\cdot}$). These radicals in turn can initiate lipid peroxidation leading to the oxidative damage of proteins and fragmentation of DNA.

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

Overall $O_2^- + H_2O_2 \rightarrow OH^- + OH^{\cdot} + O_2$

Figure 1.2. The Fenton reaction (from Hammond-Kossack and Jones, 1996)

Once formed, H_2O_2 has no unpaired electrons enabling it to pass through biological membranes into other cellular compartments. In contrast, superoxide cannot pass through membranes but instead is rapidly dismutated into H_2O_2 both non-enzymically or by superoxide dismutase (SOD). If H_2O_2 passes into other sub-cellular compartments such as the chloroplast, which contains many enzymes with metal ion co-factors, such as Cu^{2+} or Fe^{2+}, additional hydroxyl radicals would be formed, as described in the Fenton reaction in figure 1.2 (Halliwell and Gutteridge, 1990).

Of the systems postulated to form ROS in plants in response to fungal elicitation, one of the favoured mechanisms is described by the mammalian NADPH oxidase homologue which catalyses the reaction

$$NADPH + O_2 \rightarrow NADP^+ + 2O_2^- + H^+$$

A superoxide-producing complex, NADPH oxidase, consists of a catalytically active haem-binding flavocytochrome and several cytosolic regulatory proteins.

Overall, the complex passes electrons through the plasma membrane and produces O_2^- at the surface of the membrane. Although this complex has not been purified from plants, immunological studies based on the corresponding system in mammals suggest the presence of such proteins in plants (Dwyer *et al*, 1996; Tenhaken *et al*, 1995; Desikan *et al*, 1996). In addition, plant genes, encoding proteins with apparent similarity to the oxidative burst NADPH oxidase from animals, have been identified in a number of plant species (Bolwell and Wojtaszek, 1997). However, alternative mechanisms describing potential sources of the oxidative burst have been proposed. Another family of ROS-producing proteins are the pH-dependent cell-wall peroxidases which initiate ROS formation in the presence of reduced thiols such as glutathione (GSH) or cysteine (Bolwell, 1995; Pichorner *et al*, 1992). In this model, when the elicitor is recognised at the cell surface, ion transporters are activated leading to an exchange in Ca^{2+}, K^+, H^+ ions across the plasma membrane, leading to change in the pH of the extra-cellular matrix. This leads to the activation of cell wall peroxidases and the formation of O_2^-. In the presence of a reductant, this production can be sustained for a long period of time (Bolwell *et al*, 1995; Pichorner *et al*, 1992).

1.1.2 The Role of Reactive Oxygen Species in Response to Other Plant Stresses

Two types of stress, biotic or abiotic, may lead to oxidative conditions. While biotic-induced oxidative stress is generally very localised at the site of infection, often leading to the formation of visible necrotic lesions on the leaf surface, biotic oxidative stress is more likely to occur over a greater area of the plant, with the site of action often being the chloroplast (Alscher *et al*, 1997). Such general oxidative stress to the plant can result from UV light, extreme temperatures, limited water availability, or salt stress. Damage to leaves by air pollutants such as sulphur dioxide, ozone, or herbicides can also disrupt the photosynthetic apparatus, leading to production of ROS and associated oxidative damage (Mehlhorn *et al*, 1986, Foyer and Mullineaux, 1994, Foyer *et al*, 1994).

Even under non-stress conditions, the cell continually produces ROS as by-products of photosynthesis and metabolism e.g. through the action of NADPH-dependent superoxide synthetase and superoxide dismutase (SOD) (Noctor and Foyer, 1998). Superoxide is produced by the Mehler reaction in photosystem (PS) I and then rapidly dismutated to H_2O_2. As the thylakoid membrane contains proteins that bind transition metal ions, the ROS produced have the ability to initiate cascade reactions that lead to the formation of hydroxyl radicals and lipid oxyl derivatives among others. It is therefore particularly important to remove H_2O_2 from the chloroplast, as many of the enzymes involved in photosynthesis and carbon assimilation such as glyceraldehyde 3-phosphate dehydrogenase (GADPH) are sensitive to oxidising conditions (Ruelland and Miginiac-Maslow, 1999). Similarly, the stability of the highly abundant ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is dependent on the redox status of the chloroplast. Under oxidative conditions, a cysteinyl residue (Cys 247) on the large subunit of rubisco undergoes disulphide formation, leading to enzyme inhibition and rapid degradation of the protein (Mehta *et al*, 1992).

Under conditions of high irradiation and high temperatures, stomatal closure to prevent excessive water loss leads to CO_2-limiting conditions. Under these conditions, O_2 becomes an alternative electron acceptor in photosynthesis resulting in the formation of ROS (Asada and Takahashi, 1987; Robinson, 1988). Oxidative stress is also a threat during high light intensities, when photosynthetic harvesting of light energy exceeds utilisation of the electrons produced by anabolic pathways. If, for example, the plant is exposed to more photons than can be utilised for carbon assimilation, the $NADP^+$/NADPH ratio becomes a limiting factor as the $NADP^+$ pool becomes increasingly reduced. In this case, the lack of acceptors for reduced components of the electron transport chain becomes the limiting factor (Alscher, 1997) and excess energy continues to form ROS leading to membrane oxidation and consequent breakdown of plastidic proteins (Cassano and Trippi, 1992).

Most cellular components have the potential to become a source of reactive oxygen species. The overreduction of the electron transport chain is particularly susceptible as many environmental conditions such as high light limit CO_2 fixation, reducing the level of NADP+ regeneration by the Calvin cycle. Overreduction of the synthetic electron transport chain leads to the formation of singlet oxygen in the chloroplasts (Krause, 1994). Plants evolved the photorespiratory pathway to limit the effect of limited CO_2 fixation and regenerate NADP+ (Koraki and Takeba, 1996). H_2O_2 formed in the peroxisomes from the conversion of glycolate to glyoxylate in the photorespiratory pathway is removed by catalases (Smith, 1985). β-oxidation of fatty-acids, another source of reactive oxygen species, also occurs in the peroxisomes. The first step of the β-oxidation pathway involves the dehydrogenation of acyl-CoA by acyl-CoA oxidase, transferring the electrons to molecular oxygen with the formation of hydrogen peroxide (Grossi *et al*, 2003). H_2O_2 is formed following transfer of hydrogen atoms from $FADH_2$ to oxygen. The H_2O_2 is subsequently degraded by catalase. Another major source of reactive oxygen species evolved during normal metabolism is the overreduction of the electron transport chain in mitochondria. Here, the reduction of water to release protons necessary for the formation of pH gradient between the inner membrane and inner-membrane space which drives the formation of ATP from ADP and P*i* (Senior, 1988).

Although many mechanisms are in place to negate the effects of ROS generation, uncontrolled production of H_2O_2 will lead to oxidative damage in the cell. In particular, proteins are susceptible to damage through a three stage oxidation of the thiol group of component cysteinyl residues which result in a loss of protein activity (Charles and Halliwell, 1981). In the chloroplast this is followed by proteolysis by proteases associated with the thylakoid membrane (Cassano *et al*, 1992). Of particular significance is the first step of thiol oxidation which results in the conversion of an –SH group to a sulfenic acid derivative. As a sulfenic acid derivative, the thiol can be reversibly modified through the formation of a mixed disulphide with GSH to protect it from further, irreversible oxidation and

proteolysis (Barrett *et al*, 1999). This process is termed protein thiolation and will be discussed in detail later.

1.1.3 Antioxidant enzymes in Plants

Many enzymes are required to deal efficiently with the removal of superoxide (O_2^-) and H_2O_2. Superoxide is rapidly dismutated to H_2O_2 by SOD, with H_2O_2 being able to pass through membranes to other compartments. Thus, all compartments of the cell require efficient mechanisms to remove ROS. Catalases convert H_2O_2 to H_2O and molecular oxygen. These enzymes have high catalytic rates but low substrate affinities, since the reaction requires two H_2O_2 molecules simultaneously at the active site (Willkens *et al*, 1995). Protection of chloroplastic thiol-regulated enzymes is not performed by catalases as they are absent from this organelle, although reductant-requiring peroxidases, reducing H_2O_2 to H_2O are found in the chloroplast and are believed to play an important role in this process. (Noctor *et al*, 1998).

Of the protective processes available, glutathione, ascorbic acid and α-tocopherol have received major attention as antioxidants able to counteract ROS (figure 1.3) (Alscher 1997). The maintenance of pools of these three compounds in the reduced state by enzymatic regeneration is essential for the detoxification of ROS (Foyer and Mullineaux, 1994). Ascorbate and glutathione are linked in their ability to remove H_2O_2 by the ascorbate-glutathione cycle. H_2O_2 is reduced to water by ascorbate peroxidase (Apx), with the two co-substrate molecules of ascorbate being converted to two molecules of monodehydroascorbate (MDHA). Ascorbate can be regenerated enzymically or chemically from MDHA, but the rapid disproportionation of MDHA to dehydroascorbate (DHA) and ascorbate ensures recycling is required. DHA is reduced to ascorbate by dehydroascorbate reductase, using GSH as a substrate, with oxidised glutathione (GSSG) produced in the process, which in turn, is reduced by glutathione reductase (GR). Overall, no ascorbate or GSH is consumed during this process (Noctor and Foyer, 1998),

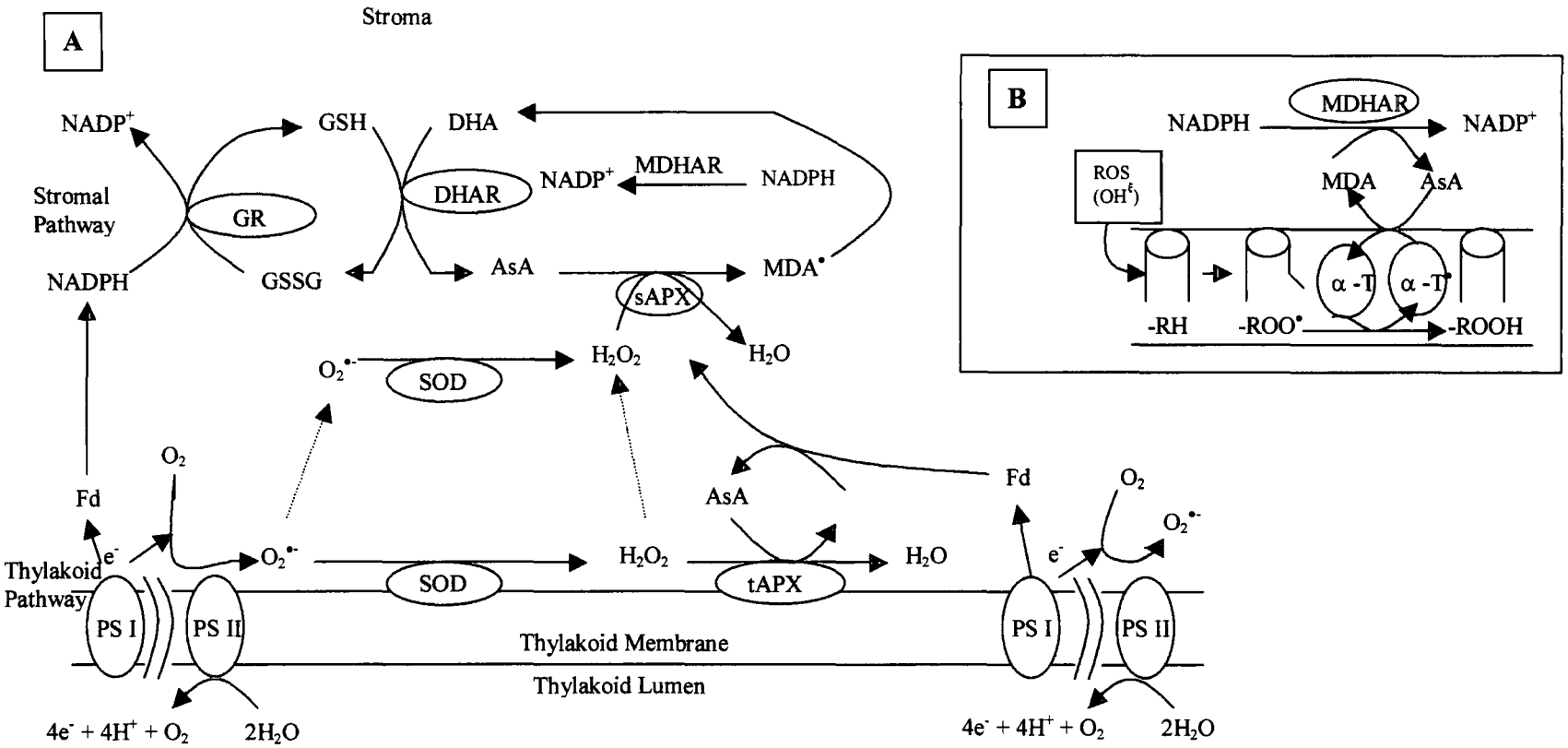

Figure 1.3. ROS scavenging pathways involving glutathione, ascorbate and α-tocopherol in A, the chloroplast and B, thylakoid associated protection involving ascorbate and ROS (From Alscher, 1997). Abbreviations used: α-t, α-tocopherol; AsA, ascorbic acid; DHAR, dehydoascorbate reductase; Fd, ferredoxin; GR, glutathione reductase; GSH, glutathione (reduced); GSSG, glutathione (oxidised); MDA, monodehydoascorbate; MDHAR, monodehydroascorbate reductase; P, peroxidation; s and t APX, stromal and thylakoid ascorbate peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

but ultimately the electrons required to maintain these compounds in their reduced state are derived from NAD(P)H (figure 1.3). Ascorbate also acts as a reductant in the regeneration of α-tocopherol. Ascorbic acid is also important in the zeaxanthin cycle in the thylakoid membrane. Under conditions of excess light, violaxanthin in photosystem II (PSII) undergoes de-epoxidation to zeaxanthin using ascorbate as reductant (Foyer, 1993). In view of their importance as antioxidants, not surprisingly, both levels of GSH and ascorbate increase in response to stress induced ROS production (Hausladen and Alscher, 1993; Foyer *et al* 1994).

1.1.4 Nitric Oxide as a Plant Stress Signalling Agent

Over the last few years, nitric oxide (NO) has been proposed as a new type of ROS (Klatt and Lamas, 2000). By reacting with oxygen, NO can form peroxynitrite, a reactive nitrogen species (RNS) which can lead to protein degradation by a similar mechanism to that caused by ROS. This new area of biochemistry has received a great deal of attention over the last 3-4 years, particularly in mammalian systems where NO is formed in response to oxidative stress by the enzyme nitric oxide synthetase (NOS). In these studies, a link between NO and thiol reductants has been uncovered with *S*-nitrosylated GSH (GSNO) identified as an important intermediate during NO-oxidative stress (Jourd'heuil *et al*, 1999), with GSNO being produced as a consequence of the antioxidant activity of GSH. In addition, several proteins have been identified in which the thiol group has been nitrosylated leading to inactivation (Klatt and Lamas, 2000). In plants, although NOS has not been purified, the role of NO as a reactive nitrogen species and as a signalling agent is slowly being revealed. Noritake (1996) first discovered that potato tubers exposed to NO-releasing compounds induced the phytoalexin response while Durner *et al* (1998) determined that NO was essential for initiating early defence signal responses in tobacco. Care needs to be exercised in interpreting these results as the chemistry of NO is particularly complex and, due to the instability of some nitric oxide derivatives, the action of individual nitrogen species is unknown.

Interestingly, there is also evidence to suggest NO can act as an 'antioxidant' compound, preventing chlorophyll degradation in potato tuber leaves when exposed to the ROS generating herbicide, diquat (Beligni and Lamattina, 1999). This particular area is advancing rapidly and much work is needed to elucidate the precise role of NO in the defence response of plants to biotic and abiotic stress.

1.1.5 Phytoalexin Response to Fungal Elicitation

One of the earliest post-elicitation responses following ROS accumulation in many plants is the formation of antimicrobial compounds or phytoalexins, which are low molecular weight, lipophilic chemicals, typically derived from phenolic or terpenoid biosynthetic pathways (Dixon, 1986). As such, phytoalexin accumulation can be used as a diagnostic marker for defence response in plants invoked by either elicitors derived from cell wall preparations or pathogens.

The major group of phytoalexins synthesised by Leguminous plants are isoflavonoids. Isoflavonoid phytoalexins are synthesised from L-phenylalanine and malonyl CoA (figure 1.4) via a series of tightly co-ordinated group of enzymes. They are induced in response to infection as a result of the upregulation of the transcript levels of each enzyme in the pathway. (Dixon *et al*, 1995a; Lawton and Lamb, 1987). Thus, in alfalfa cell cultures challenged with fungal elicitors, the induction of the genes encoding enzymes of the phytoalexin synthesis was rapid, occurring as early as 5 min post elicitation treatment. This was then followed by the closely co-ordinated induction of the respective enzyme activities, notably phenylalanine-ammonium lyase (PAL), chalcone synthase (CHS) and isoflavone reductase (IFR), three of the enzymes involved in the biosynthetic pathway, over the following 3 h (Dixon *et al*, 1995a). In addition to acting as phytoalexins, isoflavonoids are also constitutively present in other Leguominosae, such as soybean, as the respective β-D-*O*-glucoside-6"-*O*-malonate conjugates which are stored in the vacuole (Koster *et al*, 1983a). In alfalfa, a significant proportion of the phytoalexin medicarpin which accumulates in the cell in the

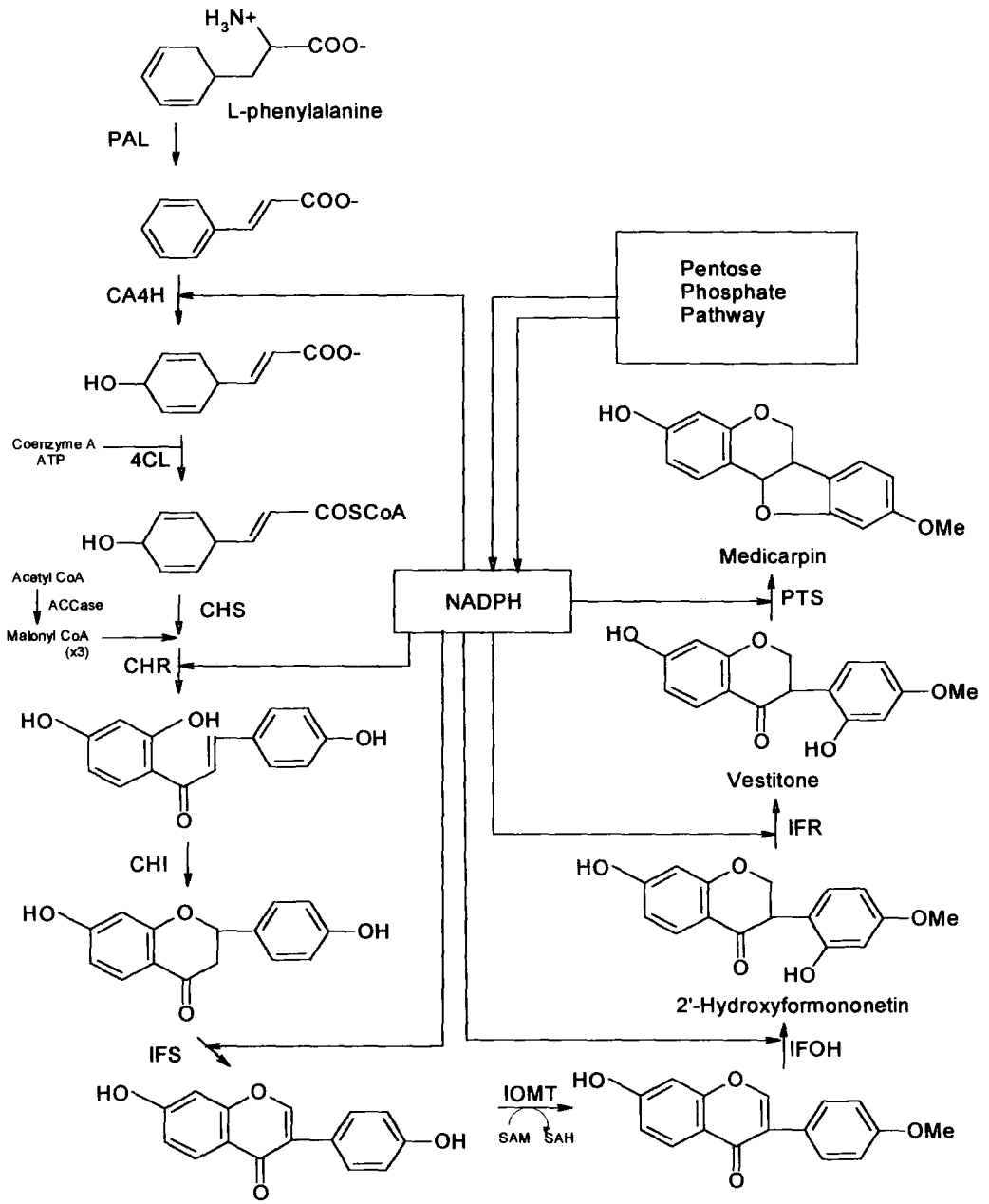

Figure 1.4 Biosynthesis of the isoflavonoid phytoalexin medicarpin from L-phenylalanine.
ACCase, acetyl coenzyme A carboxylase (EC 6.4.1.2); CA4H, cinnamic acid 4-hydroxylase (EC 1.14.13.11); CHI, chalcone isomerase (EC 5.5.1.6); CHR, chalcone reductase (EC 1.1.1.4); CHS, chalcone synthase (EC 2.3.1.74); 4CL, 4-coumaryl CoA ligase (EC 6.2.1.12); IFOH, isoflavone 2'hydroxylase (EC1.14.13.52); IFR, isoflavone reductase (EC 1.3.1.45); IFS, isoflavone synthase (EC 5.4.9.9); IOMT, isoflavone 4'-*O*-methyltransferase; PAL, phenylalanine ammonium lyase (EC 4.3.1.5); PTS, pterocarpan synthase (EC 1.1.1.246); SAM, *S*-adenosyl L-methionine.

early stages of elicitation can be derived from the hydrolysis of the respective conjugates (Edwards *et al*, 1997). However, newly synthesised medicarpin was the major source of phytoalexin accumulation over the longer term (Edwards *et al*, 1997).

Significantly, in French Bean and parsley, it has been demonstrated that GSH can elicit a rapid and selective activation of the transcription of defence genes in a manner analogous to that observed after elicitation by a fungal cell wall preparation (Conrath *et al*, 1989; Wingate *et al*, 1988), suggesting that increased thiol accumulation can act as an elicitation signal. However, later studies concluded that increased synthesis of endogenous thiols is a response to elicitor treatment as opposed to a signal for the induction of phytoalexin synthetic pathway (Edwards *et al*, 1991).

1.2 Cellular Thiols in Plants

1.2.1 Glutathione

The tripeptide glutathione, γ-glutamyl-cysteinyl-glycine (GSH) is the most abundant low molecular weight thiol in plant and mammalian cells (Klapheck, 1988). In plants, its major roles include the transport and storage of sulphur (Schupp *et al*, 1992) and the detoxification of xenobiotics through enzyme-mediated conjugation catalysed by glutathione transferases (Marrs, 1996; Alscher, 1989). GSH and associated precursors are also needed to synthesise phytochelatins, which are involved in heavy metal detoxification (Grill *et al*, 1990). GSH can also act as a regulator of defence gene expression (Wingate *et al*, 1988) and plant development (Ogawa *et al*, 2001). Recent work has also highlighted the importance of GSH in initiating and maintaining cell division during post embryonic root development. Arabidopsis mutants *root meristemless1* (*rml1*) / *cadmium sensitive2* (*cad2-1*) have short mature roots composed of the same number of cells as the embryonic root, failing to initiate

cell division when germinated (Cheng *et al*, 1995). *Cad2-1*, encoding the γECS gene (Cobbet *et al*, 1998) is allelic to *rml1* resulting in the *rml1/cad2-1* mutants being devoid of GSH. Studies of these mutants have shown GSH to be essential for both initiation and maintenance of cell division (Vernoux et al, 2000) .

In animals, GSH can form mixed disulphides with cysteine residues in proteins which can regulate enzyme activity (Dafré, 1996) as well as protecting susceptible protein thiol groups from irreversible oxidation during stress conditions. In both plants and animals, GSH acts as the major redox buffer within the cell, forming disulphides with other GSH molecules to produce oxidised glutathione (GSSG), (Noctor *et al*, 1998). The γ-peptide bond between glutamate and cysteine is believed to protect GSH from cellular amino-peptidase, enabling it to fulfil its many roles (Sies, 1999).

The concentration of GSH in plants ranges from 0.1 mM to 10 mM under normal conditions, but this can be increased up to five fold by increasing the concentrations of the major precursors, or by altering the activities of the biosynthetic pathway (May *et al*, 1998).

1.2.2 Glutathione Homologues

Several homologues of glutathione are found throughout the plant kingdom. Leguminous species such as soybean, contain homoglutathione (hGSH), where β-alanine replaces glycine in the tripeptide, as the major thiol (> 95 % total thiol) and in wheat, serine replaces glycine to give hydroxymethyl-glutathione (hmGSH) (Klapheck, 1988). In some instances more than one thiol is present. For example, in alfalfa both GSH and hGSH are present in significant amounts, although it is still unclear as to the individual roles each play. In alfalfa plants, the differential level of GSH or hGSH synthesised was dependent on the abundance of transcripts for *gshs1* and *gshs2*, encoding glutathione synthetase (GSHS) and homoglutathione synthetase (hGSHS) respectively (Frendo *et al*, 1999). GSH was

the only thiol present in seeds, suggesting it was the major thiol for sulphur storage. GSH and *gshs1* were also present in the roots, leaves and all organs of mature and young plants. hGSH and *gshs2*, on the other hand were only found in the roots of 48 h old plantlets and in the nodules and shoots of mature plants (Frendo *et al*, 1999). These results suggest that the two thiols have differing roles in plant growth and development in alfalfa.

1.2.3 Glutathione Biosynthesis and Regulation

GSH is synthesised via a two-step ATP-requiring enzymatic process instead of through traditional protein synthesis via the translation of mRNA. Initially described in animal systems (Meister, 1983), the biosynthetic pathway is now well established in plants (see reactions 1 and 2 below). The first step of the synthesis is the ligation of L-glutamate to L-cysteine, via the γ carboxyl group of glutamate, to form γ-glutamylcysteine (γ-EC) catalysed by γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2). The second reaction involves the production of glutathione by the formation of a peptide bond between the α-carboxyl of cysteine in γ-EC and α-amino group of glycine, catalysed by glutathione synthetase (GSHS; EC 6.3.2.3). In both cases, the catalytic mechanism involves an acylphosphate intermediate resulting from the transfer of the γ-phosphate of ATP to the respective carboxyl group, followed by the formation of a peptide bond and the release of inorganic phosphate (May *et al* 1998).

1 L-Glu + L-Cys + ATP → L-γ-Glu-L-Cys + ADP + Pi

2 L-γ-Glu-L-Cys + ATP → L-γ-Glu-L-Cys-Gly + ADP + Pi

In legumes, where hGSH is the major thiol, the final step of synthesis is catalysed by homoglutathione synthetase (hGSHS) (Klapheck, 1988). In wheat, the synthetic pathway for hmGSH is still unknown.

Efficient regulation of the glutathione pool is thought to be particularly important in metabolism. In the chloroplast, GSH provides the redox-buffering capacity vital for efficient photosynthesis, as it is involved in reducing the oxidised species which are formed as a result of light capture and subsequent electron transport events (Foyer and Halliwell, 1976; Kunert and Foyer, 1993). The multifunctional nature and importance of glutathione has led to a great deal of interest in the regulation of glutathione metabolism and many biochemical and molecular studies have been carried out (Noctor *et al*, 1998a). Of particular interest, has been to determine whether changes in the pool size and ratio of GSH:GSSG are the causes or the effect of defence-related responses.

Control of the GSH pathway has been studied in several species, with transgenic plants being produced to elucidate the role of each step under differing conditions. Glutathione synthesis has been found to vary according to substrate availability, activity of pathway enzymes and environmental conditions. The formation of γ-EC by γ-ECS is the major rate limiting step under normal growth conditions (May *et al*, 1998), although this, in turn, is limited by the availability of cysteine (Strohm, 1995). To determine the roles of GSH biosynthetic enzymes, cDNAs for *gsh1* and *gshs1*, encoding for γ-ECS and GSHS respectively, were cloned from poplar and their expression in poplar leaves was then manipulated by genetic engineering (Noctor, 1998b). In transformed poplars over-expressing *gsh1*, an increase in γ-ECS enzyme activity of between 18 and 60 fold was determined, resulting in a 2 to 4 fold increase in the level of foliar GSH. GSHS activity determined simultaneously was similar to that detected in the untransformed poplars, suggesting the changes resulted from the increase in γ-ECS activity alone (Noctor *et al*, 1996; Arisi *et al*, 1997). These increases in GSH were accompanied by an increase in the size of the GSSG pool, thus maintaining the redox balance and suggesting that endogenous glutathione reductase (GR), levels were sufficient to cope with the increase and able to recycle GSSG back to the thiol active GSH. Although the levels of both γ-EC and GSH increased, no decrease in the cysteine pool was observed and, when both transformed and non-transformed plants were

supplied with cysteine, the level of GSH doubled. This increase in cellular GSH following feeding of cysteine has also been observed in roots and leaves of several plant species (Buwalda *et al*, 1990; Farago and Brunold, 1994; Strohm *et al*, 1995) suggesting that cysteine availability plays a major role in determining total GSH content.

The identity and regulation of Arabidopsis γECS has only recently been clarified. Early expression of γECS sequence in *E. coli* did not raise the GSH level above that found in wild type *E. coli* as anticipated. Also, the *At*γECS sequence is highly divergent compared to other eukaryotes, unlike GSHS sequence which is extensively homologous with other eukaryotes (May *et al*, 1998). Only recently was the GSH level of *cad2-1* mutants restored by the complementation of *At*γECS sequence (Xiang *et al*, 2001).

During cysteine biosynthesis, adenosine 5'-phosphosulphate reductase and serine acetyl transferase may limit the availability of the cysteine precursor *O*-acetylserine. The activity of these enzymes increased 2 and 4 fold respectively in response to the expression of γ-ECS in poplar, leading to an improved substrate supply for the enzyme (Noctor *et al*, 1998a). The co-ordinated control of GSH synthesis through cysteine availability is consistent with the role of GSH as a major storage and transport form of sulphur (Rennenberg, 1995). Expression of *gshs1* targeted to the chloroplast did not result in any significant increase in GSH levels, suggesting that at the biochemical level, γ-ECS was more important in regulating overall GSH content.

Even though γ-ECS enzyme activity is the rate-limiting step in GSH biosynthesis, a major limitation on flux through the GSH pathway is the effect of feedback inhibition on γ-ECS by GSH (May *et al*, 1998). This has been readily demonstrated *in-vitro* with GSH having a K_i value of 0.42 mM towards γ-ECS (Hell and Bergmann, 1990). However, as shown with the transgenic poplar

overexpressing γ-ECS, this feedback inhibition can be overcome *in-vivo* (Noctor *et al*, 1997).

1.2.4 Changes in Thiol Metabolism Due to Stress

When the plant cell is oxidatively stressed, the redox homeostasis is perturbed, partly by the rapid oxidation of GSH combating the stress. In response, plant cells have to maintain the GSH:GSSG ratio by a combination of synthesising more GSH to maintain a high GSH:GSSG ratio and by reducing the GSSG pool through the action of glutathione reductase. As GSH is considered the major redox buffer in the cell, any change in the level of GSH may be important. In contrast to other cellular redox couples (e.g. $NADP^+$:NADPH), the absolute levels of GSH and GSSG have an impact on the reduction potential. For example, if the GSH:GSSG ratio remained the same but the absolute concentration decreased 10 fold, using the Nearnst equation, a 59.1 mV change in the redox couple would be determined (Shafner and Buettner, 2001). This change in redox potential may effect the cellular redox state and the that of any redox dependant enzymes.

Studies monitoring the overall levels of GSH in arabidopsis cell cultures have observed an increase in the total GSH pool after oxidative stimulation by H_2O_2 following catalase inhibition (May and Leaver, 1993). Exposure to ozone also leads to an accumulation of GSH in poplar (Sen Gupta *et al*, 1991; Luwe, 1996) and in alfalfa cell culture challenged with an elicitor preparation from *Colletotrichum lindermuthianum*, both GSH and hGSH accumulated (Edwards *et al*, 1991). In soybean cell cultures, treatment with an elicitor preparation from *Colletotrichum lindermuthianum*, increased the hGSH content 5 fold (Edwards *et al*, 1991). Although GSH represents only approximately 5 % of cellular thiol in soybean, no major change in its accumulation was observed. (Edwards *et al*, 1991). In addition to increases in the total thiol pool, ranging from 1 to 4 fold, large increases in the proportion of the thiol present as oxidised GSSG have also been reported. While GSSG normally comprises around 5 % total cellular thiol,

this can increase to more than 50 % following treatment with the herbicide aminotriazole (a catalase inhibitor), as determined in barley and tobacco plantlets (Smith, 1985). This increase is not uniform in plants, however as no increase in GSSG was detected in arabidopsis cell cultures during H_2O_2 induced stress (May and Leaver, 1993). This increase in the proportion of GSSG is believed to be a response to the generation of ROS during severe stress, which oxidises GSH directly as well as due to increased use of GSH in maintaining the ascorbate pool through the ascorbate-GSH cycle, the latter being increasingly active following the oxidative burst (Noctor *et al*, 1998). Glutathione peroxidases and lipid peroxidation also contribute to changes in GSH metabolism. GSH peroxidases in plants induced following the elicitation of the defence response and catalyse the reduction of organic hydroperoxides to their corresponding less toxic alcohols, using GSH as a substrate for this reaction forming GSSG and H_2O_2 (Dixon *et al*, 1998). This peroxidation differs slightly from that seen in mammalian cells whereby GSH peroxidases require selenium co-factor and are constitutively expressed (Eshdat *et al*, 1997). Arabidopsis plants containing reduced levels of GSH were also unable to accumulate anthocyanins, a process believed to be catalysed by GSTs, in the vacuoles (Xiang *et al*, 2001).

By alleviating feedback inhibition of γ-ECS by reducing the pool of GSH, either through its conversion to GSSG, or depletion through other means, γ-ECS activity is increased and the synthesis of GSH enhanced. During severe oxidative stress, the levels of mRNA transcripts encoding γ-ECS and GSHS steadily increase, resulting in the production of more enzyme through *de-novo* synthesis, further elevating the GSH content. The initiating signals, which induce the expression of the genes of GSH are unknown (Xiang and Oliver, 1998). However, jasmonic acid may play a role in the signalling for GSH biosynthesis and recycling. Arabidopsis plants treated with jasmonic acid accumulated transcripts encoding *gsh1*, *gshs1* and *gr1* (cytosolic glutathione reductase) (Xiang and Oliver, 1998). However, the accumulation of transcripts in this instance was not associated with increased activity of the associated enzymes. Also no changes in GSH:GSSG ratio

were observed suggesting that ROS generation through the oxidative burst was not involved. Interestingly, there is also a link between jasmonic acid and cross-resistance to different types of oxidative stress. In tobacco for example, jasmonic acid treatment lead to cross-tolerance to ozone and other environmental stresses (Orvar *et al*, 1997).

The availability of glycine is also important in regulating the accumulation of GSH, notably during stress which interferes with photorespiration. Under conditions of high light or treatment with the herbicide paraquat, photorespiration is increased and the availability of photorespiratory generated glycine leads to an up-regulation of GSH biosynthesis (Noctor *et al*, 1999).

When challenged with either Cd^{2+} or Cu^{2+} plants accumulate GSH-derived phytochelatins (γ-glu-cys$_{2\text{-}12}$-gly), which act to chelate heavy metals and their removal to the vacuole (Xiang *et al*, 2001). As the GSH pathway is used in for phytochelatin synthesis, the GSH pool initially declines following exposure to heavy metals due to phytochelatin synthesis. Subsequently GSH synthesis increases with GSH levels recovering to pre-stress levels (Xiang and Oliver, 1998).

GSH is also important in the regulation of plant cell cycle as progression through the cell cycle is tightly controlled by an ordered series of events. In studies of Arabidopsis root development, high levels of GSH were associated with epidermal and cortical initials whilst cells with an extended G1 phase had markedly lower GSH levels (May *et al*, 1998). Also, flowering of Arabidopsis wild type has been showed to be delayed by addition of the γECS inhibitor buthionine sulphoximine to reduce GSH levels and the delayed flowering effect of the cad2-1 (γECS deficient) mutant has been shown to be alleviated by addition of GSH (Ogawa *et al*, 2001).

1.2.5 Glutathione and Herbicide Metabolism

Glutathione plays an important role in the detoxification of herbicides and xenobiotics forming the respective GSH-conjugates as catalysed by the large protein family of glutathione *S*-transferase (GST). This conjugation occurs by the direct attack of the GSH thiolate anion to an elecrophilic substrate (X-Z), normally displacing a leaving group in the process

$$X\text{-}Z + GSH \rightarrow X\text{-}SG + HZ$$

(Z; e.g. a halide)

The conjugation reactions are catalysed both by constitutively-expressed GSTs and by GSTs which are induced by a range of stress treatments including exposure to xenobiotics, fungal pathogens and extreme environmental conditions (Marrs *et al*, 1996). GSTs, like GSH, are known to accumulate following oxidative stress, GSTs in soybean, for example, accumulate after exposure to ROS (Flury *et al*, 1998). Following the action of GSTs, the glutathione conjugates are transported into the vacuole by an ATP-dependent GSH-conjugate transporter (Tommasini *et al*, 1998). GSTII from *Zea mays* also has peroxidase-type activity (Dixon et al, 1997).

1.3 Protein Thiolation in Animals

The process of ROS production, either during a period of externally applied stress or due to 'normal' metabolism of the cell provides reactive species, which would lead to phytotoxicity and the oxidation and decomposition of protein if left unchecked. The reactive sulphydryl group of cysteines residue in proteins is particularly vulnerable to oxidative modification. Irreversible oxidation leads to denatured proteins, which are actively degraded by proteases within the cell, several of which are present in the thylakoid membrane (Cassano *et al*, 1994).

Protein *S*-glutathionylation, also referred to as thiolation, is the formation of mixed disulphides between the protein thiol group and the thiol group of GSH (Thomas *et al*, 1995). Regulation of metabolic pathways via redox-sensitive cysteine residues on key enzymes mediated through reversible interactions with GSH, is thought to be an important mechanism of cellular control (Vivekanandan and Edwards, 1987). Thus, microsomal GSTs were reportedly activated through thiolation of their Cys 49 residue (Dafré *et al*, 1996).

The sulphydryl group of cysteine can exist in multiple oxidation states. Under normal conditions, the cysteinyl thiol group is fully reduced (Cys-SH). In this state, the possible formation of disulphide bonds is avoided and protein activity is retained if the free cysteine is required for catalysis, regulation or structural integrity. There follows three progressive oxidation states of the thiol group, sulphenic acid, sulphinic acid and sulphonic acid. The sulphinic or sulphonic acid states are stable, irreversible oxidation states with proteins containing such modified residues undergoing denaturation and degradation by proteases. In contrast, the formation of mixed disulphides with glutathione whilst in the sulphenic acid state protects the cysteine residues and prevents further oxidation to the sulphinic acid state; providing a mechanism by which the protein is subsequently able to become re-reduced following the period of stress.

The process of thiolation is dynamic and the rates of thiolation and dethiolation (the reduction of the mixed disulphide bond to restore the protein SH group with the release of GSH) determining the overall protein thiolation status. Although the roles of thiolation are relatively poorly understood, it is believed that the antioxidant role of *S*-thiolation is distinct from its potential role in metabolic regulation (Thomas *et al*, 1995). The potential for protein thiolation is significant. In mammalian cells, the concentration of reactive protein sulphydryls is at least as high as the total glutathione pool (DiSimplicico *et al*, 1998). Proteins which can undergo thiolation *in-vitro*, have been placed into 3 categories relating to their likely state of thiolation *in-vivo* (Thomas *et al*, 1995). The first group of proteins

consist of those that are readily dethiolated under normal cellular conditions. It is therefore unlikely that these proteins will be observed in a thiolated state *in-vivo*. Secondly, those proteins which are readily thiolated under normal growth conditions i.e. in the absence of ROS or other stimuli; an increase in thiolation state is, therefore, unlikely to be observed following oxidative stimuli. Thirdly, proteins whose thiolation state increases following exposure to ROS and are, therefore, detectable as such during *in-vivo* labelling studies. The third group of proteins is believed to be the largest group, although the difficulty of detection of the first two groups makes it impossible to confirm this.

In addition to roles in protecting protein SH groups and regulating metabolic enzymes, thiolation is also emerging as a key regulator of signalling events in mammalian cells. For example, in non-phagocytic cells, growth factors induce the intracellular production of ROS and the elevation of tyrosine phosphorylation, which can also be induced by activating kinases or inactivating phosphatases (Barrett *et al*, 1999). In this example, the ROS-mediated signal mechanism involves the highly abundant protein-tyrosine phosphatase 1B (PTP-1B). This is believed to be regulated by the active-site Cys-215 since oxidation by H_2O_2 leads to irreversible inactivation and loss of activity. In the presence of GSH, a mixed disulphide is formed, protecting Cys-215 with the modification reversed by the thiol-reducing enzyme glutaredoxin (Barrett *et al*, 1999).

It has also been determined recently that thiolation can regulate whole protein complexes. In eukaryotic cells, the proteasome, an essential proteolytic complex responsible for the rapid degradation of abnormal proteins generated through mutations or oxidative damage (Coux *et al*, 1996; Grune *et al*, 1995; Grune *et al*, 1997) is now thought to be under thiol-modulated redox control (Demasi *et al*, 2001).

1.3.1 Mechanisms

The mechanism of protein *S*-thiolation (Pr-SSG) *in-vivo* is still under some debate, especially with the discovery of the potential involvement of nitric oxide. However, *in-vitro* it is believed to operate through one of two mechanisms.

As previously mentioned, during oxidative stress, the GSH redox state alters, decreasing the ratio of GSH:GSSG from approximately 100:1 down to 10:1 or even 1:1 (Klatt and Lamas, 2000). This change in ratio can lead to disulphide exchange, as shown in the reaction below

$$\text{Pr-SH} + \text{GSSG} \rightarrow \text{Pr-SSG and GSH}$$

Although it is an energetically-viable mechanism, this reaction is unlikely to proceed in many proteins, because the K_{mix} value, the concentration at which 50 % of the available protein cysteinyl residues become thiolated, of most proteins is around 1. This means that in order to achieve 50 % thiolation, the ratio of GSH:GSSG must be about 1, a ratio unlikely to be achieved *in-vivo*. One known exception to this is the thiolation of the transcription factor cJun from rat liver cells which has a calculated the K_{mix} value of approximately 13 (Klatt *et al*, 1999). Although this does not prove that the thiolation mechanism involves disulphide exchange it does show that in some instances that it is possible.

The second proposed thiolation mechanism involves the partial oxidation of the protein cysteine residue to the unstable sulphenic acid derivative, allowing direct mixed disulphide formation with glutathione without the need for GSSG. This is shown below

$$\begin{aligned} & \text{Pr-SH} + \text{OH}^{\cdot} && \rightarrow \text{Pr-S}^{\cdot} + \text{HOH} \\ \Rightarrow\ & \text{Pr-S}^{\cdot} + \text{GSH} && \rightarrow \text{Pr-S-GS}^{\cdot-}\text{H} \\ \Rightarrow\ & \text{Pr-S-GS}^{\cdot-}\text{H} + \text{O}_2 && \rightarrow \text{Pr-SSG} + \text{O}_2^{\cdot-} \end{aligned}$$

To facilitate the thiolation of cysteine residues by either mechanism, various structural criteria need to be taken into consideration. To allow for the disulphide exchange mechanism to thiolate cysteine, the sulphydryl site should have proton-donating groups in close proximity to stabilise the anionic form of the sulphydryl. Direct thiolation by GSH needs structural requirements with proton withdrawing groups to aid formation of thiyl radical (Thomas *et al*, 1995). The only known thiolated protein with structural properties conducive to stabilise such a thiolate anion is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Eriksson and Liljas, 1993). In GAPDH, the amide protons of residues 151-152 are positioned such that they interact directly with the SH of Cys 151. Residues 150-153 define a loop in which the backbone amides of 150-151 are orientated toward the SH of the cysteine, suggesting a local environment favourable to the binding of negatively charged compounds. A nearby positively charged histidine then stabilises any thiolate anion formed at Cys 151 and enhances the ionisation of the sulphydryl residue. However, it is uncertain whether this mechanism operates under physiological conditions as the ionisation state of the histidine can not be deduced from the X-ray crystal structure of GAPDH (Thomas *et al*, 1995).

The conformation of glutathione varies according to whether it is being used for mixed disulphide protection or as a co-substrate. When glutathione binds as a substrate, e.g. in GSTs, nearly all the polar atoms of glutathione are recognised by the enzyme. In GSTs, salt links are formed with the α-amino and α-carboxyl groups of the γ-glutamyl residue and the α-carboxyl of the glycyl residue. The amide nitrogen of the peptide bond between the γ-glutamyl and cysteinyl residues then hydrogen bonds to the GST. In contrast, during thiolation GSH has been found to exist in several conformations at different cysteine residues within the same protein as demonstrated with carbonic anhydrase III (CA III) (Eriksson and Liljas, 1994). CA III formed mixed disulphides with GSH at two sites, Cys 183 and Cys 188, without resulting in any conformational change to the protein. As the only interaction between protein sulphydryl and GSH is via the covalent

disulphide bond, the interaction appears to be disorganised. Thus, when bound by thiolation there is low specificity of interaction between GSH and the CA III which may improve accessibility to glutaredoxin to bind to the site during dethiolation (Thomas *et al*, 1995).

Thiolation often occurs when the cell is exposed to oxidative stress and since GSSG accumulates together with OH radicals, it was believed that thiolation occurred non-enzymatically (Thomas *et al*, 1995). However, subsequent studies on human GAPDH suggest that the enzyme glutaredoxin, normally associated with the reverse reaction, dethiolation, was also capable of catalysing thiolation (Lind *et al*, 1998). In these studies oxidised GAPDH was incubated with GSSG, resulting in several thiolated forms of GADPH. When incubated in the presence of recombinant human glutaredoxin 3 (Grx), the rate of thiolation was enhanced (Lind et al, 1998). Thiolation of GADPH was not found to affect its enzyme activity and it was assumed that the thiolated cysteinyl residues were not essential for activity, thiolation simply protecting the protein from oxidative damage. Other studies with GAPDH required nitrosylated GSH (GSNO) to thiolate the enzyme, as GSH alone would not do so. In this case, thiolation led to a loss of dehydrogenase activity which could only be recovered by the addition of DTT (Mohr *et al*, 1999). From this example it is clear that thiolation may occur with or without the intervention of a catalytic protein and that different thiolating agents have different biological effects on protein activity.

1.3.2 Dethiolation

The process of dethiolation is better understood than that of thiolation. Several enzymes have the ability to reduce mixed disulphides and these have been purified from both mammalian and plant sources (Jung and Thomas, 1996; Cho *et al*, 1998a). Although non-enzymatic dethiolation can occur via changes in the redox status leading to an increased ratio of GSH to GSSG, dethiolation is greatly increased in the presence of glutaredoxin (also known as thiol-transferase; Grx)

(Chrestensen *et al*, 2000). Grx is a member of the thiol-disulphide oxidoreductase (TDOR) family present in all prokaryote and eukaryotic organisms.

Grx participates in a pathway that couples the oxidation of NADPH with reduction of many substrates including ribonucleotides (ribonucleotide reductase), inorganic sulphates and methionine sulphoxide (Cho *et al*, 2000). Grx also shows some dehydroascorbate reductase (DHAR) activity as illustrated by Grx isolated from Arabidopsis seeds, rice and Chinese cabbage (Cho *et al*, 1998b; Sha *et al*, 1997). Grx can also reduce non-thiol substrates such as alloxan (2,4,5,6[1H,3H]-pyrimidinetetrone) to dialuric acid (Washburn and Wells, 1997). The re-reduction of Grx uses GSH, resulting in the formation of GSSG. It is, therefore, essential that the functioning of Grx as a reductant is coupled to GR to maintain the redox balance and ensure a continual supply of GSH. The NADPH-dependent reduction of ribonucleotides by Grx is highly sensitive to inhibition by GSSG (Holmgren, 1989), possibly through the reversible thiolation of the active-site cysteines present in glutaredoxin (Lind *et al*, 1998).

There are many sequence homologues of glutaredoxin. In Arabidopsis there are thought to be 2 distinctive types, termed S and L types, with a total of at least 20 Grx-like sequences in the GenBank database in this species (Meyer *et al*, 1999). So far, two of these have been purified from Arabidopsis representing the S and L-types of glutaredoxin respectively. The S-type was a 22 kDa heat-stable protein found in the seed (Cho *et al*, 1998a), while a 26.6 kDa L-type glutaredoxin was present in leaves (Kim *et al*, 1999a).

Other enzymes potentially able to dethiolate proteins include thioredoxin and protein disulphide isomerase, with both of these proteins having low activity toward protein mixed disulphides as compared with glutaredoxin (Jung and Thomas, 1996). Glutaredoxin, thioredoxin and protein disulphide isomerase are related proteins with partially overlapping functions, similar protein structure and a prominent redox active site (Ecklund *et al*, 1984). Thus, both Grx and

thioredoxin have a pair of cysteine residues in the conserved catalytic motif -Cys-Xaa-Xaa-Cys- (Xaa being any amino acid), enabling them to reduce the disulphide bridges of the target protein through the direct transfer of electrons from NADPH.

Grx plays a central role in the protection *of Saccharomyces cerevisiae* against damage to proteins by hydrogen peroxide and the chemical oxidant, menadione (Rodriguez-Manzaneque *et al*, 1999). Thus, the induction of the GRX genes *GRX*1 and *GRX*2 by oxidative, heat or osmotic stress has been reported (Grant *et al*, 2000). When exposed to diamide, an oxidant that readily oxidises GSH, a 5 fold increase in GRX2 was observed within 60 min whilst GRX1 was increased 2 fold (Grant *et al*, 2000).

Since glutaredoxin and thioredoxin both contain active site disulphides it has been hypothesised that they may themselves be susceptible to irreversible oxidative damage and loss of activity. *In-vitro* assays of pure glutaredoxin and thioredoxin in the presence of hydroxyl-radicals lead to a loss in their respective enzyme activity at physiological protein concentrations with the presence of 1 mM GSH preventing this inhibition (Starke *et al*, 1997). It was concluded that significant inactivation of Grx was only likely to occur during severe oxidative conditions.

1.4 Disulphide Bond Functionality and Regulatory Properties

The post-translational process of disulphide bond formation is essential for the correct folding and stability of many proteins, the process being carried out in the endoplasmic reticulum (ER). The minimal requirements for efficient refolding *in-vitro* have been defined as consisting of a redox buffer containing oxidising and reducing equivalents and an enzymatic catalyst for thiol-disulphide exchange. This enzymatic catalysis is provided through the action of a protein disulphide isomerase which depends on a pair of cysteines with the motif Cys-Xaa-Xaa-Cys within a homologous thioredoxin domain (Frand *et al*, 2000). The redox potential in the ER is more oxidising than the cytoplasm, leading to an increased ratio of

GSH:GSSG of between 1:1 to 1:3. The high proportion of GSSG lead to the conclusion that GSSG was the oxidising equivalent responsible for maintaining the highly oxidised status of PDI. Recent work using *E. coli* mutants lacking *gsh1*, the gene coding for γ-ECS, discovered that the absence of GSH did not inhibit disulphide bond formation, though the mutants were susceptible to oxidative stress (Cuozzo and Kaiser, 1999).

Many chloroplastic enzymes are regulated via disulphide oxidation/reduction by the well-characterised ferredoxin-thioredoxin system, linked to photosynthetic electron transport (Ruelland and Miginiac-Maslow, 1999) (figure 1.5). This regulation via disulphide bond exchange extends to the chloroplastic enzymes phosphoribulokinase, G3PDH, fructose 1,6-*bis*-phosphatase (FBPase), and sedoheptulose-1,7-*bis*-phosphatase (SBPase) of the Calvin Cycle, glucose-6-phosphate dehydrogenase in glycolysis and NADP-malate dehydrogenase (NADP-MDH) (Ruelland and Miginiac-Maslow, 1999).

The electron acceptor thioredoxin (Trx), has the ability to interchange with various protein disulphides due to a reactive dithiol active site, consisting of Cys-Xaa-Xaa-Cys. The heterodimeric enzyme ferredoxin-thioredoxin reductase (FTR) then plays a key role in linking photosynthetic electron transport to protein redox conditions (Ruelland and Miginiac-Maslow, 1999).

Thioredoxins differ from glutaredoxins in their ability to reduce disulphide bridges (Prinz *et al*, 1997). The major substrates of thioredoxin are oxidised intramolecular protein disulphides, with the enzymes being very poor reductant of mixed disulphides with GSH as compared with glutaredoxin (Jung and Thomas, 1996). The role of thioredoxin in plants under stress conditions is poorly understood, although a novel chloroplastic thioredoxin, CDSP 32, has recently been discovered (Broin *et al*, 2000). CDSP 32 is inducible under drought or oxidative conditions in potato plants and is believed to play a role in the protection of chloroplasts from oxidative damage. This is achieved through the reduction of

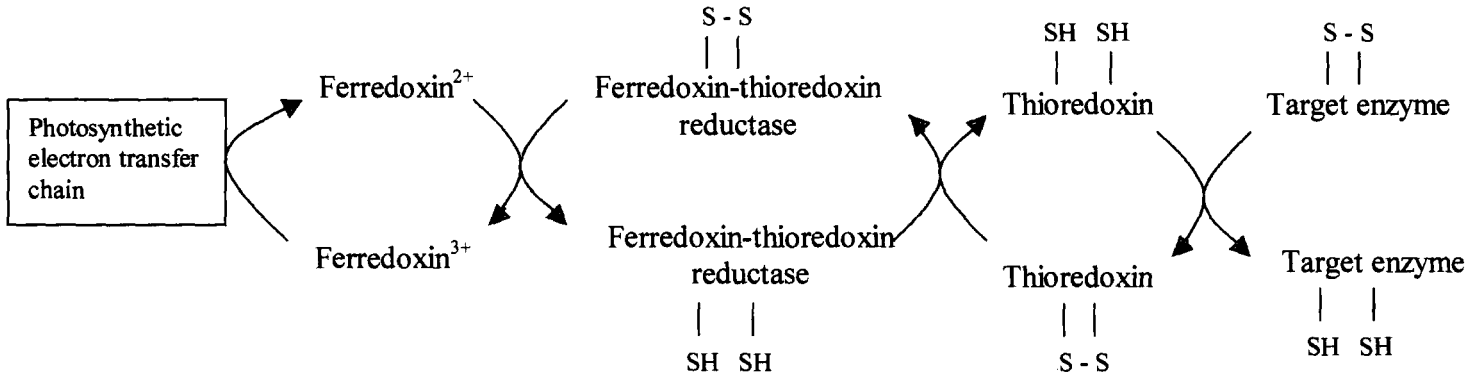

Figure 1.5. The chloroplast ferredoxin-thioredoxin system.
Upon capture of photons by chlorophyll molecules the membrane bound photosynthetic electron transfer chain first reduces ferredoxin. A small fraction of reduced ferredoxin serves as an electron donor to ferredoxin-thioredoxin reductase, whose disulphide bond is reduced and undergoes thiol-disulphide interchange with oxidised thioredoxin. Reduced thioredoxin can reduce disulphides of several enzymes, most of which are weakly active or inactive in the oxidised form (Ruelland and Miginiac-Maslow, 1999).

internal disulphide bonds in target proteins inactivated by redox change, or by supplying electrons to a thioredoxin-dependent protein (Broin *et al*, 2000).

The role of disulphide bond regulation has been widely studied in mutant strains of *E. coli* which lack part of the thioredoxin system. Under conditions of oxidative stress, disulphide regulation activates several transcription factors which regulate the genes necessary for the elicitation of the defence response to redox stress. The transcriptional activators SoxR and OxyR in *E. coli* are examples of systems which require the formation of disulphide bonds for activation (Toledano *et al*, 1994; Ding and Demple, 1996; Gaudu and Weiss, 1996). Under normal growth conditions, the active site cysteines are present in a reduced, inactive state. These cysteinyl residues then undergo disulphide formation following exposure to H_2O_2 (OxyR) or O_2^- (SoxR) which activates the transcription factor and leads to gene activation and increased cellular resistance to ROS (Demple, 1998). Activation of OxyR results in the induction of the defence-related genes for glutathione reductase and glutaredoxin (Prieto-Alamo *et al*, 2000; Aslund *et al*, 1999). The transcription factors are subsequently inactivated through the action of glutaredoxin (Aslund *et al*, 1999).

Aims and Objectives of the Project

The primary aim of this project was to investigate the effects of biotic elicitation on plant thiol content and to determine the related changes in protein thiolation. The system chosen for study were suspension-cultured cells of alfalfa (*Medicago sativa* cv. Europe) and *Arabidopsis thaliana* respectively. Alfalfa cell cultures were selected as they have a rapid and well defined phytoalexin response following treatment with fungal elicitors. Alfalfa also produce both GSH and hGSH and it was of interest to determine the differential use of the thiols during oxidative stress induced by elicitation, especially the formation of mixed disulphides between GSH or hGSH and protein sulphydryl groups. In order to characterise the importance of thiolation in more detail, Arabidopsis cell cultures were used as a prelude to using proteomics to identify modified proteins. In particular, it was of interest to determine whether or not GSH-dependent enzymes were subject to thiolation and the effect of this modification on their activity. This was investigated by determining the effect of thiolation on the activity of purified recombinant GSH-dependent enzymes.

The major aims of the project were to:

1. Determine the effects of elicitation on thiol content of plant cell cultures (Alfalfa and Arabidopsis).
2. Identify and quantify the range of proteins thiolated during elicitation.
3. Determine the rate of thiolation/dethiolation.
4. Determine the effect of thiolation on the activity of selected enzymes.

2.0 Materials and Methods

Chemicals

All chemicals were obtained from BDH or Sigma-Aldrich Co. Ltd and were of 'AnalR' grade or higher. Radiochemicals were obtained from ICN U.K. with L-[^{35}S]cysteine having a specific activity of 1 mCi mmol^{-1}.

2.1 Plant Material

Cell suspension cultures of alfalfa (*Medicago sativa* cv *Europe*) were derived from previous studies (Tiller *et al*, 1994). Cell suspension culture *Arabidopsis thaliana* (cv Columbia) were derived from studies described by May and Leaver (1993). For comparative purpose, soybean suspension cultures (*Glycine max* cv. *Mandarin*) were obtained from Syngenta, Jealott's Hill, Bracknell.

2.1.1 Alfalfa Cell Cultures

Alfalfa callus tissue was grown on modified Schenk and Hildebrandt media supplemented with 1 g L^{-1} *myo*-inositol, 30 g L^{-1} sucrose, 5 g L^{-1} agar-agar. The media consisted of :-

Component	mg L^{-1}
KNO_3	2500
$MgSO_4.7H_2O$	400
$NH_4H_2PO_4$	300
$CaCl_2.2H_2O$	200
$FeSO_4.7H_2O$	15
Na_2EDTA	20
$MnSO_4.H_2O$	10
KI	1
H_3BO_3	5

$ZnSO_4.7H_2O$	1
$CuSO_4$	0.2
$NaMoO_4.2H_2O$	0.1
$CoCl_2.6H_2O$	0.1
Thiamine-HCl	5
Nicotinic acid-HCl	5
Pyridoxine-HCl	0.5
Kinetin	0.108
2-4, D	0.45
PCPA	1.87

The media was adjusted to pH 5.8, then sterilised by autoclaving for 20 min at 121 °C. Callus tissue was sub-cultured every 4-5 weeks on to fresh agar and grown in the dark at 25 °C.

Cell suspensions (50 ml) were grown in the above liquid media (without agar), on an orbital rotary shaker (130 rpm) at 25 °C in the dark. Routine sub-culturing was carried out every 7-9 days with a 15 % (v/v) inoculum. Fresh suspension cultures were initiated from callus culture every 6 to 8 weeks by planting calli into 25 ml liquid medium and straining off finely dividing cells from the supernatant after 7 days.

2.1.2 Soybean Cell Cultures

Soybean callus tissue was grown on Gamborg-B5 medium (Sigma), supplemented with 1 mg L^{-1} 2-4-D, 20 g L^{-1} sucrose, and 6 g L^{-1} agar at pH 5.5 and sterilised as above.

Component	mg L^{-1}
KNO_3	3000
$MgSO_4.7H_2O$	500

$(NH_4)_2SO_4$	134
$CaCl_2.2H_2O$	150
$NaH_2PO_4.H_2O$	150
Sequestrene 330 Fé	28
$MnSO_4.H_2O$	10
KI	0.75
H_3BO_3	3
$ZnSO_4.7H_2O$	2
$CuSO_4$	0.025
$NaMoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
Myo-inositol	100
Thiamine-HCl	10
Nicotinic acid	1
Pyridoxine-HCl	1

Cell suspension cultures and callus cultures were grown under identical conditions to alfalfa.

2.1.3 Arabidopsis Cell Cultures

Arabidopsis thaliana cell cultures were grown on Murashige and Skoog Basal salts with minimal organics media supplemented with 0.1 g L^{-1} *myo*-inositol and 0.4 mg L^{-1} thiamine The media consisted of :-

Component	mg L^{-1}
KNO_3	1900
$MgSO_4.7H_2O$	370
$NH_4H_2PO_4$	1650
$CaCl_2.2H_2O$	440
KH_2PO_4	170
$FeSO_4.7H_2O$	278
Na_2EDTA	373

$MnSO_4.H_2O$	22.3
KI	0.83
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H2O$	0.025
$NaMoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
Kinetin	0.05
Naphthalene acetic acid	0.5

The media was adjusted to pH 5.7, then sterilised by autoclaving for 20 min at 121 °C.

Cell suspension cultures were grown in the above media on an orbital rotary shaker (130 rpm) at 25 °C under constant illumination (550 μE m^2 s^{-1}). Routine sub-culturing was carried out every 7-9 days. Callus tissue was grown on the above media supplemented with 3 g L^{-1} agar-agar and sub-cultured on to fresh media every 4-5 weeks.

2.2 Biotic Elicitation of Plant Cell Cultures

2.2.1 Preparation and Testing of Fungal Elicitor

Bakers yeast (1 kg) was suspended in 1.5 litres of 20 mM sodium citrate buffer (pH 7.5) and autoclaved for 60 min at 121 °C. After cooling, the suspension was centrifuged at 10000 g for 20 min. Over 1 h, one volume of ethanol was added to the supernatant and the slurry stirred slowly overnight at 4 °C prior to centrifugation. After discarding the pellet, the supernatant was treated with, one volume of ethanol to give a 75 % ethanol:water (v/v) solution and stirred slowly overnight at 4 °C. After centrifugation, the supernatant was discarded and the pellet taken up in a minimal volume of distilled water. The content of the

reducing sugar was assayed by the acid phenol assay (2.2.2) and after dilution, the elicitor preparation was stored in 1 ml aliquots at –20 °C.

2.2.2 Acid Phenol Assay for Reducing Sugars

The concentration of elicitor was measured in reducing sugar equivalents using D-glucose standards which were prepared in the range 0-50 μg ml^{-1}, with 1 ml reacted with 25 μl of 80 % (w/v) phenol and 2.5 ml concentrated sulphuric acid. After cooling, the absorbance was determined at 490 nm and a standard curve constructed of D-glucose (μg) against A_{490}. This curve was then used to quantify the reducing sugar content of the elicitor preparation, which was similarly treated.

2.2.3 Treatment of Cell Cultures with Fungal Elicitor

To determine the effect of fungal elicitor, suspension cell cultures were grown to mid-log phase (5 days after inoculation) before application of 500 μl yeast elicitor (equivalent to 100 μg of sugar equivalent per ml culture).

2.2.4 Treatment of Cell Cultures with Organic Hydroperoxides

Arabidopsis and alfalfa cell cultures were grown to mid-log phase before the addition of 500 μl aqueous *t*-butyl hydroperoxide (*t*-HP) to give a final concentration of 1 mM. Cells were harvested at timed intervals over an 8 h period by vacuum filtration and frozen in liquid nitrogen and stored at –20 °C until required. Controls consisted of treatment with 500 μl sterile distilled water.

2.2.5 Harvesting Suspension-Cultured Cells

At timed intervals after treatment, the suspension cultures were filtered through a nylon mesh under vacuum. The media was frozen at –20 °C and after washing with water, the cells were weighed and frozen in liquid nitrogen prior to storage at –80 °C.

2.2.6 HPLC analysis of Isoflavonoid Phytoalexins

The isoflavonoids were extracted from frozen cells in 5 v/w cold acetone (-20 °C) to prevent hydrolysis of conjugates and peroxidative destruction of phenolics. After homogenisation with a pestle and mortar using acid washed sand as abrasive, the extracts were filtered under vacuum. The residue was then re-extracted in 5 v/w cold acetone + methanol (1:1 v/v). The combined filtrate was concentrated under vacuum, and the residue made up in methanol to give an equivalent of 1 ml extract per gram fresh weight of tissue extracted The extract was injected (50 µl) into a C-18 reversed-phase column (5 µm particle size, 4 mm i.d. x 250 mm) using the following solvent conditions :-

After equilibrating the system in 80 % solvent A (1 % aqueous phosphoric acid): 20 % solvent B (100 % acetonitrile) the percentage solvent B was increased to 60 % over 45 min using a linear gradient at a flow rate of 0.8 ml min^{-1}. The elution of the isoflavonoids was monitored by their UV absorbance at 287 nm.

2.2.7 Assay of Phenylalanine Ammonium Lyase (PAL)

The basis of this assay was the spectrophotometric measurement of the formation of *trans*-cinnamic acid from L-phenylalanine with a correction applied for non-specific changes in absorbance applied using a D-phenylalanine control, based on the method from Lamb *et al* (1979). The assay solution (final volume 1 ml) consisted of 900 µl buffer containing 12.1 mM L-phenylalanine in 50 mM Tris-

HCl (pH 8.5) and 100 µl enzyme extract. Parallel incubations were run with buffer containing 12.1 mM D-phenylalanine acting as a control. Formation of cinnamic acid was monitored by taking absorbance readings in quartz cuvettes at 290 nm at timed intervals over a 2 h period.

2.3 Protein Quantification

The protein content of the extracts was determined using a dye reagent (Bio-Rad) based on the method of Bradford (1976). Calibration curves were prepared from γ-globulin standards in the range 0-1 mg ml^{-1} protein.

2.4 Thiol Quantification in Plant Cell Cultures

2.4.1 Extraction of Crude Extract

To extract the total soluble low molecular weight thiols, 1 g cell material was homogenised in 3 ml 1 M Tris-HCl, pH 7.5 and left on ice. After 30 min, the homogenate was centrifuged at 13000 g to remove cell debris and stored at –20 °C until required. In a parallel extraction, *N*-ethylmaleimide (NEM) was added to a final concentration of 5 mM during the initial homogenisation. NEM reacts with the available reduced thiols present. In the subsequent procedure, which involved reduction of the oxidised thiols with DTT, only the oxidised thiols were quantified in the NEM treated samples, as opposed to the non-NEM treated samples where total thiol (reduced + oxidised) was measured.

2.4.2 Derivatisation of Soluble Thiols using Fluorescent Bimane Probe

To account for losses during derivatisation, an internal standard of GSH was used in all determinations of total thiols. A 100 µl aliquot of supernatant was treated in the presence of either 10 µl 0.1 mM GSH (internal standard) or 10 µl distilled water. The samples were then treated with 10 µl 0.1 M NaOH and 10 µl 50 mM

DTT and left for 10 minutes at room temperature prior to addition of 10 µl of 3.6 M HCl. After centrifugation to remove insoluble material, 100 µl supernatant was derivatised with 10 µl 30 % (v/v) *N*-ethyl morpholine and 10 µl 5 mg ml^{-1} monobromobimane (mBBr) prepared in anhydrous acetonitrile and the mixture incubated in the dark for 20 min. The sample was then made up to 1 ml with 5 % (v/v) acetic acid prior to HPLC analysis.

2.4.3 Extraction and Derivatisation of Protein Bound Thiols.

After extraction of the cells (1 g) with 3 ml 1 M Tris-HCl pH 7.5, the proteins present were precipitated on ice by the addition of 100 % (w/v) trichloroacetic acid (TCA) to give a final concentration of 10 % TCA (w/v). The protein pellet was collected by centrifugation (13000 g, 5 min) and washed twice in 300 µl 5 % (v/v) perchloric acid to remove contaminating low molecular weight thiols. To release thiols bound to the protein, the pellet was incubated with 300 µl 10 mM DTT in 0.1 M Tris-HCl pH 7.5 for 15 min, and then centrifuged at 13000 g to remove the protein precipitate. The supernatant was stored at –20 °C until required and then derivatised with mBBr as described in 2.4.2.

2.4.4 Separation of Bimane Labelled Thiols by HPLC

After derivatisation, thiols (50 µl) were injected on a Gilson 306 HPLC fitted with a 5 µm particle size, C-18 reverse phase HPLC column (250 x 4.6 mm) (Phenomenex, UK). Separation of thiols was achieved using buffer A (15 % methanol : 85 % 50 mM K_2HPO_4, pH 6.0) at a flow rate of 1 ml min^{-1}. At the end of each analysis, any remaining bound material was washed from the column using 100 % methanol. Detection was by fluorescence (Gilson Model 121 fluorimeter); using excitation and emission wavelengths of 305-395 nm and 430-470 nm respectively.

2.4.5. Thiol Derivatisation by 2, 4-Dinitrofluorobenzene (FDNB)

The derivatisation with 2, 4-dinitrofluorobenzene (FDNB) was based on the method of Ji *et al* (1999). Briefly, following extraction in 3 v/w 0.1 M Tris pH 7.4, proteins were precipitated by adding perchloric acid to a final concentration of 5 % v/v. After centrifugation, 100 µl of the supernatant was treated with 10 µl of 60 mM iodoacetamide and adjusted to pH 8.5 by adding 100 µl 2.5 M KCO_3. After incubation in the dark for 15 min, 100 µl of 1.5 % (v/v) FDNB in methanol was added and left at 4 °C for 16 hours. Reaction was terminated upon the addition of 310 µl of acetic acid (5 % v/v).

2.4.6 Separation of Cellular Thiols By HPLC Following Derivatisation by FDNB

After derivatisation with FDNB, thiols (50 µl) were injected on a Gilson 306 HPLC fitted with a 5 µm particle size, amino column (150 x 4.6 mm) (Phenomenex, UK). Separation of thiols was achieved using buffer A (70 % methanol in 500 mM sodium acetate pH 4.2) and buffer B (80 % methanol) at a flow rate of 1.2 ml min^{-1} with the following gradient: 0 to 5 min 20 % buffer A, 20 % to 99 % A in 7 min, 99 % A for 5 min, 99 % to 20 % in 3 min, equilibrating the column in 20 % A for 7 min. Detection was by UV absorbance (Gilson Model 116) at 365 nm.

2.5 Quantification of Thiols Rabiolabelled with L-[^{35}S]cysteine

2.5.1 *In-vivo* Labelling of the Thiol Pool with L-[^{35}S]cysteine

Plant cell cultures were grown to mid-log growth phase as described in section 2.1. Cell cultures (10 ml) were incubated for 1 h with 100 µg ml^{-1} cycloheximide to prevent incorporation of cysteine into proteins via protein synthesis (Padgett

and Whorton, 1998). L-[^{35}S]cysteine (25 μCi, specific activity 1 μCi mmol^{-1}) was then added and the cells incubated for 4 h prior to the addition of the elicitor.

To determine the specific activity (dpm nmol^{-1}) of the thiol pools, the mBBr derivatised thiols were resolved by HPLC and quantified using fluorescence detection (section 2.5). Fractions corresponding to each thiol peak (1 ml) were collected and 4 ml of a liquid scintillation cocktail 'Econoscint' added. The radioactivity present was then quantified using a Packard 1600TR Liquid Scintillation Analyzer operating with an external standard to correct for quenching.

2.5.2 Quantification of L-[^{35}S]Cysteine Incorporation into Proteins

To determine the cysteine incorporation into proteins by mixed disulphide formation, 1 ml cell free extract was incubated in the presence or absence of 10 mM DTT. The DTT was required to release disulphide bound [^{35}S]-label from the proteins in order to determine the radioactivity which was present due to protein thiolation rather than due to incorporation of L-[^{35}S]cysteine into protein via protein synthesis. Protein preparations were precipitated on ice with 10 % (v/v) TCA and filtered through a Whatman GF/A filter disk which was further washed three times with 1 mM cysteine in 10 % (v/v) TCA to remove any residual L-[^{35}S]cysteine. TCA precipitated protein bound thiols were then quantified by liquid scintillation counting after adding 4 ml of Econoscint.

2.5.3 Pulse-chase Radiolabelling of Protein Bound Thiols with L-[^{35}S]Cysteine

Plant cell cultures (10 ml) were incubated with 100 μg ml^{-1} cycloheximide for 1 h prior to a 4 h incubation with L-[^{35}S]cysteine (25 μCi) in the presence of either elicitor treatment or a water control. After incubation for 4 h to label the intracellular thiol pool, the cell cultures were filtered and washed with sterile media to remove unabsorbed L-[^{35}S]cysteine. L-[^{35}S]cysteine containing media

was retained and the counts determined to determine the uptake of L-[^{35}S]cysteine. The cells were then resuspended in sterile media supplemented with 1 mM L-cysteine and harvested at timed intervals over a 24 h period.

2.5.4 Short Term Labelling of Thiol Pool During Fungal Elicitation

To study incorporation and turnover of L-[^{35}S]cysteine into soluble thiols and into protein-bound mixed disulphides during elicitation, cell cultures were labelled with 25 μCi L-[^{35}S]cysteine at set times after the addition of elicitor (= time 0) as detailed in the following table :-

Table 2.1. Details of short term labelling studies during an elicitation time course. Time 0 h refers to the addition of elicitor in each case with negative times referring to events occurring prior to the addition of elicitor.

Elicitation Time/Harvest time point	Time	Action
0	-3 h	Add cycloheximide
	-2 h	Add L-[^{35}S]cysteine
	0	Harvest cultures
2	-1 h	Add cycloheximide
	0 h	Add L-[^{35}S]cysteine
	+2 h	Harvest cultures
4	+1 h	Add cycloheximide
	+2 h	Add L-[^{35}S]cysteine
	+4 h	Harvest cultures
8	+5 h	Add cycloheximide
	+6 h	Add L-[^{35}S]cysteine
	+8 h	Harvest cultures

2.5.5 Synthesis of L-[^{35}S]cysteine-labelled Glutathione

L-[^{35}S]cysteine containing glutathione and homoglutathione was synthesised using crude *E. coli* BL 21 extract containing a recombinant homoglutathione synthetase

derived from soybean together with endogenous bacterial glutathione synthetase using a procedure developed in our laboratory by Dr Mark Skipsey.

E. coli BL 21 cells expressing soybean homoglutathione synthetase were grown in Luria-Bertani (LB) broth to mid-growth phase (A_{600} = 0.6 O.D.) before the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 2 h, cells were harvested and resuspended in 250 mM Tris-HCl pH 8.0 containing 1 mM DTT before being disrupted by sonication (5 x 20 s^{-1}). Low molecular weight compounds were removed using a PD 10 Sephadex G25 column (Pharmacia) equilibrated with re-suspension buffer as recommended by the manufacturer.

Protein content was determined and 50 µl of the lysate (500 µg protein) was incubated with 50 µl L-[^{35}S]cysteine (100 µCi) in 250 mM Tris-HCl containing 50 mM KCl, 20 mM $MgCl_2$, 5 mM DTT, 10 mM ATP, 1 mM cysteine and 5 mM glutamic acid in a total volume of 182.5 µl. After a 30 min incubation at 30 °C either 10 mM glycine or 10 mM β-alanine was added together with 17 mM ATP to give a final volume of 200 µl. Reactions were terminated after a further 60 min by placing on ice and 20 µl of the final preparation reacted with 200 µl 200 mM Tris-HCl pH 8.0 containing 1.2 mM monobromobimane. The derivatisation was terminated after 20 minutes by the addition of 5 % (v/v) acetic acid to a final volume of 1 ml and the *S*-bimane derivatives analysed by HPLC (section 2.5.1). Fractions corresponding to the glutathione or homoglutathione peak were collected and counted by liquid scintillation and the specific activity determined.

After the optimal conditions for thiol synthesis were deduced, synthesis was carried out as above, except the incubation volumes were increased two fold and the reaction terminated upon the addition of 8 µl 3.6 M HCl to reduce the pH to pH 2.

2.6 Protein Analysis

2.6.1 One-Dimensional SDS-PAGE

To determine ^{35}S-labelled thiolated proteins in cell extracts, extracts were resolved by SDS-PAGE under non-reducing conditions to prevent reduction of mixed disulphides and radioactive polypeptides visualised by fluorography.

Samples were prepared by heating (80 °C) an equal volume protein extract with 6 x non-reducing loading buffer (0.139 M Tris-HCl pH 6.8 containing 22.2 % (v/v) glycerol, 4.4 % (w/v) SDS and 28 mg ml^{-1} bromophenol blue) for 5 minutes and then allowing to cool.

Protein separation was carried out on a Mini-Protean II kit (Bio-Rad) following manufacturers guide lines based on the method of Laemlli (1970) using 0.75 mm thick gels. Resolving gels were polymerised from 12.5 % (w/v) acrylamide, 0.33 % (w/v) *N'N'*-bis-methylene-acrylamide, in 0.375 M Tris-HCl, pH 8.8 containing, 0.1 % (w/v) ammonium persulphate, 0.1 % SDS and 0.05 % (v/v) TEMED (*N,N,N',N'*-tetramethylethylenediamine). Stacking gels were polymerised from 4 % (w/v) acrylamide, 0.11 % (w/v) *N'N'*-bis-methylene-acrylamide in 0.125 M Tris-HCl pH 6.8 in 0.1 % (w/v) SDS and 0.05 % (v/v) TEMED. For electrophoresis the running buffer was 25 mM Tris-HCl, pH 8.3 containing 192 mM glycine, 0.1 % (w/v) SDS. Following electrophoresis (120 V for 60 – 90 min) the gels were washed three times for 5 min each with distilled water then the polypeptides present stained with GelCode blue as detailed by the manufacturer (Pierce, U.K.).

To visualise radiolabelled polypeptides, following electrophoresis, gels were fixed (water:methanol:acetic acid 6:3:1 v/v) for 30 min and then immersed in Amplify (Amersham) fluorographic reagent for 30 min before drying on a Gel Dryer (BioRad) at 60 °C for 2 h. The dried gel was exposed to X-ray Hyperfilm ECL

(Pharmacia) for 2 to 5 days and the film developed using an automatic compact X-4 developer (X-Ograph visualisation system).

For reference, ^{14}C-methylated protein standards (Amersham) (molecular weight 97 kDa, 69 kDa, 55 kDa, 46 kDa, 30 kDa, 12 kDa) were resolved on the SDS-PAGE gels to determine the molecular masses of radioactive polypeptides.

2.6.2 Two-Dimensional Gel Analysis

Protein extracts (up to 250 μg protein), were precipitated on ice using an equal volume of ice-cold 80 % (v/v) acetone on ice for 5 min. The resulting precipitate was pelleted by centrifugation (11500 g) for 10 min at 0 °C and the supernatant discarded. Protein pellets were then washed twice in 80 % (v/v) acetone and then resuspended in 250 μl loading buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS containing 0.8 % (v/v) pharmampholyte pH range pH 3-10 (Pharmacia, UK). The resuspended protein was agitated for 1 h at room temperature before addition of 0.1 % (w/v) bromophenol blue and application of the sample on to DryStrip (Pharmacia, UK) IEF strip pH 3-10. After covering with a layer of mineral oil to prevent drying the sample was left overnight at room temperature.

After loading the protein on to the DryStrip, the oil and unbound protein was removed by gently washing with distilled water, before loading in the Immobiline IEF tray and covering in a thin layer of mineral oil.

The first separation was run on a Multiphor II IEF System (Pharmacia, UK), under the following conditions for each strip:-

Phase 1	500 V 0.05 mA	5W	0.01 h	1 Volt hours (Vh)
Phase 2	3500 V 0.05 mA	5W	2.30 h	2800 Vh
Phase 3	3500 V 0.05 mA	5W	2.30 h	3700 Vh

After electrophoresis, the mineral oil was washed from the DryStrip and the gel gently agitated for 30 min at room temperature in buffer B, consisting of 50 mM Tris-HCL, pH 8.8, 6 M Urea, 30 % (v/v) glycerol, 2 % (w/v) bromophenol blue.

To determine the molecular mass of the protein, an SDS-PAGE gel was used, as described in section 2.7.1, with the following changes:- The spacer thickness was increased to 1.5 mm to accommodate the gel strip and no stacking gel was required. After loading the DryStrip, it was overlaid with a layer of 1 % agarose containing 1 % SDS and bromophenol blue dye. Molecular weight markers were loaded on to a thin strip of Whatman MM (Whatman, UK) filter paper and placed at the side of the IEF DryStrip.

2.6.3 Visualisation of Separated Proteins by Silver Staining

Where protein extracts contained low quantities of protein and the GelCode reagent was insufficiently sensitive to visualise the polypeptides, the more sensitive silver staining method was employed.

Following electrophoresis, proteins were fixed by constantly agitating the gel in acetic acid: methanol: water (10:30:60 v/v) for 1 h. The gel was then washed twice in acetic acid: ethanol: water (10:10:80 v/v) for 30 min each and then incubated in oxidiser reagent before applying the silver reagent as recommended by the manufacturer (BioRad). After briefly washing in water, the developing solution containing 95 mM sodium carbonate and 0.0016 % (v/v) formaldehyde, was applied and incubated until the protein bands had developed to sufficient intensity. Developing was terminated by the addition of 10 % (v/v) acetic acid.

2.6.4. Visualisation of Radiolabelled Proteins by Phosphorimaging

Following separation of the polypeptides by SDS-PAGE, the polypeptides were transferred onto Hybond-C nitrocellulose membrane (Amersham International plc, UK) using a tank electroblotter (mini Trans-Blot cell, Bio-Rad) and the manufacturers protocol. Electroblotting was performed at 100 V for 30 min in 16 mM Tris-HCl, 120 mM glycine, pH 9.5. After allowing to dry, the membrane was transferred to a phosphorimager beta emitting particle detector (BioRad, UK) and the phosphor image developed over exposed for 16 h.

2.7 Nitric Oxide Metabolism

2.7.1 Preparation of *S*-Nitrosoglutathione (GSNO)

GSNO was prepared according to the method by Hart (1985). Briefly, an ice-cold solution of GSH (5 mmol) in 10 ml of 2 M HCl was added to a sodium nitrite solution (5 mmol). After stirring for 40 min at 4 °C, the red solution was treated with an equal volume of ice cold acetone and stirred for a further 10 min. The resulting red precipitate was removed by filtration and washed successively three times with 3 volumes of ice cold water, acetone and ether to produce *S*-nitrosoglutathione. The red precipitate was transferred to an air tight tube and stored at –20 °C until required.

2.7.2 Determination of Nitric Oxide (NO) with Nitric Oxide Probe

Nitric oxide formation was determined using a nitric oxide probe using the facility in the Department of Chemistry (Professor Lyn Williams). The nitric oxide level was determined by monitoring the release of NO following treatment of extracts with 1 mM ascorbate. Standards consisting of GSNO were treated under identical conditions to derive a standard curve.

2.8 *In-vitro* Thiolation

2.8.1 *In-vitro* Labelling of Alfalfa and Arabidopsis with [^{35}S]-Glutathione

Crude protein extracts from alfalfa and arabidopsis cell cultures were obtained by homogenising cells in 3 v/w 20 mM Tris-HCL, pH 7.5 and centrifuging to remove cell debris. Standard assay conditions involved subjecting 10 µl extract containing 30 µg protein to 1 µl 20 mM (*t*-HP) in the presence of 10 µl 1 µCi L-[^{35}S]cysteine labelled GSH ([^{35}S]-GSH) for 10 min on ice. Any remaining free thiols were then alkylated with 5 mM NEM to terminate any further reaction.

Protein separation was achieved using SDS-PAGE analysis under the non-reducing conditions described (section 2.6), with the 2-mercaptoethanol being omitted from the loading buffer.

2.8.2 *In-vitro* labelling of Pure Recombinant Proteins

2.8.2.1 Recombinant Glutathione-Dependent Enzymes Used

A number of GSH-dependent enzymes from a variety of plant sources were available as the respective cDNA clones expressed in *E. coli* from several on-going studies in our laboratory. The sources of the enzymes are as detailed below.

Protein	Plasmid : Accession Number	Origin	Reference/Source
DHARI	*Atp*ET24D-*dhar*1	*A. thaliana*	Dixon *et al*, in press
DHARII	*Atp*ET24D-*dhar*3	*A. thaliana*	Dixon *et al*, in press
DHARIII	*Atp*ET24D-*dhar*4	*A. thaliana*	Dixon *et al*, in press
Esterase D	*AtpET24DSFGH*	*A. thaliana*	Kordic *et al*, 2002

Glyoxalase	pET-*Gm*GlyoxI : AJ010423	*Glycine max*	Skipsey *et al*, 2000
*Zm*GSTF1	*Zm*GSTI : P12653	*Zea mays*	Dixon *et al*, 1999
*Zm*GSTU1	*Zm*GSTV : Y12862	*Zea mays*	Dixon *et al*, 1999
*Zm*GSTU2	*Zm*GSTVI : AJ010439	*Zea mays*	Dixon *et al*, 1999
*At*GSTF8		*A. thaliana*	De Ridder *et al*, 2002
theta GST	*At*GSTTI : AB010072	*A. thaliana*	De Ridder *et al*, 2002
zeta GST	*At*GSTZI : AJ278293	*A. thaliana*	Dixon *et al*, 2000
lambda GST1	*At*GSTLI	*A. thaliana*	Dixon *et al*, in press
lambda GST2	*At*GSTL2	*A. thaliana*	Dixon *et al*, in press

2.8.2.2 Preparation of Glutathione-Sepharose Affinity Matrix

Glutathione Sepharose was prepared following the method of Simons and Vander Jagt (1981). Epoxy-activated Sepharose CL-6B (2 g) was added to 20 ml of distilled water and allowed to swell for 25 min. The resultant gel was washed on a sintered glass funnel with 200 ml distilled water and resuspended in 10 ml 44 mM potassium phosphate, pH 7.0 in a 25 ml flask. Oxygen was removed by bubbling the slurry with nitrogen for 5 min before the addition of 6 ml of 100 mM GSH which had been adjusted to pH 7.0 dissolved in 1 M NaCl adjusted to pH 7. The gel suspension was incubated for 24 h to allow the GSH to react with the activated Sepharose and then washed with 100 ml of water and incubated with 1 M ethanolamine for 4 h at 30 °C to block any unreacted groups on the Sepharose. The derivatised gel was then subsequently washed with 100 ml 0.1 M sodium acetate, pH 4.0 containing 0.5 M KCl, 100 ml 0.1 M sodium borate, pH 8.0 containing 0.5 M KCl and finally with 10 mM potassium phosphate, pH 7.4. The glutathione Sepharose was then packed into a 10 mm (i.d.) chromatography column for affinity chromatography.

2.8.2.3 Purification of *A. thaliana* Glutathione Transferases

Following extraction of crude proteins from Arabidopsis as described in section 2.8.1, the extract was applied to the GSH-Sepharose column equilibrated with 20mM Tris-HCl, pH 7.4 at a flow-rate of 1 ml min^{-1}. Proteins with no affinity for GSH were eluted by 20mM Tris-HCl, pH 7.4. The affinity bound proteins were then recovered by the addition of 5 mM GSH to the elution buffer. Fractions containing the GSH-binding proteins were collected and GST activity determined (see 2.8.2.1).

2.8.2.4 Purification of Recombinant Glutathione Transferase from *E. coli* BL21-DE3

The bacterial pellets were resuspended in 20 mM Tris-HCl, pH 7.5 and lysed by 4 x 20 s^{-1} treatments of sonication at an amplitude of microns (Soniprep 150, MSE) with 1 min between each cycle. The cell debris was then removed and discarded following centrifugation at 1000 g for 5 min.

All protein purification work was carried out on a GradiFrac (Pharmacia) low-pressure liquid chromatography system with a flow rate of 1 ml min^{-1} at 4 °C. Protein elution was determined by monitoring the change in absorbance at 280 nm with the fractions collected to determine activities of interest.

A column containing GSH-Seharose 4B was equilibrated with 10 column volumes of 20 mM Tris-HCl pH 7.5. After loading the protein extract on the column, any unbound protein was removed by washing with 10 column volumes of 20 mM Tris-HCl, pH 7.5. To elute the bound protein, 5 mM GSH was added to the wash solution. To ensure all protein was removed from the matrix, 5 column volumes of Tris-HCl, pH 7.5 containing 500 mM NaCl was then used to wash the affinity column.

2.8.2.5 Expression and Purification of Dehydroascorbate Reductase I, II and III

E. coli BL21-DE3 harbouring the pET24D vector containing the DNA sequence for Arabidopsis dehydroascorbate reductase (DHAR) I, II, III (pET24D-DHARI, pET24D-DHARII and pET24D-DHARIII) respectively were obtained from Dr David Dixon. The DHARs each contained a C-terminal fusion of 6 His residues to facilitate affinity purification on a nickel chelate column (Dixon *et al*, in press). *E. coli* containing the pET24D-DHARs were grown and treated with IPTG as described in section 2.10.8, using kanamycin as the selective agent, and lysed as described in section 2.8.1.3. After determining protein content, DHAR was purified using a column of chelated nickel to bind the His tagged fusion protein. A 20 ml iminodiacetic acid-Sepharose 6B affinity column, previously charged with 0.1 M $NiSO_4$ was equilibrated with 10 column volumes of 20 mM Tris-HCl, pH 7.8 containing 0.5 M NaCl and 20 mM imidazole. The crude bacterial lysate was loaded on to the column and the unbound proteins washed off 60 ml equilibration buffer. The imidazole concentration was then increased to 150 mM and fractions collected and assayed for enzyme activity and protein content. Any further bound protein was removed by washing the column in 300 mM imidazole in 20 mM Tris-HCl, pH 7.8 with 0.5 M NaCl, followed by a re-equilibration period of 10 column volumes of the original buffer.

2.8.3 Effect of Thiolation on Enzyme Activity

A stock solution of GSH (100 mM) was prepared in distilled water and the pH adjusted to pH 7.0 using 1 M NaOH. GSH solutions were stored at –20 °C to minimise oxidation, and thawed solutions were discarded after use for the same reason.

To invoke thiolation, the pure recombinant proteins in either 20 mM Tris-HCl, pH 7.5, were incubated in 1 mM GSNO, or 2 mM *tert*-butyl hydroperoxide in the

presence of 1 mM GSH for 5 min on ice. Controls consisted of incubating the enzyme preparation with an appropriate volume of water. In addition the proteins were incubated with 2 mM *tert*-butyl hydroperoxide, or 1 mM GSH alone. To determine the effect of *S*-alkylation on enzyme activity, the proteins were also incubated with either 1 mM *N*-ethyl maleimide, or 1 mM iodoacetamide in the dark.

All spectrophotometric assays of enzyme activity were performed using a Unicam Helios α dual-beam spectrophotometer. Assays determined over 1 min or less were monitored continuously. Absorbance measurements at wavelengths below 290 nm were performed using quartz cuvettes (1 cm path length), while at higher wavelengths plastic cuvettes (1 cm path length, Sarstedt Ltd, UK) were used. To correct for the non-enzymic rate of reaction, protein was omitted from assays.

2.8.3.1 Glutathione Transferase Activity toward 1-Chloro-2,4-dinitrobenzene (CDNB)

Glutathione transferase activity toward CDNB was determined in a total volume of 1 ml as described by Dixon *et al* (1997). Assays consisted of 900 µl 100 mM phosphate buffer pH 6.5 and 25 µl 40 mM CDNB (in ethanol) pre-incubated for 10 min at 30 °C, after which time 25 µl enzyme extract and 50 µl 100 mM glutathione, pH 7.0 was added. The increase in absorbance at 340 nm was monitored over a 30 s^{-1} period. Enzyme activity was then determined using an extinction coefficient of 9.6 mM^{-1} cm^{-1}. The chemical rate of conjugation was obtained using a heat-treated protein extract and subtracted from the rate determined in the presence of enzyme to determine the true activity.

2.8.3.2 Determination of the Dechlorination of Dichloroacetic Acid

The dechlorinating activity of zeta class GSTs was determined by colorimetric assay using dichloroacetic acid as a substrate (Dixon *et al*, 2000).

In a final volume of 1 ml, 200 µl of 500 mM potassium phosphate buffer, pH 7.4, 10 µl 100 mM glutathione pH 7.0 (prepared as in section 2.8.2), and 25 µl enzyme preparation was pre-incubated at 37 °C and 25 µl 20 mM dichloroacetic acid (DCA) added. Following incubation for 20 min at 37 °C, the reaction was terminated by the addition of 50 µl TFA and chilled on ice for 10 min. The precipitated protein was removed by centrifugation for 5 min at 13000 g, 850 µl of the supernatant transferred to a plastic test tube and 500 µl 1 M NaOH, 300 µl 800 mM potassium phosphate buffer, pH 6.8 and 400 µl 0.66 % (v/v) phenylhydrazine added with thorough mixing. Following incubation at room temperature for 10 minutes and subsequent cooling on ice for 5 min, 1 ml concentrated HCl was added and mixed thoroughly. The reaction mixture was then treated with 400 µl 3.33 % (w/v) potassium ferricyanide and after exactly 15 min at room temperature, the absorbance at 535 nm was determined. Calibration was achieved by incubating with glyoxylic acid (0 to 250 µl) replacing the DCA with the tube containing no glyoxylic acid used to blank the instrument.

2.8.3.3 Determination of Dehydroascorbate Reductase Activity

The assay contained 900 µl 100 mM potassium phosphate buffer, pH 6.5, 0.5 mM dehydroascorbate, 5 mM GSH (prepared as described in section 2.8.2) and 10 µl enzyme preparation. After incubating the buffer at 30 °C for 5 min, the substrates and enzyme extract were added and activity determined by monitoring the increase in absorbance at 265 nm over 1 min. Enzyme activity was then determined, assuming an extinction coefficient for dehydroascorbate of 14 M^{-1} cm^{-1}. Control reactions where the enzyme was omitted showed there was no appreciable chemical rate for this reaction.

2.8.3.4 Determination of Glutathione Peroxidase Activity

The glutathione peroxidase activity of the Arabidopsis theta class GST was determined toward cumene hydroperoxide using a spectrophotometric method (Edwards, 1996).The resulting oxidation of GSH to GSSG was quantified using a coupled GSH reductase assay to recycle GSSG back to GSH with the concomitant oxidation of NADPH to NADP. The decline in absorbance at 340 nm was then determined over 30 s^{-1}.

2.8.3.5 Determination of Glyoxalase I activity

Glyoxalase I activity was determined using a spectrophotometrically (Skipsey *et al*, 2000) by measuring the isomerisation of the hemithioacetal adduct of methylglyoxal and glutathione to the respective thioester *S*-D-lactoylglutathione. The assay (1 ml) contained 40 μM GSH pH 7.4, prepared as described in section 2.8.2.1 and 3.5 mM methylglyoxal in 100 mM sodium phosphate buffer, pH 7.5 containing 16 mM $MgSO_4$. After pre-incubating for 5 min at 30 °C to allow for hemithioacetial substrate formation, 25 μl enzyme extract was added and activity determined by measuring the difference in absorbance at 240 nm over 30 s^{-1}. Enzyme activity was then determined assuming an extinction coefficient for the *S*-D-lactoyl-glutathione of 3370 M^{-1} cm^{-1}. Control reactions where the enzyme was omitted showed there was no appreciable chemical rate for this reaction.

2.8.3.6 HPLC determination of γ-Glutamylcysteine Synthetase Activity

γ-Glutamylcysteine synthetase (γECS) activity was determined by monitoring the synthesis of γ-glutamylcysteine based on the assay described by May and Leaver (1994) with quantification of the FDNB derivatised product by HPLC.

A. thaliana cell cultures were grown to mid-log phase, and harvested as previously described (section 2.1.3). Following extraction in 3 volumes 0.1 M

Tris-HCl, pH 7.5, the extract was passed through a PD-10 column (Pharmacia) equilibrated with the extraction buffer, to remove any low-molecular compounds.

The assay mixture contained 50 µl extract containing 250 µg of protein, in a total volume of 200 µl containing 100 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 10 mM ATP, 0.8 mM cysteine and 30 mM glutamic acid. After 30 min incubation at 30 °C, reactions were terminated by the addition of 10 µl 3.6 M HCl. Following derivitisation with FDNB, the thiols were separated and quantified by HPLC as described in section 2.4.5.

2.8.3.7 Determination of Esterase D activity

Pure recombinant esterase D from Arabidopsis was obtained from a previous study carried out in our laboratory (Kordic *et al*, 2002).

Esterase D activity was determined using a spectrophotometric method which measured the hydrolysis of *p*-nitrophenol acetate. The enzyme (10 µg) was incubated in a final volume of 1 ml in an assay containing 100 mM potassium phosphate, pH 7.2, and 1 mM *p*-nitrophenol acetate was incubated at 37 °C. After addition of the recombinant protein, the increase absorbance at 400 nm was monitored for 30 s^{-1}. Enzyme activity was then determined assuming an extinction coefficient for *p*-nitrophenol of 17 M^{-1} cm^{-1} after correcting for hydrolysis rate in the absence of esterase D.

2.8.3.8 Spectrophotometric determination of mGST activity in Arabidopsis microsomal preparations.

Arabidopsis cell cultures were harvested in exponential phase and proteins extracted as per section 2.3.1. Microsomal pellets were recovered by ultracentrifugation at 72,000 g for 1 h after an initial centrifugation at 10,000 g. The supernatant was removed and the resulting pellet resuspended in 0.1 M Tris-

HCl pH 7.4 containing 1 % (v/v) Triton X-100. mGST activity towards CDNB was determined before and after an activation treatment with 2 mM NEM for 1 min.

2.9 Analysis of Polypeptides for *S*-Thiolation

2.9.1 Preparation of Proteins for Electrospray Ionisation Time-of-Flight Mass Spectrometry (ESI-TOF-MS)

The pure proteins treated -/+ thiolation treatments (as described in section 2.8.2) were desalted through a HiTrap Desalting column (5 ml) (Pharmacia, UK) in 10 mM Tris-HCl, pH 7.5 and the protein content determined by measuring the OD_{280}.

2.9.2 Preparation of Peptide Fragments for HPLC ESI-TOF-MS

To aid digestion, the protein was denatured by incubating the protein at 45 °C in the presence of 5 % (v/v) acetonitrile. After 1 h, the protein was cooled to 37 °C and endoproteinase Lys-C (Promega, UK) added to a ratio of 1:100 (content of endoproteinase : protein) and incubated for 18 h. The reaction was terminated by the addition of 0.1 % TFA and the samples stored at – 20 °C until required.

2.9.3 Separation of Peptides by HPLC

Purified proteins were prepared as in section 2.9.1. A known amount of protein was injected on the System Gold (Beckman, UK) HPLC and separation achieved using a C-18 reverse-phase column 300 μm particle size, 2 x 125 mm length with the following elution profile:-

After equilibrating the system in 90 % solvent A (= 0.1 % trifluoroacetic acid (TFA) in water): 10 % solvent B (= 0.1 % TFA in acetonitrile) the percent solvent

B was increased to 100 % over 20 min using a linear gradient at a flow rate of 0.25 ml min^{-1}. The eluent was monitored for absorbance at 214 nm.

2.9.4 ESI-TOF-MS Analysis

In order to quantify the numbers of protein cysteinyl residues undergoing thiolation during the period of oxidative stress, ESI-TOF-MS was used to monitor the change in protein molecular mass following treatment with *t*-HP and GSH.

After purification of proteins and thiolation (+/-) treatment as described in section 2.9.1, the protein samples were mixed with tetrahydrofuran (THF) : water (1:1) and formic acid (0.1 % v/v) added prior to injection. Their molecular masses were determined by direct injection of the protein sample into a time of flight mass spectrometer (Micromass, UK) using by electrospray ionisation with the cone-voltage set at 35 V and with a desolvation temperature of 360 °C. The sample was introduced at a flow rate of 10 µl min^{-1}. The ESI-TOF-MS raw data for polypeptides consisted of multiple peaks due to multiply charged mass ions. After calibrating the multiply charged peaks of myoglobin over a range of 200 – 1700 Da the data was then processed with Mass Lynx 2.0 software to obtain deconvoluted mass spectra showing the parent protein mass giving rise to the multiply charged species. The mass determined was ± 1 Da. The thiolated (-/+) polypeptides were then simultaneously analysed.

To identify which of the peptide fragments obtained following Lys-C treatment were thiolated, the peptide digests prepared as described in section 2.9.2 were resolved by HPLC as described in section 2.9.3 and the eluting fragments analysed by ESI-TOF-MS. Data was collected over the course of the HPLC run (30 min).

2.10 Molecular Biology Methods

Unless otherwise stated, the methods used for cloning and expression were as described in Sambrook (1989).

Custom oligonucleotides were synthesised commercially by MWG, UK.

2.10.1 Bacterial culture

Bacteria were cultured in Luria-Bertani (LB) broth (1 % w/v bacteriological peptone, 1 % w/v NaCl and 0.5 % w/v yeast extract) from a single colony picked from a fresh agar plate. Liquid cultures were grown at 37 °C on an orbital shaker (200 rpm), in the presence of the appropriate antibiotics for selection. For bacterial culture on agar, of 1.5 % agar select containing LB broth was used, and inoculated plates were incubated at 37 °C.

2.10.2 PCR (Polymerase Chain Reaction)

The PCR reaction contained 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, two oligonucleotide primers (each 1 mM), template and 1 unit of *Taq* DNA polymerase (where 1 unit catalyses the incorporation of 10 nmol of dNTP into acid-insoluble form in 30 min at 74 °C). PCR amplifications consisted of 30 cycles in the conditions: 94 °C for 90 s^{-1}, 50 °C for 120 s^{-1} and 72 °C for 120 s^{-1}. Reaction tubes were heated to 94 °C before addition of *Taq* DNA polymerase to minimise mis-priming and therefore non-specific amplification of non-target sequences. Reactions were carried out on a Techne Progene thermal cycler.

2.10.3 DNA ligation

Ligation of DNA was carried out in 10 µl reactions using T4 DNA ligase in ligation buffer. Digested purified insert and vector were added in an approximate

ratio 3:1, and made up to volume with sterile distilled water. Ligation reactions proceeded for up to 16 h before heat inactivation of the DNA ligase at 65 °C for 15 min prior to electroporation into *E. coli*.

2.10.4 Transformation of *E. coli*

Routine transformation work used electro-competent cells. A liquid culture (500 ml) was inoculated with a single colony and incubated at 37 °C until the cell turbidity increased to 0.6 A U at 600 nm. Cells were then chilled on ice for 1 h and pelleted by centrifugation (5000 g for 10 min). The cell pellet was resuspended and re-pelleted in chilled, sterile water 6 times, until the media had been washed from the cells. The cell pellet was finally resuspended in 2 ml of chilled 15 % (v/v) glycerol solution and dispensed in 50 µl aliquots in chilled 500 µl microfuge tubes, flash frozen in liquid nitrogen and stored at –80 °C until required.

To transform the electro-competent *E. coli* cells, a 50 µl aliquot of competent cells was thawed on ice and 1 µl of ligation product added gently mixed, and the mix transferred to a chilled electroporation cuvette (BioRad). The plasmid was electroporated into the cells using a BioRad electroporation apparatus, set at 2.5 kV, 200 Ω, and 25 µFD capacitance. Immediately following electro-transformation, the cells were transferred to a fresh tube containing, 1 ml SOC media (20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 20 mM glucose in 1 L^{-1} water) and incubated at 37°C. After a 1 h period, 100 µl of transformed cells were spread on to LB agar plate containing the appropriate selective agent.

2.10.5 Plasmid Purification

A single colony of transformed *E. coli* XL1 Blue was grown in 10 ml LB containing the appropriate selecting agent overnight at 37 °C. The cell suspension was centrifuged and the plasmid purified. Routine plasmid purification was

achieved using a Wizard Plus Miniprep DNA purification system (Promega, UK), following the manufacturers protocol.

2.10.6 DNA digestion with Restriction Enzymes

Digestion of DNA was carried out using commercially available enzymes and buffer systems (Promega, UK). Typically 1 –2 μg plasmid DNA was digested with 1 U restriction enzyme (where 1 U is the amount of enzyme required to cut 1 μg DNA in a total volume of 50 μl at 37 °C in 1 h) in the appropriate buffer system for 1 – 4 h, and analysed by gel electrophoresis.

2.10.7 DNA Agarose Gel Electrophoresis

Agarose gels were prepared by melting 1 % (w/v) agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.6). After allowing to cool slightly, a few drops of ethidium bromide were added to the gel and thoroughly mixed. The gel was then poured in to a casting gel with the appropriate well forming comb. After allowing to set, the comb was removed and the gel transferred to the electrophoresis tank and immersed in TAE buffer. DNA samples (5 μl to 24 μl) in 6 x loading buffer (30 % glycerol, 0.25 % bromophenol blue, 0.25 % xylene cyanol FF) were applied and run at 120 V for 30 min, or until required separation was achieved.

2.10.8 Purification of DNA from Agarose Gels

Following separation, the gel was transferred to a UV trans-illuminator and briefly exposed to UV light to determine the position of the DNA fragments. This period of exposure was kept to a minimum to prevent cross-linking of DNA strands. DNA fragments of interest were then cut out of the agarose gel and transferred to a clean tube where the agarose was dissolved with 3 x (v/w) gel buffer (90.8 % (w/v) sodium iodide solution saturated with sodium sulphite) with incubation at

50 °C. To precipitate the DNA, 10 µl 50 % (w/v) silica fines were added, mixed thoroughly, incubated at room temperature of 15 min and spun at 14000 g; the supernatant being discarded. The DNA bound silica fines were washed with 0.5 ml of 70 % (v/v) ethanol before being dried down with a gentle stream of air to ensure all ethanol was removed, resuspended in 20 µl water and incubated at 37 °C for 10 min to release the DNA from the silica fines. Following centrifugation, the solution was frozen at –20 °C until required.

2.10.9 Expression of Recombinant Protein

Following insertion of the correct plasmid into *E. coli* BL21 cells, a single transformed colony was transferred to 10 ml LB containing the appropriate selecting agent and incubated overnight as described in section 2.9.1. Typically, 5 ml of culture were inoculated in 500 ml fresh LB media containing the selective agent, and grown to an $O.D._{600}$ = 0.6. Recombinant protein production was induced by the addition of 1 mM IPTG and after 3 h, the cells were harvested by centrifugation at 5000 g for 10 min and the pellet resuspended in 10 ml of appropriate buffer for purification. Cell suspensions were flash frozen and stored at –20 °C until required.

2.10.10 DNA Sequencing

DNA sequencing was performed on double stranded DNA using ABI 373A or ABI 377A automated fluorescent sequencing machines (Durham University Sequencing Service).

2.10.11 DNA Sequence Analysis

Analysis of the DNA sequences was performed using 'DNA for Windows!' software. Multiple sequence alignments were performed using the CLUSTAL W programme (Thompson *et al*, 1994). Similarity searches were carried out using

BLAST suite of programs (Altschul *at al*, 1990) on the National Center for Biotechnology Information's BLAST WWW Server.

3.0 Changes in Thiol Metabolism in Alfalfa and Soybean Cell Cultures on Elicitation

3.1 Introduction

Elicitation of the defence response in plants can be induced through biotic stress, for example, by challenge with a microbial pathogen or through the addition of cell wall preparations from microbial pathogens, which are termed biotic elicitors. Abiotic elicitors of the defence response include UV light, xenobiotics and heavy metal salts (Hammond-Kossack and Jones, 1996). Biotic or abiotic elicitation invariably induces an oxidative burst leading to the production of reactive oxygen species (Wojtaszek, 1997), which alter cellular redox homeostasis and biochemistry. The production of oxygen species is thought to be integral to the signalling mechanism for the induction of defence-related genes and the hypersensitive response, which leads to localised cell death (Levine *et al*, 1994).

In alfalfa, a primary response to treatment with biotic elicitors, such as cell wall preparation from yeast, is the production of the isoflavonoid antimicrobial phytoalexin medicarpin. Phytoalexins are present in unchallenged cells at basal concentrations and upon elicitation, the enzymes involved in synthesis are rapidly induced, resulting in their accumulation (Dixon *et al*, 1995). Phenylalanine ammonium lyase (PAL) is the first enzyme of the medicarpin biosynthetic pathway and under non-stressed conditions shows minimal activity. In response to elicitation, levels of PAL and other enzymes of the isoflavonoid synthetic pathway such as chalcone synthase and isoflavone reductase, are enhanced with almost identical induction kinetics (Dixon *et al*, 1995). As such, the inducibility of these enzymes make them suitable markers of the elicitation response.

Using the induction of PAL activity and medicarpin accumulation as markers of the defence response, it was then of interest to determine changes in thiol metabolism associated with the defence response. Several studies have shown

changes in soluble reduced and oxidised thiols in response to elicitation in plants (Smith, 1985; May and Leaver, 1993). However the associated changes in the protein bound thiols in plants has not been reported. In this study the reduced and oxidised soluble thiols and protein bound thiols were monitored in alfalfa cell cultures during elicitation by a fungal cell wall preparation, with the intention of obtaining a comprehensive overview of changes in thiol metabolism and redox status in a well characterised elicitation system.

3.2 Results

3.2.1 Effect of Growth of Alfalfa Cell Suspension Cultures on Thiol Content

The thiol content of plant tissue is known to be variable, dependent on factors such as plant age, tissue type and environmental conditions (May *et al*, 1998; Rennenberg, 1995). In cell cultures, external environmental conditions are kept virtually constant throughout the course of the experiments, so the direct effects of culture growth only on thiol content could be determined. This experiment also revealed the optimal age for the use of the cultures for future experimentation. Alfalfa cell suspensions were sub-cultured and then harvested over a 12 day period. At each harvest point, the fresh weight and thiol content was determined. A standard growth curve for alfalfa, showed an initial lag phase of 2-3 days followed by a period of exponential growth for 7 days. By day 10 the cells were approaching the stationary phase, resulting in little further gain in fresh weight (figure 3.1). Over this period of growth, the thiol composition changed markedly (figure 3.2), with levels of both glutathione (GSH) and homoglutathione (hGSH), the major thiols in alfalfa cell cultures (Edwards *et al*, 1991), changing in response to growth. Differences were observed between the two thiols, with the GSH concentration increasing rapidly over the first 3 days followed by a steady decline over the following 7 days, while hGSH increased steadily over the first 8 days. Both thiols then declined as the cells entered the stationary phase. For

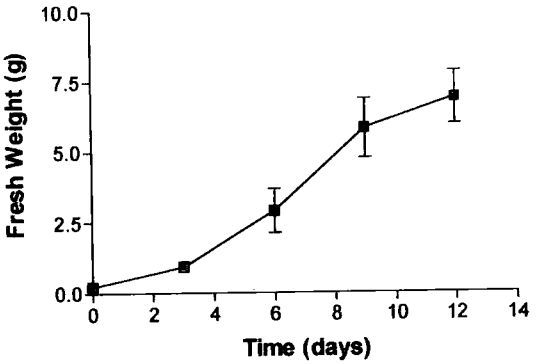

Figure 3.1. Increase in fresh weight of alfalfa cell suspension cultures after sub-culturing.
Data is a mean of 2 samples with error bars showing variation between replicates. In the instance where no error bars are visible, the variation lies within the extent of the graphical symbol.

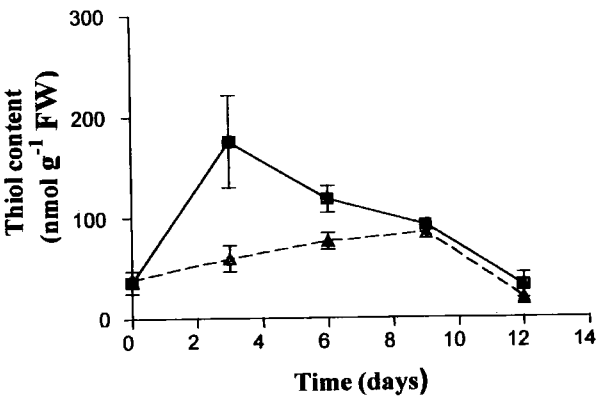

Figure 3.2. Thiol content of alfalfa after sub-culturing into fresh media.
Values refer to total levels of the respective thiols (■ GSH; ▲ hGSH) (oxidised + reduced form). Data is a mean of 2 samples with error bars showing variation between replicates. In the instance where no error bars are visible, the variation lies within the extent of the graphical symbol.

future experiments, alfalfa cell cultures were used in mid-logarithmic phase, 6 days after sub-culturing as this ensured an adequate mass of actively multiplying cell material and readily detectable levels of thiols.

3.2.2 Effect of Elicitor Concentration on PAL and the Phytoalexin Response in Alfalfa Cell Cultures

Alfalfa cell suspensions were treated with a range of concentrations of a cell wall elicitor preparation derived from bakers yeast, or a control treatment which consisted of sterile distilled water. The range of elicitor concentrations used was defined from their reducing sugar equivalent content with 0, 70, 140 and 210 μg glucose equivalents ml^{-1} culture being tested. In each case, the cell cultures were harvested 24 h after treatment.

The elicitor treated cell suspensions responded by inducing PAL activity. In cell suspension cultures treated with sterile water only, PAL activity was low (0.44 ± 0.33 μkats kg^{-1} protein). Following treatment with 210 μg glucose equivalents ml^{-1} elicitor, activity increased approximately 10 fold to 4.36 μkats/kg protein. However, cell cultures treated with 70 μg ml^{-1} or 140 μg ml^{-1} of elicitor resulted in an increase in activity to 14.1 and 15.2 μkats kg^{-1} protein respectively, giving a maximal 37 fold increase in activity as compared to controls (figure 3.3).

To determine the content of the phytoalexin medicarpin and its glycolsile-conjugate precursors, the cell-cultures were extracted with acetone and the resulting concentrate analysed by HPLC. Following separation on a reversed phase column, the isoflavonoids present were detected from their UV absorbance. A typical HPLC chromatogram of extracts from control and elicitor treated cell cultures is shown in figure 3.4. The compound eluting at 32 min in the elicited extracts was identified from its chromatographic behaviour and UV absorbance spectrum as medicarpin. Similarly, the compounds eluting at 19 min and 22 min

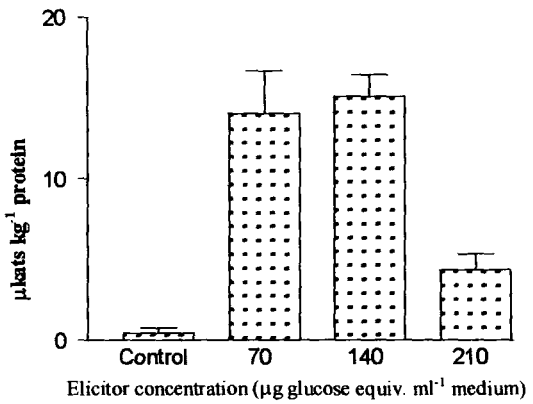

Figure 3.3 Enhancement of phenylalanine-ammonium lyase (PAL) activity in alfalfa cell cultures 24 h after treatment with a fungal elicitor preparation.
Data is a mean of 2 samples with error bars showing the variation between replicates

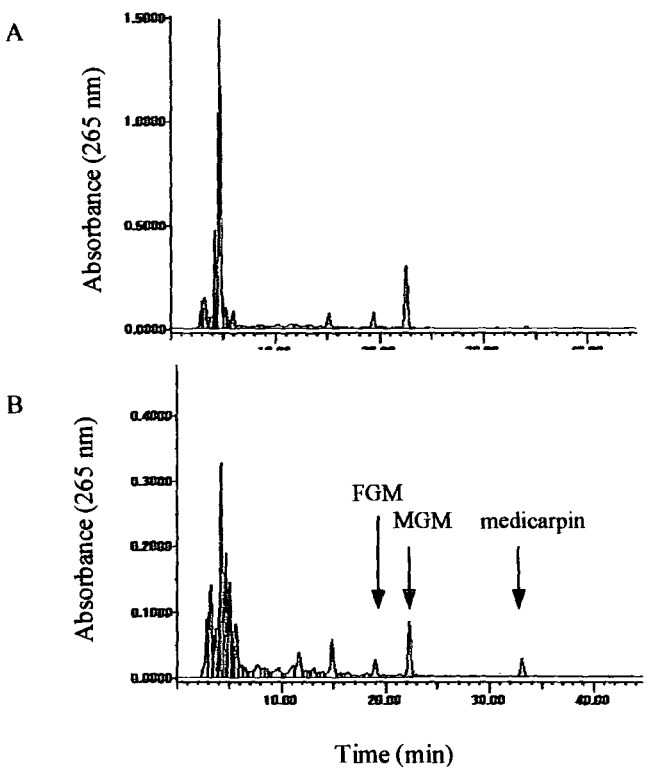

Figure 3.4. HPLC analysis of phenolic metabolites from alfalfa cell cultures before (A) and after (B) treatment for 24 h with a yeast cell wall preparation.
The peak eluting with a retention time of 32.7 min in the elicitor treated cell extracts is the isoflavonoid phytoalexin medicarpin. The peaks eluting at 19 min and 22 min are the medicarpin precursors formononetin-7-*O*-glucoside 6"-*O*-malonate (FGM) and medicarpin-3-*O*-glucoside 6"-*O*-malonate (MGM) respectively.

were identified as formononetin 7-*O*-glucoside-6"-*O*-malonate (FGM) and medicarpin-3-*O*-glucoside-6"-*O*-malonate (MGM) respectively. FGM is the glycosylated form of formononetin, a precursor of medicarpin, which constitutively accumulates in the vacuole. Following elicitation, hydrolysis of the conjugate results in the release of formononetin to feed into the medicarpin biosynthetic pathway. MGM is the detoxified medicarpin-conjugate. In the absence of elicitors, basal medicarpin synthesis results in a slow accumulation of MGM in the vacuole which upon elicitation is hydrolysed to release the antimicrobial medicarpin. Following hydrolysis of MGM to release medicarpin during elicitation, *de novo* synthesised surplus phytoalexin then results in the accumulation MGM (Edwards *et al*, 1997; Tiller *et al*, 1994).

Medicarpin accumulation mirrored the PAL activities in the cells treated with the differing concentrations of elicitor. In the control cell suspension, there was no detectable medicarpin present. Following treatment with 210 μg ml^{-1} glucose equivalents of the yeast cell wall preparation, medicarpin accumulation was still low (2.1 nmol g^{-1} fresh weight (FW)). However, in cultures treated with 70 μg ml^{-1} or 140 μg ml^{-1} glucose equivalents of the elicitor preparation of 68.6 nmol g^{-1} FW and 63.9 nmol g^{-1} FW of the phytoalexin accumulated respectively (figure 3.5). The isoflavonoid conjugates, formononetin 7-*O*-glucoside-6"-*O*-malonate (FGM) and mmedicarpin-*O*-glucoside-6"-*O*-malonate (MGM) were present in cells under non-stressed conditions at around 20 nmol g^{-1} FW and 100 nmol g^{-1} FW respectively. In response to treatment with 70 μg ml^{-1} or 140 μg ml^{-1} of the elicitor, the level of these conjugates increased slightly as compared with the control cells along with the increase in medicarpin. When treated with 210 μg ml^{-1} glucose equivalents, FGM and MGM levels almost trebled while the accumulation of medicarpin was very minor, showing the concentration of elicitor applied to the cell cultures was important in determining the nature of the phytoalexin response. Thus, at the lower concentration of elicitor used the formation of medicarpin and the associated increase in PAL activity lead to the

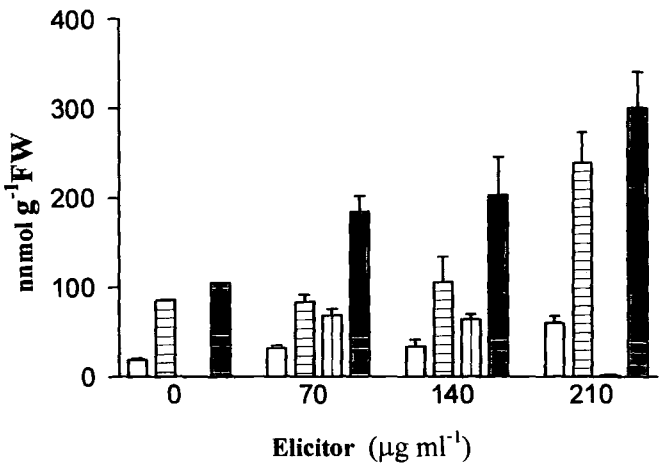

Figure 3.5 Phytoalexin and isoflavonoid conjugate accumulation in alfalfa 24 h after treatment with differing concentrations of yeast elicitor preparation.

□ = formononetin 7-*O*-glucoside-6"-*O*-malonate (FGM); ⊟ = medicarpin-3-*O*-glucoside-6"-*O*-malonate (MGM); ▥ = medicarpin; ■ = total isoflavonoids. Data is a mean of 2 samples with error bars showing the variation between replicates. In the instance where no error bars are visible, the variation lies within the extent of the graphical symbol.

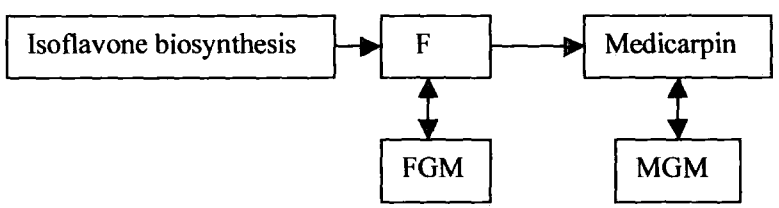

Figure 3.6 Metabolic relationship between medicarpin and associated conjugates. The isoflavonoid biosynthetic pathway synthesises the medicarpin precursor formononetin (F) and the conjugated form (FGM; formononetin-7-*O*-glucoside-6"-*O*-malonate) accumulates constitutively. Under normal conditions, medicarpin is detoxified and stored in the conjugated form, medicarpin-3-*O*-glucoside-6"-*O*-malonate (MGM). Upon elicitation, hydrolysis of FMG and MGM release formononetin and medicarpin respectively, to provide a rapid antimicrobial response.

direct formation of free medicarpin rather than the conjugated form. At the higher concentration of elicitor, when PAL activity was relatively low, the accumulation of the FGM and MGM predominated with little of the aglycone medicarpin accumulating. For future experiments, 100 μg ml^{-1} glucose equivalents of elicitor were used, as at this concentration a high level of induced defence response was invoked, in terms of PAL activity and medicarpin accumulation.

After determining the optimal elicitor concentration for the induction of the defence response, exponentially growing alfalfa cultures were treated with the yeast elicitor and the PAL and phytoalexin responses monitored over a 24 h period. PAL activity in control cultures was almost undetectable. Within 4 h of elicitor treatment, PAL activity increased almost 50 fold and remained high during the experimental time course (table 3.1). Maximum medicarpin accumulation (46.4 nmol g^{-1} FW) occurred 8 h after elicitation and by 24 h, the level had declined almost to basal level. This transient accumulation resulted from *de-novo* synthesis of medicarpin as opposed to release from its conjugate as the levels of the MGM conjugates remained stable over the 0-8 h period. During this period there was a minor decrease in FGM content suggesting that some turnover of the formononetin precursor of medicarpin had occurred. However, the release of formononetin could not account for the majority of medicarpin accumulation. After 8 h, medicarpin content declined and this was associated with a corresponding accumulation of MGM. The results give further evidence of the relationship between medicarpin and isoflavonoid conjugates in legumes such as alfalfa, undergoing a phytoalexin response (figure 3.6).

These results differ from those previously described by Mackenbrock *et al* (1993), whereby in chickpea cell cultures elicited with a yeast cell wall preparation, the formation of the isoflavonoid conjugates predominated when low concentration of elicitor were applied. As the elicitor content increased, phytoalexin aglycen accumulation increased due to the turnover of isoflavonoid conjugate. In alfalfa, the total amount of phytoalexin did increase with the concentration of elicitor

Elicitor Treatment (h)	PAL activity (μkats kg^{-1}) Ave	SD	Isoflavonoid Content (nmol g^{-1} fresh weight) Medicarpin Average	S.D.	FGM Average	S.D.	MGM Average	S.D.	Total isoflavonoid content
0	0.4	0	0.0	0.0	51.9	0.7	65.0	0.4	116.9
4	19.5	0.7	3.1	0.5	41.7	0.7	65.9	3.2	110.7
8	18.8	1.7	46.4	3.2	34.3	1.4	69.0	2.6	149.8
24	16.5	3.2	2.3	0.3	40.3	3.3	138.2	0.5	180.8

Table 3.1. PAL activity and isoflavonoid content of alfalfa cell cultures.
Alfalfa cell cultures were treated with 100 μg ml^{-1} reducing sugar equivalents fungal elicitor for the times indicated. Data are an average of duplicate cell cultures with a standard deviation given.

applied, but the increase was due primarily to *de-novo* medicarpin synthesis. Although the increase could be attributed to increased turnover. When treated with the highest dose of elicitor, the phytoalexin conjugate MGM selectively accumulated. The different phytoalexin responses to elicitation in alfalfa and chickpea maybe due to the differences in isoflavonoid synthesis, as chickpea cell cultures accumulate both medicarpin and the related phytoalexin maackiain (Weidermann *et al*, 1991).

As a model for studying the effects of elicitation in alfalfa, the suspension culture has advantages over a whole plant system. The phytoalexin response seen in intact tissues can be quantitatively reproduced in cell suspension cultures (Kessmann and Barz, 1987) coupled with the ability to produce a large mass of cells under controlled conditions in a short period of time, make cell cultures a useful model system. Previous studies comparing the cell cultures to whole plants have revealed that in alfalfa, the cultures are most similar to the roots in the composition of phytoalexin conjugates and response to elicitation (Tiller *et al*, 1994).

3.2.3 Effect of Elicitation on Soluble Thiol Content in Alfalfa Cell Cultures

Alfalfa cell cultures were selected as they also represented a novel system for studying thiol metabolism in plants following elicitation by a yeast cell wall preparation. Alfalfa cell cultures contain both glutathione (GSH) and homoglutathione (hGSH) in relatively large amounts (Edwards *et al*, 1991) and offered the opportunity to investigate the metabolism of two thiols *in planta* during elicitation.

To monitor the glutathione flux during the period of elicitation, the GSH and hGSH pools were labelled *in-vivo* with L-[^{35}S]cysteine. Protein synthesis was inhibited by the addition of 100 μg ml^{-1} cycloheximide prior to the addition of 25 μCi L-[^{35}S]cysteine for 24 h. Inhibition of protein synthesis increased the

potential for incorporation of L-[^{35}S]cysteine in to soluble thiol pools rather than newly synthesised proteins.

After *in-vivo* labelling GSH and hGSH with L-[^{35}S]cysteine for 4 h, exponentially growing alfalfa cell suspensions were treated with 100 μg ml^{-1} elicitor and harvested over a 20 h period. Washed cells were extracted in 0.1 M Tris-HCl, pH 7.4 with and without treatment with NEM and then derivatised by the thiol-specific fluorescent probe monobromobimane. The *S*-bimane conjugates were separated by HPLC as described in section 2.4.4 and the amounts (nmol) and specific activity (dpm nmol^{-1}) of the radiolabel quantified.

In alfalfa cell cultures, GSH and hGSH metabolism responded differently to treatment with the fungal elicitor (table 3.2). After a 24 h treatment with elicitor, GSH accumulated almost 3 fold increase as compared with the water treated control. The increase in the reduced GSH was mirrored in the GSSG pool, in which the content increased approximately 4 fold between 8 and 24 h. The accumulation of GSH was in contrast to hGSH which remained constant. Any slight elevation in hGSH content was also seen in the water treated cultures, reflecting minor non-elicitor specific changes in thiol metabolism. Even though hGSH biosynthesis did not significantly increase during the period of elicitation, a 4 fold increase in oxidised hGSH was formed in response to the elicitation response.

Interestingly, the specific activity of GSH decreased during the period of elicitation. Following elicitor treatment in the presence of L-[^{35}S]cysteine, the GSH content increased however, the decrease in specific activity during this period suggests the source of free cysteine required for *de-novo* GSH synthesis came from sources other than the L-[^{35}S]cysteine supplied. It was not possible to identify the origin of this alternative cysteine pool without further investigation. During the period of treatment with the elicitor, the specific activities of hGSH and the oxidised thiols GSSG and hGSSG, remained relatively constant.

		Reduced Thiol								Oxidised Thiol							
Time	Elicitor	GSH				hGSH				GSSG				hGSSG			
(h)	+/-	nmol g^{-1}		dpm $nmol^{-1}$		nmol g^{-1}		dpm $nmol^{-1}$		nmol g^{-1}		dpm $nmol^{-1}$		nmol g^{-1}		dpm $nmol^{-1}$	
		Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
0	+	52	4			98	12			2	0			2	1		
	-	52	4			98	12			2	0			2	1		
4	+	62	4	149	94	112	14	102	29	4	1	165	18	4	1	85	17
	-	59	5	278	165	98	5	298	123	2	1	180	12	2	0	185	29
8	+	103	31	83	4	101	14	84	12	10	3	132	22	6	2	97	12
	-	61	12	269	14	97	41	195	36	3	0	188	34	2	0	126	28
24	+	151	4	90	12	119	3	109	10	13	3	119	26	8	3	96	19
	-	67	4	258	16	132	20	226	21	3	1	165	12	2	1	146	21

Table 3.2 Soluble thiol levels and specific radioactivity of GSH and hGSH following labelling with L-[^{35}S]cysteine in alfalfa after treatment with 100 μg ml^{-1} fungal cell wall elicitor as determined following derivatisation with monobromobimane.

Alfalfa cell cultures (10 ml) were treated with 100 μg ml^{-1} for 1 h prior to labelling of the thiol pools with 2.5 μCi L-[^{35}S]cysteine. After 4 h, cell cultures were treated with 100 μg ml^{-1} cell wall elicitor (+) or water (-) and harvested at the specified time points. Values are from duplicate cell cultures with standard deviation given.

The differential accumulation of the two thiols suggested that GSH and hGSH synthesis and turnover were differentially regulated. To determine if hGSH accumulation was limited by β-alanine availability, in a subsequent study the medium was supplemented with 1 mM β-alanine. This resulted in no increase in hGSH or GSH in alfalfa (table 3.3), whereas media supplemented with 1 mM cysteine, the rate limiting substrate of thiol biosynthesis in mono-thiol systems (Strohm *et al*, 1995), resulted in a 7 fold increase in GSH content over 24 h. No such increase was observed in the hGSH content (table 3.3). This suggested that increased accumulation of GSH could be selectively mediated by increasing cysteine content in cells undergoing the elicitation response.

3.2.4 Effect of Elicitation on Thiol Metabolism in Soybean Cell Cultures

The studies in alfalfa had demonstrated that GSH, but not hGSH accumulated in elicited legume cell culture containing both thiols. It was then of interest to determine if hGSH would be similarly unresponsive in a cell culture where it was the only thiol.

In soybean cell cultures, hGSH is the major thiol (> 95 % total thiols) (Edwards *et al*, 1991). When exponentially growing soybean suspension cultures were treated with the fungal cell wall elicitor, the hGSH content significantly increased within 4 h of treatment and remained elevated for the experimental time (figure 3.7). To determine whether β-alanine or cysteine were limiting hGSH biosynthesis, the media was supplemented with either 1 mM β-alanine or 1 mM cysteine. As in alfalfa, after 4 h, β-alanine was not found to be limiting biosynthesis, though addition of 1 mM cysteine did increase hGSH over a 16 h period (table 3.4) suggesting there are differences between hGSH biosynthesis in alfalfa and soybean.

Plant	Treatment (time, h)	GSH (nmol g^{-1}FW)	hGSH (nmol g^{-1}FW)
Alfalfa	none	66.9 ± 1.3	136.4 ± 4.6
	1 mM β-alanine, 4 h	84.8 ± 0.4	140.2 ± 6.6
	1 mM cysteine, 4 h	271.3 ± 79.5	145.0 ± 31.3
	1 mM cysteine, 8 h	335.3 ± 3.9	112.6 ± 9.1
	1 mM cysteine, 24 h	547.7 ± 65.3	106.4 ± 2.8

Table 3.3. Thiol content of alfalfa cell cultures following treatment with 1 mM β-alanine or 1 mM cysteine.
Data is a mean of 2 experiments with the standard deviation given.

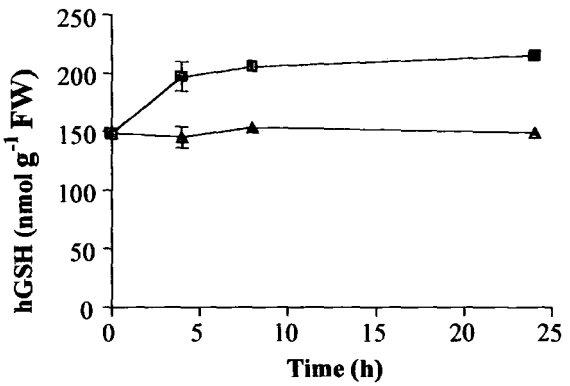

Figure 3.7. hGSH content in soybean following treatment with either (■)100 μg ml^{-1} yeast cell wall elicitor or sterile water (▲).
Data is a mean of 2 samples with error bars showing variation between replicates. In the instance where no error bars are visible, the variation lies within the extent of the graphical symbol.

Plant	Treatment	hGSH content (nmol gFW^{-1})
Soybean	none	336.3 ± 72.8
	1 mM β-alanine, 4 h	323.3 ± 30.6
	1 mM cysteine, 4 h	469.3 ± 71.2
	1 mM cysteine, 16 h	630.0 ± 18.9

Table 3.4. hGSH content of soybean cell culture following treatment with 1 mM β-alanine or 1 mM cysteine for the time (h) specified.
Data is a mean of duplicate experiments with the standard deviation given.

3.2.5 Effect of Elicitation on Protein Thiolation in Alfalfa Cell Cultures

To monitor the formation of mixed disulphides with protein thiol groups in elicitor treated alfalfa, the protein bound thiol pool was determined after treating acid precipitated protein extracts with a reducing agent. The thiols were derivatised by mBBr and separated by HPLC as described for the soluble thiols. Unfortunately, the level of protein bound thiols was insufficient for accurate quantification by this method so an alternative method was devised. Quantification of the protein bound thiol pool involved labelling the thiol pools with L-[^{35}S]cysteine followed by separation by HPLC in order to determine the thiol identity. To reduce the incorporation of L-[^{35}S]cysteine into protein and maximise incorporation into GSH/hGSH, protein synthesis was inhibited by the addition of 100 μg ml^{-1} cycloheximide.

Alfalfa cell cultures growing in the mid-logarithmic phase, were treated for 1 h with cycloheximide before the addition of 2.5 μCi ml^{-1} L-[^{35}S]cysteine. After 4 h, the cultures were treated with 100 μg ml^{-1} yeast cell wall elicitor, or an equal volume of sterile distilled water in the presence of the L-[^{35}S]cysteine for up to 24 h.

Following elicitation, the protein bound thiols were quantified by reducing covalently bound thiols from washed, acid precipitated protein fraction. In addition, the free thiols were also quantified as their *S*-bimane conjugates. In both cases, the level of incorporated L-[^{35}S]cysteine into GSH and hGSH was determined by scintillation counting the fractions (figure 3.8). It was then possible to quantify the amount of both free and protein bound thiols.

Further analysis of the cell culture extracts revealed that following elicitation, the major protein bound thiol was found to be GSH. This reflected the changes seen with the soluble thiol pool (table 3.5). In water-treated cultures, approximately 1 nmol GSH formed mixed disulphides with 1 mg protein over the 24 h period.

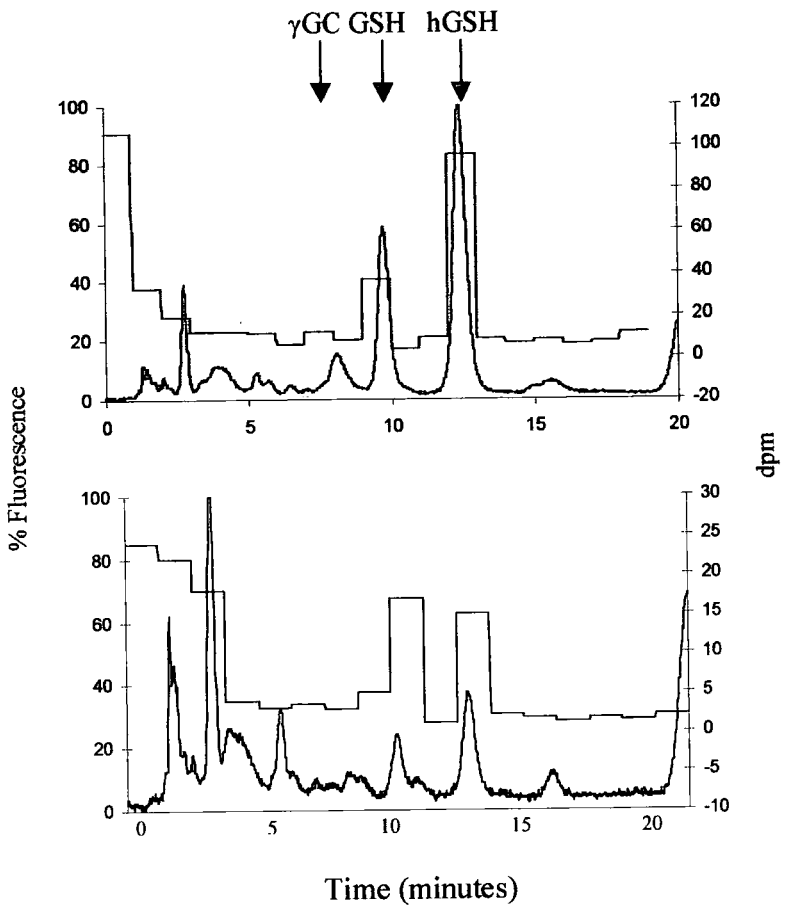

Figure 3.8 HPLC analysis of ^{35}S-labelled *S*-bimane derivatives of unincorporated thiols from alfalfa cell cultures treated for 4 hr with elicitor and L-[^{35}S]cysteine . The relative fluorescence is shown together with the histogram showing the radioactivity associated with 1 min fractions. A. Analysis of total thiols present in the extract. B. Analysis of oxidised thiols present in the NEM-treated extract after reduction with DTT.

Protein Bound Thiols					
Time (after addition of elicitor)	Elicitor	GSH (nmol mg^{-1})		hGSH (nmol mg^{-1})	
(h)	+/-	Ave	SD	Ave	SD
0	+	0.9	0.2	0.7	0.1
	-	0.9	0.2	0.7	0.1
4	+	0.8	0.4	0.9	0.3
	-	1.1	0.1	0.8	0.1
8	+	5.8	1.1	0.2	0.2
	-	1.2	0.5	1.4	0.4
24	+	2.3	0.6	0.6	0.3
	-	0.6	0.4	0.7	0.2

Table 3.5. Protein bound thiol content of alfalfa at timed intervals following elicitation with 100 μg ml^{-1} cell wall elicitor.
For the corresponding change in free ^{35}S-labelled thiols, refer to table 3.2. Alfalfa cell cultures (10 ml) were treated with 100 μg ml^{-1} cycloheximide for 1 h prior to labelling of the cellular thiol pool with 2.5 μCi ml^{-1} L-[^{35}S]cysteine. After 4 h in the presence of the label, alfalfa cell cultures were treated with 100 μg ml^{-1} fungal cell wall elicitor (+) or water (-). The amounts of protein-bound radioactive thiols were then calculated from the known specific activities of labelling of GSH and hGSH based radioassaying the bimane derivatives resolved by HPLC. Values are from duplicate experiments with the standard deviation given.

In the culture treated with fungal elicitor, protein thiolation was increased such that 8 h after elicitor treatment thiolation was quantified at 5.8 nmol GSH mg^{-1} protein. Maximal thiolation by GSH was detected after 8 h, after which, the level of protein bound GSH decreased to 2.3 nmol GSH mg^{-1} protein by 24 h. This level was still almost 4 times the level detected after a treatment with water only. The increase in protein bound GSH occurred during the period of accumulation of GSH in the alfalfa. However, unlike the free GSH (table 3.2), the level of Pr-SSG declined after 8 h, whereas GSH accumulated over the 24 h period. In both water treated and elicitor-treated cultures, the level of protein bound hGSH remained around 1 nmol mg^{-1} protein, over the 24 h period (table 3.5), suggesting that either hGSH does not form mixed disulphides with protein thiols, or that the rate of dethiolation of protein bound hGSH is equal to the thiolation rate. The latter proposal seems unlikely in view of the relatively constant specific activity of labelling of the hGSH pool over this period (table 3.2).

3.2.6 Determination of Protein Thiol Turnover in Alfalfa Cell Cultures

The radiolabelling studies clearly demonstrated that protein thiolation occurred during biotic elicitation in alfalfa, with GSH being the major thiol detected. To determine the turnover of protein-bound thiols, the incorporated radiolabel was chased out by incubating the cells in fresh media supplemented with 1 mM cysteine.

Following elicitor treatment, after 4 h the level of protein bound GSH detected in the cells was approximately twice that detected in non-elicited alfalfa cell cultures. Both sets of cell cultures were then transferred to fresh media containing +/- unlabelled cysteine (figure 3.9). The lower protein-bound level of [^{35}S]-GSH level detected following the addition of unlabelled cysteine is due to the turn-over of protein-bound [^{35}S]-GSH. The level of protein-bound GSH in water treated alfalfa cell cultures remained at basal levels in the presence or absence of non-labelled cysteine. After a further 16 h in the presence

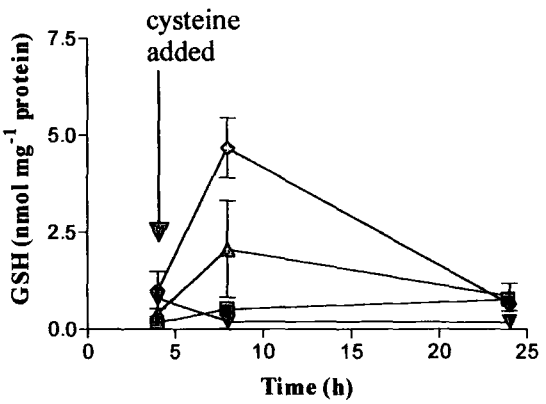

Figure 3.9 Analysis of mixed disulphides released following reduction of protein extracts from alfalfa cell cultures 'pulse-chase' labelled with L-[^{35}S]cysteine after elicitation with 100 μg ml^{-1} yeast elicitor.

Alfalfa cell cultures were pulse labelled for 4 h with L-[^{35}S]cysteine (2.5 μCi ml^{-1}) in the presence of cycloheximide with or without elicitor treatment. At 4 h, the control (▼) or elicited (◆) cells were transferred to fresh media alone or the control (■) and elicited (▲) cells were transferred to fresh media supplemented with 1 mM unlabelled cysteine. Data are mean of 2 samples with error bars showing variation between replicates. In the instance where no error bars are visible, the variation lies within the extent of the graphical symbol.

of the elicitor, the level of protein-bound GSH declined to a similar level to that determined in the water treated alfalfa cell cultures.

3.2.7 Thiols in Alfalfa Cell Cultures

Recent reports have implicated nitric oxide (NO) as an important compound in the post elicitation response in plants, acting as a signalling compound in a similar manner to reactive oxygen species (Durner *et al*, 1998). It was therefore important to determine whether accumulation of GSNO occurred during the elicitation response in alfalfa.

3.2.8 Development of an HPLC Based Detection System for Alfalfa Thiols Based on Derivatisation with 2,4-Dinitroflurobenzene (FDNB)

The thiol specific probe monobromobimane conjugates GSH via the free thiol group. Therefore, any thiols which do not possess a free thiol group, such as GSNO would remain undetected as the NO is bonded to the sulphydryl group. To detect GSNO and other thiols simultaneously, an HPLC based assay was developed using (FDNB), a UV absorbing probe. Similar methods have previously been used by Ji *et al* (1999) to detect the presence of GSNO in mammalian tissue. Optimisation of the labelling procedure for plant extracts by varying the concentration, conditions and reaction termination conditions resulted in a relatively quick, reproducible procedure as described in section 2.4.5. Initially, known concentrations of thiol standards were derivatised for separation by HPLC. Commercially synthesised peptides GSH, hGSH and GSSG were derivatised, whereas GSNO was prepared in the laboratory as described in section 2.7.1.

The derivatisation of thiols with FDNB revealed a more complex HPLC trace then the *S*-bimane derivatised extracts (figure 3.10). Addition of iodoacetamide aids separation by alkylating free sulphydryl groups and FDNB conjugates to free

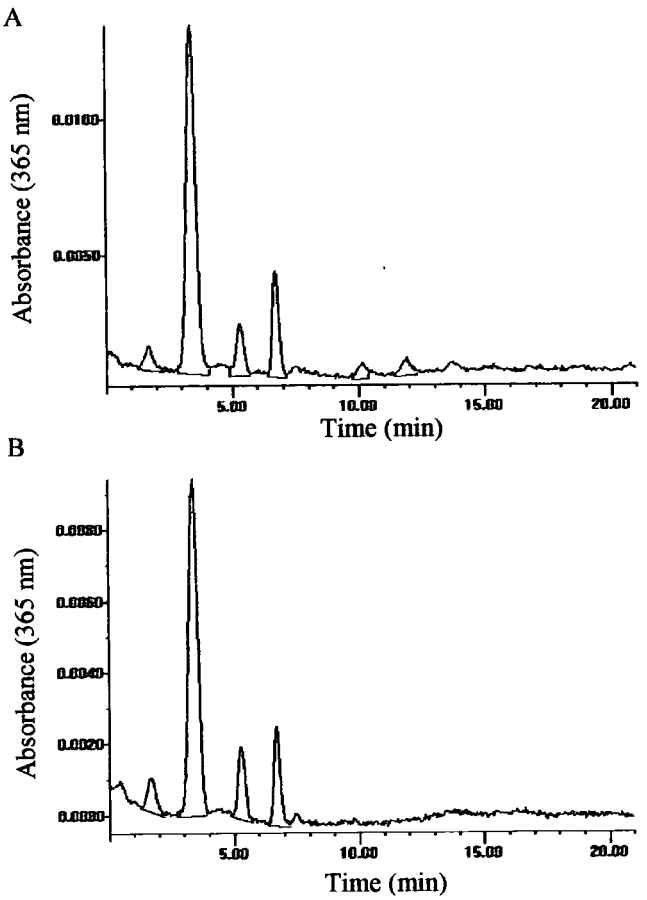

Figure 3.10 Separation of alfalfa cell culture extracts by HPLC following derivatisation by FDNB.
A, derivatisation of thiols in the absence of DTT; The peak at 1.5 min is hGSH, 3.5 min = UN 1, 5.5 min = GSH, 7 min = UN 2, 10 min = hGSSGh, 12 min = GSSG; B, derivatisation of thiols following pre-treatment with DTT.

amino groups allowing the detection of non-thiol containing amino acids, peptides and other GSH adducts.

3.2.9 Identification of Metabolites derivatised by FDNB in Alfalfa

After achieving separation of the standards, alfalfa cell cultures were grown to mid-exponential growth phase and the thiol pools were labelled with L-[^{35}S]cysteine in the presence of cycloheximide and treated with the fungal elicitor over a period of 8 h. Soluble extracts were derivatised by FBNB and following separation, the traces were found to contain several unknown peaks alongside the known thiol peaks. A UV absorbing compound (termed UN 1) eluted at the same time as the GSNO standard, being several times more abundant then GSH (figure 3.10). Also present was a second abundant UV absorbing compound (termed UN 2) (table 3.6). This initially suggested that GSNO was a major metabolite in the alfalfa cell culture. However, treatment of the alfalfa extract with DTT prior to FDNB derivatisation did not convert UN 1 in to GSH as would have been expected if it been GSNO. Incorporation of L-[^{35}S]cysteine into UN 1 was low as compared with GSH and hGSH which suggested its rate of biosynthesis from L-[^{35}S]cysteine was low compared with GSH and hGSH (table 3.6). However, despite poor incorporation of L-[^{35}S]cysteine, both UN 1 and UN 2 appeared to be as major *S*-containing metabolites as compared with GSH and hGSH (table 3.7).

In the absence of convincing evidence that the alfalfa cultures contained GSNO, it was of interest to determine if GSNO could be determined in elicited cells by detecting NO following GSNO reduction. Soluble extracts from elicited cell cultures were tested using a nitric oxide probe. Using the prepared GSNO standard, the technique was shown to be viable as when GSNO was reduced with either DTT, ascorbate or Cu^{2+}, nitric oxide was released and detected at a concentration as low as 1 μM. A similar result was observed when alfalfa protein extracts were spiked with known concentrations of GSNO. However, when

Time	Elicitor	Unknown 1				Unknown 2			
(h)	+/-	nmol g^{-1}		dpm $nmol^{-1}$		nmol g^{-1}		dpm $nmol^{-1}$	
		Ave	SD	Ave	SD	Ave	SD	Ave	SD
0	+	876	98	35	7	452	50	347	31
	-	876	98	35	7	452	50	347	31
2	+	562	199	25	11	99	42	172	52
	-	699	205	39	8	285	132	408	17
4	+	647	237	26	9	122	25	141	7
	-	644	191	22	2	334	52	200	61
8	+	979	74	12	2	218	39	68	20
	-	672	118	27	15	365	140	83	0

Table 3.6 Content and L-[^{35}S]cysteine incorporation into unknown metabolites detected in alfalfa following treatment with water or yeast cell wall elicitor preparation.

Alfalfa cell cultures were exposed to L-[^{35}S]cysteine for 2 h in the presence of cycloheximide prior to harvesting and soluble thiols derivatised with FDNB derivatised and quantified by HPLC.

Time (after addition of elicitor)	Elicitor	Reduced Thiols								Oxidised Thiols							
		GSH				hGSH				GSSG				hGSSGh			
(h)	+/-	nmol g^{-1}		dpm $nmol^{-1}$		nmol g^{-1}		dpm $nmol^{-1}$		nmol g^{-1}		dpm $nmol^{-1}$		nmol g^{-1}		dpm $nmol^{-1}$	
		Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
0	+	162	30	772	82	104	19	2413	165								
	-	162	30	772	82	104	19	2413	165								
2	+	114	29	264	127	62	18	762	441	22	1	176	32	23	3	302	152
	-	131	37	807	5	63	10	2564	398	18	5	577	82	13	3	1156	92
4	+	82	31	241	42	39	18	2186	377	37	15	395	67	26	5	292	138
	-	91	33	723	198	28	5	2765	544	29	3	1587	79	23	1	1723	467
8	+	233	14	355	77	95	16	793	2	25	12	188	57	14	8	246	38
	-	129	24	867	56	77	15	2497	297	19	2	740	284	17	1	1262	40

Table 3.7 Thiol content and L-[^{35}S]cysteine incorporation into GSH, hGSH, GSSG, hGSSGh detected in alfalfa following treatment with water (Elicitor -) or a fungal cell wall elicitor (Elicitor +).
Alfalfa cell cultures were exposed to L-[^{35}S]cysteine for 2 h in the presence of cycloheximide prior to harvesting and soluble thiols derivatised with FDNB derivatised and quantified by HPLC.

alfalfa cell extracts were treated with DTT, ascorbate or GSH, no NO was detected.

Having ruled out the possibility that UN1 was GSNO, changes in its concentration over the elicitation time course were determined. The level of UN 1 was approximately 4 fold greater (875 nmol g^{-1} FW) than GSH over the time course, remaining relatively stable in the elicitor treated cells. A slight transient decrease was detected following treatment with water. The second unknown peak (UN 2) eluted after 7 min and was highly abundant relative to GSH and hGSH. This second peak responded differently to treatment with yeast elicitor as compared with UN 1. Following treatment with the yeast elicitor, the abundance of UN 2 decreased within 2 h and over the following 6 h, the level remained low. When treated with water, the abundance of UN 2 in alfalfa decreased slightly but was significantly greater then the level in elicitor-treated extracts.

The specific radioactivity of UN 1 was low in both water and elicitor treated cultures (approximately 30 dpm $nmol^{-1}$ UN 1). A transient decrease was observed in specific activity following treatment with the yeast elicitor, whereas treatment with water had little effect (table 3.6). The specific activity of UN 2 was significantly greater than that detected in UN 1 (350 dpm $nmol^{-1}$ UN 2) and fluctuated significantly during the experimental period. Following treatment with the yeast elicitor, the specific radioactivity of UN2 mirrored its relative abundance by decreasing significantly. The specific radioactivity of the water-treated cell cultures also decreased significantly during the time course, although at a slower rate than the yeast elicitor treated extract.

The identification of UN 1 and UN 2 was further investigated by attempting to develop methods suitable for HPLC mass-spectrometry analysis. However, time constraints meant identification was not possible. From the data obtained, it can be surmised that both UN1 and UN2 contained sulphur but did not contain a free sulphydryl group as the peaks were not detected during *S*-bimane derivatisation.

3.2.10 Changes in Soluble and Protein Bound Thiols in Alfalfa

By labelling the peptide containing thiol pools with L-[^{35}S]cysteine during the elicitation response, it was possible to monitor changes in the pool as well as quantifying GSH, hGSH, GSSG, oxidised hGSH (hGSSGh), Pr-SSG and protein bound hGSH (Pr-SSGh) simultaneously (table 3.8).

In comparison with the untreated alfalfa cell cultures, the total thiol pool increased by approximately 50 % after only 8 h elicitation treatment and this increase can be attributed to an increase in GSH biosynthesis (table 3.8). Conversely, the proportion of hGSH decreased during this period. Before the 8 h time point, there was little difference between the total GSH and total hGSH present. However, relative proportions of oxidised and reduced forms of each thiol and the protein bound forms were seen to vary during this period. After 2 h, the proportion of soluble GSH remained fairly stable in the control culture whereas in the elicited cultures, soluble GSH accounted for less than 50 % of the total thiol pool due to the increase in GSSG and hGSSGh. Although after 2 h of treatment, the total pool size was not significantly greater, the proportion of GSH forming mixed disulphides with proteins had increased from 0 % to 6 % of the total thiol pool. This agreed with the previous studies with bimane derivatives which showed that GSH was the major thiol involved in the formation of mixed disulphides with proteins. Protein thiolation was most apparent within 2 h of elicitation, with protein bound GSH accounting for 6 % of the total thiols. By 4 h the proportion of total thiol present as GSH bound to protein had declined to 1 %. Although the results generated describe a trend of thiol metabolism following elicitation, care needs to be taken when looking at the absolute level of thiols as the alfalfa cell culture passage number influences the total soluble thiol level.

The specific radioactivities of each thiol following labelling with L-[^{35}S]cysteine can be used to determine changes in the thiol pools during elicitation. In terms of total thiol contents, under non-stressed conditions, the level of GSH transiently

decreased over the time course, whereas when treated with elicitor, the level of GSH increased (table 3.7). Although increasing slightly when treated with elicitor, the level of GSSG remained similar to that detected in the water treated cell cultures. Levels of hGSH responded as observed previously, with no difference between the water treated and elicited cell cultures, similarly little difference was observed in the hGSSGh pool during elicitation.

Under non-stressed conditions, the overall specific activity of labelling of GSH remained around 800 dpm $nmol^{-1}$ over the time course (table 3.7). Although between 4 h and 8 h the GSH specific activity increased in parallel with increased GSH content. In comparison, when treated with the yeast cell wall elicitor, the specific activity of GSH was greatly reduced over the first 4 h. This is a surprising result, suggesting that much of the initial increase in GSH content was not due to the incorporation of freshly supplied radiolabelled cysteine but rather an unknown secondary unlabelled cysteine source. After 8 h, GSH content was almost double that detected in the water treated cultures and although the specific activity increased from the level detected after 4 h, it was still only half the specific activity in control cultures. The specific activity of GSSG in the water-treated control was also greater than that detected in the elicited cultures. Unlike the soluble hGSH content, the specific activities varied greatly between treatments. In the water treated cultures, the specific activity remained around 2000 dpm $nmol^{-1}$ hGSH during the time course, whereas when treated with the elicitor, the specific activity decreased by over 50 % within 2 h only to recover by 4 h. However, after 8 h, the specific activity then decreased to the level detected after 2 h (table 3.7). Similarly in the hGSSGh pool, when treated with elicitor, the specific activity decreased compared with the water treated alfalfa cell cultures.

		Total	Percentage of Total Thiol Pool					
		Thiol pool	Soluble	Soluble	Soluble	Soluble	Protein	Protein
Time (h)	Treatment	nmol g^{-1}FW	GSH	hGSH	GSSG	hGSSGh	GSH	hGSH
0	water	266	61	39	0	0	0	0
2	water	224	58	28	8	6	0	0
2	elicitor	237	48	26	10	10	6	0
4	water	175	47	22	17	14	0	0
4	elicitor	182	50	16	20	13	1	0
8	water	245	53	32	8	7	0	0
8	elicitor	368	63	26	7	4	0	0

Table 3.8 Changes in thiol pools in alfalfa cell cultures following treatment with a fungal cell wall elicitor.
Alfalfa cell cultures were exposed to L-[^{35}S]cysteine for 2 h in the presence of cycloheximide prior to harvesting and thiols derivatised with FDNB and quantified by HPLC. Reduced, oxidised and protein bound thiol contents were quantified following FDNB derivatisation. Values are rounded to the nearest whole number.

3.3 Discussion

The alfalfa cell cultures were shown to be a viable cell system for monitoring the elicitation response in a leguminous species. By determining the elicitation response by monitoring PAL activity and medicarpin accumulation, it was possible to ensure that the cell cultures were viable and responding to the presence of the elicitor. Previous work suggested increased GSH concentration in suspension cell cultures may elicit the defence response (Yamada *et al*, 1989). However, this was subsequently proven no to be the case in bean and alfalfa cell cultures by the addition of the GSH precursor oxothiazolidine 4-carboxylate. The resulting increase in GSH level was detected without the concomitant induction of PAL or phytoalexin biosynthesis (Edwards *et al* 1991). This information demonstrated that addition of GSH alone to the cell culture would not induce the defence response, which was of interest in the current study.

The majority of work carried out on plant thiol metabolism has been conducted in species with GSH as the major thiol, or in the case of leguminous species, hGSH. Relatively little work has been carried out where two thiols are present in similar proportions, as is found in alfalfa. Questions remain as to whether the two thiols perform similar roles during oxidative stress. In plants containing either GSH or hGSH similar functions have been observed in the protection against heavy metals (Grill *et al*, 1990) and xenobiotics (Skipsey *et al*, 1997). In plants with two thiols, recent studies have suggested different roles may be assigned to the thiols. Frendo *et al* (1999) indicated that in *Medicago truncatula*, different roles may be assigned to GSH and hGSH after observing that only GSH was present in the seed, suggesting a role as the major sulphur storage compound. Also the translocation of GSH through the vascular system but not hGSH which remained in the roots and nodules of this legume suggests that GSH was the major transportable form of cysteine required for protein synthesis in developing plants. This is in agreement with Klapheck (1988) who observed that GSH was the major source of sulphur for protein synthesis in plants containing more than one thiol.

As GSH was the dominant thiol in leaves of whole alfalfa plantlets, it has been suggested that this thiol has a greater role in the protection against oxidative stress caused during photosynthesis (Alscher *et al*, 1997). The results in alfalfa cell cultures indicate that GSH and hGSH metabolism differs both in the accumulation and use of the individual thiols. Exact concentrations of GSH and hGSH in alfalfa cell cultures depended on the number of passages since cell culture initiation and to a lesser extent stage of growth (Edwards *et al*, 1991). Therefore some variation in the ratio of GSH:hGSH may have been due to the age of the cultures rather than experimental conditions. Following elicitor treatment of alfalfa cell cultures, hGSH did not accumulate, nor did the associated oxidative stress result in the formation of mixed disulphides between hGSH and proteins. In contrast, the amount of GSH bound to protein thiols increased 6 fold (table 3.5). The lack of metabolic plasticity in hGSH in the alfalfa cell cultures was also suggested by the cysteine feeding studies. The availability of cysteine is considered a major rate-limiting factor in thiol biosynthesis (May et al, 1998). However, when 1 mM cysteine was added to the growth media only GSH accumulated in the alfalfa cell cultures. In contrast, the addition of cysteine to soybean cell cultures resulted in a significant increase in hGSH levels. Interestingly addition of β-alanine did not increase hGSH synthesis in either alfalfa or soybean, suggesting this compound is not limiting in hGSH biosynthesis. The study confirms previous findings of the importance of cysteine as the rate limiting substrate (Noctor *et al*, 1998). However, even though biosynthesis of hGSH did not increase following elicitation, the ratio of hGSSGh:hGSH increased in a similar manor to that seen with GSSG:GSH. During oxidative conditions, the proportion of oxidised to reduced thiol (approximately 5 % under non-stressed conditions) increased to 20 % as a result of exposure to ROS with both thiols. In alfalfa cell cultures, the increase in oxidised hGSH shows that if any compartmentalisation of the hGSH biosynthesis pathway did exist, then the product, hGSH, was still exposed to the oxidative conditions. Interestingly, this increase in oxidised hGSH did not lead to the formation of protein mixed disulphides as the level of protein bound hGSH

remained the same as the control cultures. It could be that protein thiolation is catalysed enzymatically and the substrate affinity favours the use of GSH. Significantly, glutaredoxin has been shown to increase thiolation of GADPH *in-vitro* (Lind *et al*, 1998), though the significance of this *in-vivo* has not been determined. The different levels of protein thiolation may also be due to cell to cell differences. For example, although care was taken to ensure alfalfa cells remained friable, it is possible that some clumping of cells may have occurred. Any clumping of cells could have reduced the elicitation response subsequently determined. Also, minor changes in the protein thiolation levels may occur due to isolation procedure, however, addition of NEM to the extraction media would have minimised these changes.

Following derivatisation by FDNB, it was hoped that the role of GSNO during the elicitation response in alfalfa could be deduced. In mammalian systems, GSH is known to react with NO via the thiol group to form *S*-nitrosoglutathione (GSNO) and can induce protein thiolation in mammalian GADPH following the formation of the glutathione thiyl radical resulting from NO cleavage (Mohr *et al*, 1999). The formation of glutathione disulphide *S*-dioxide (GS(O)SG) following the spontaneous breakdown of GSNO has also been identified as an active thiolating compound (Li *et al*, 2001). It is possible that any GSNO formed during the elicitation response in alfalfa was degraded too rapidly to be detected using the FDNB derivatisation method. However, the negative results of the NO electrode suggested NO to be a minor metabolite during the elicitation response in alfalfa.

Although working with cell culture suspensions has many advantages and in many respects, reflects the biochemistry e.g. the phytoalexin defence response, of whole plants, there are drawbacks to using this system. One of these is in determining the location and compartmentalisation of biosynthetic pathways. As indicated previously, in *M. truncatula* plant the location of GSH and hGSH differs, the location of GSH or hGSH is dependent on the expression of *gshs1* and *gshs2*, the genes putatively identified as encoding GSH synthetase and hGSH

synthetase respectively (Frendo *et al*, 1999). Similarly in *Trifolium incarnatum*, which synthesises GSH and hGSH, hGSH predominates in the roots and GSH in the seeds and foliage (Klapheck, 1988). The lack of hGSH in the foliage suggests a minor role in the oxidative stress response. In cell cultures, tissue specific regulation of biosynthetic pathways should be removed as the cell culture contains a homogenous cell type. It would therefore be of interest to determine the effect of infection on thiol metabolism in different organs in legumes where the accumulation of GSH and hGSH is compartmentalised.

4.0 Study of Thiolation in Suspension Cell Cultures of Alfalfa

4.1 Introduction

Oxidative conditions imposed on living cells in response to pathogens, environmental or chemical stress can lead to protein modifications resulting in a loss of their function and degradation by proteases. During oxidative stress, sulphydryl groups in cysteine residues are vulnerable to progressive oxidative modification, resulting in loss of activity (Reddy *et al*, 2000). Oxidation of sulphydryl groups is a three step process leading to the sequential formation of sulphenic, sulphinic acid and sulphonic acid derivatives. Prevention of the sulphinic acid derivative through the formation of a mixed disulphide between the sulfenic acid derivative of the protein cysteinyl residue and free GSH effectively prevents irreversible oxidation. The formation of mixed disulphides between protein and soluble thiols is termed *S*-glutathionylation, or thiolation (Thomas *et al*, 1995). The mixed disulphide can be reversed though the action of the disulphide bond reducing enzyme glutaredoxin, or through changes in the cellular environment resulting in a restoration of reducing conditions.

Mammalian studies have revealed a number of proteins that are thiolated during periods of oxidative stress, these include the transcription factor c-Jun (Klatt *et al*, 1999) whose activity is reversibly inhibited upon mixed disulphide formation. A number of similar transcription factors contain regulatory cysteine residues and is speculated that these are redox regulated in a similar fashion (Klatt and Lamas, 2000). Through this mechanism, *S*-thiolation may represent a general path for transducing oxidative stress into regulation of gene expression.

In mammals many proteins have been identified as undergoing thiolation and more is known about the role of thiolation in cellular regulation. In contrast, in plants few proteins have been identified as being thiolated during oxidative conditions and the effects of thiolation on the plant cells is unknown.

In the previous chapter, alfalfa cell suspensions were shown to respond to yeast cell wall elicitor by increasing the content of GSH bound to proteins in the form of mixed disulphides. After determining the evidence for protein thiolation, it was then necessary to identify the proteins which were thiolated during oxidative stress in alfalfa cell cultures following the addition of yeast cell wall elicitor. It was then of interest to compare thiolation in alfalfa with thiolation in other plants, identifying similarities and differences in these reactions in different species. To identify proteins thiolated in alfalfa cell cultures, the intracellular thiol pools were labelled with L-[^{35}S]cysteine *in-vivo* and *in-vitro* under control and oxidative conditions, the latter induced by treatment with a yeast cell wall elicitor.

4.2 Results

4.2.1 Identification of Thiolated Proteins Following *in-vivo* Labelling of Alfalfa Cell Cultures with L-[^{35}S]Cysteine

Alfalfa cell cultures were pre-treated with cycloheximide to prevent the incorporation of L-[^{35}S]cysteine directly into protein via *de-novo* synthesis. The thiol pool was then labelled with L-[^{35}S]cysteine for 4 h before addition of elicitor and the cells harvested over a 24 h time course. The proteins were extracted on ice in buffer containing *N*-ethyl maleimide (NEM). NEM alkylated available cysteinyl residues preventing the further formation of mixed disulphides during extraction. Protein extracts were separated by SDS-PAGE and exposed to X-ray film to determine the number of proteins modified by thiolation. However, separation of the proteins by SDS-PAGE revealed that the level of incorporated L-[^{35}S]cysteine via thiolation by L-[^{35}S]GSH was too low to clearly visualise protein thiolation during elicitation *in-vivo*. Figure 4.1 shows the typical labelling of protein extracts from control and elicited cell cultures. In these studies, there was no immediately apparent differences in the [^{35}S]-GSH thiolated proteins from the control and elicitor treated alfalfa cells. Increasing the time of film exposure to the gel only increased the background intensity and did not lead to an increase in the number of bands visualised. Previous results had determined that a quantitative

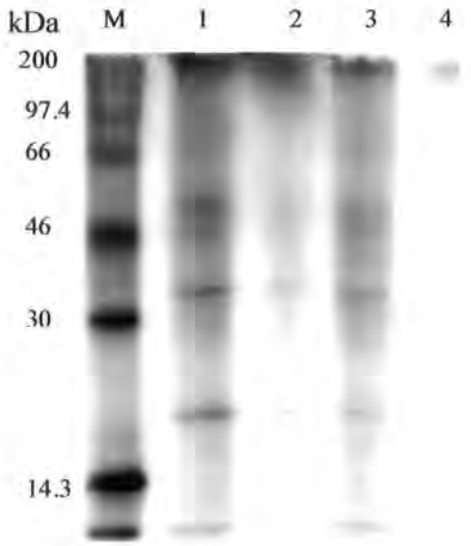

Figure 4.1 Analysis of L-[^{35}S]cysteine labelled alfalfa protein extracts labelled in presence or absence of elicitor ± cycloheximide treatments.

Cell cultures (10 ml) were treated with or without 100 μg ml^{-1} cycloheximide for 1 h, followed by a 4 h labelling with 25 μCi L-[^{35}S]cysteine. The cell cultures were then either treated with yeast cell wall elicitor for a further 4 h or left untreated. Proteins were extracted and separated by SDS-PAGE. Lanes: 1, extracts from unelicited cultures which had not been treated with cycloheximide; 2, control alfalfa labelled after cycloheximide treatment; 3, elicited alfalfa which had not been exposed to cycloheximide; 4, elicited alfalfa with cycloheximide treatment; Lane M, markers whose molecular masses are shown on the left of the figure in kDa. Each lane (lanes 1-4) was loaded with 40 μg protein.

increase in protein thiolation *in-vivo* followed treatment with a fungal elicitor. However, following the resolution of the polypeptides by SDS-PAGE, it was clear that the level of radiolabelled incorporation was inadequate in identifying specific proteins. An alternative, *in-vitro* method was developed therefore to increase the specific activity of labelling of the proteins thiolated.

4.2.2 *In-vitro* Thiolation of Alfalfa Protein Extracts with [^{35}S]-Glutathione

In-vitro thiolation of protein extracts allowed greater sensitivity of labelling of the protein by increasing the specific activity of [^{35}S]-thiol present in the assay. Whereas *in-vivo* labelling used endogenous thiol biosynthesis to produce [^{35}S]-labelled GSH and hGSH, *in-vitro* thiolation required the addition of pre-formed L-[^{35}S]cysteinyl-GSH or L-[^{35}S]cysteinyl-hGSH ([^{35}S]-GSH or [^{35}S]-hGSH). The radiolabelled thiol derivatives were synthesised enzymatically to a high specific activity of labelling by a procedure developed by Dr Mark Skipsey, using an extract from *E. coli* BL21 expressing pET24D containing the DNA sequence of hGSH synthetase (provided by Mark Skipsey), the final enzyme in hGSH biosynthesis. Briefly, in the preparation, the endogenous GSH synthetase from *E. coli* was used to synthesise [^{35}S]-GSH using γ-glutamyl-[^{35}S]-cysteine and glycine as substrates. [^{35}S]-hGSH was prepared using the recombinant hGSH synthetase using γ-glutamyl-[^{35}S]-cysteine and β-alanine as substrates. A crude protein preparation was prepared from the recombinant *E. coli* after removing low molecular weight compounds using a Sephadex gel filtration column. Non-radioactive cysteine was added to the reaction to regulate the final specific activity of the labelled GSH/hGSH. To determine the optimal assay conditions, varying concentrations of cysteine were used and the conversion of γ-glutamyl-cysteine to GSH or hGSH determined.

The biosynthesis of [^{35}S]-GSH or [^{35}S]-hGSH was carried out in a two step reaction. The first step used glutamate and L-[^{35}S]cysteine to synthesise γ-glutamyl-[^{35}S]-cysteine. The second step synthesising [^{35}S]-GSH or [^{35}S]-hGSH after the addition of glycine or β-alanine respectively and fresh ATP for a further

1.5 h. In the presence of 0.1 mM cysteine, on overall > 95 % conversion of cysteine to GSH was observed. In contrast increasing the cysteine concentration to 1 mM resulted in only 60 % incorporation of cysteine into radioactive GSH or hGSH. In the latter case, extending the incubation time did not improve the final yields of [^{35}S]-GSH/[^{35}S]-hGSH. To ensure the only thiols present were in the tripeptide form, the assay was spiked with 0.1 mM cysteine only.

The assay was terminated with 50 mM HCl and the protein precipitate removed by centrifugation. Levels of L-[^{35}S]cysteine incorporation into GSH were determined by counting the radioactivity in fractions corresponding to the thiol peaks and separated by HPLC after derivatisation with monobromobimane. After confirming that the radiochemical purity of [^{35}S]-GSH or [^{35}S]-GSH exceeded 95 %, the reaction products were stored in aliquots in liquid nitrogen.

To determine the degree of protein thiolation *in-vitro*, alfalfa cell cultures were harvested in mid-log phase and proteins extracted in 20 mM Tris-HCl, pH 7.4 and stored at –20 °C until required. The alfalfa protein extracts were subjected to oxidative stress generated by the addition H_2O_2 or *t*-HP both at a final concentration of 1 mM, in the presence of 1 μCi [^{35}S]-GSH. Both the oxidative treatments resulted in an increase in thiolation as determined by fluorography of the radiolabelled polypeptides after resolution by SDS-PAGE. Initial studies demonstrated that there was little difference in thiolation in terms of the number of proteins which underwent labelling or in the degree of thiolation observed using the two reagents (data not shown). In future work, *t*-HP was used to generate oxidative stress as this reagent has been used extensively in mammalian thiolation studies (Lii *et al*, 1994).

The effect of varying the exposure time to the oxidative stress on thiolation with [^{35}S]-GSH was also determined. In each case, the assay was terminated by the addition of NEM (final concentration 2 mM) before analysis by non-reducing SDS-PAGE. The addition of NEM was essential to alkylate free protein and

residual soluble thiol groups, preventing artefactual protein thiolation during sample preparation and electrophoresis. The time course of thiolation of alfalfa protein extracts in the presence of *t*-HP revealed a time-dependent increase in labelling of polypeptides over a 15 min treatment (figure 4.2). At time 0, the reaction was terminated immediately by the addition of NEM. A radiolabelled polypeptide of 16 kDa was visible suggesting a rapid reaction with [^{35}S]-GSH. However, the polypeptide at 16 kDa was present throughout the time course at similar intensity suggesting the polypeptide was derived from the *E. coli* protein extract used to synthesise [^{35}S]-GSH. Upon addition of *t*-HP, polypeptide bands of approximately 20 kDa and 28 kDa were visible after 5 min. As the assay time increased, polypeptides at 38 kDa, 46 kDa, 50 kDa and 90 kDa also became visible. The reaction was terminated after 15 min to help minimise loss of polypeptide resolution as the background labelling intensified. The minor polypeptides at the bottom of the gel are likely due to contaminating polypeptides derived from the preparation of [^{35}S]-GSH and un-reacted [^{35}S]-GSH respectively.

The *in-vitro* [^{35}S]-thiolation assays of alfalfa total protein extracts identified a number of polypeptides that are modified in the presence of *t*-HP. However, by only separating the proteins on the basis of their molecular weight, there is always the possibility of there being more than one protein present at a given molecular mass. To overcome this possibility and to separate all the proteins, further analysis by 2D gel electrophoresis separating the proteins on the basis of their pI was performed. However, in these early studies it was not possible to obtain cleanly resolved radiolabelled proteins, probably due to the low specific activity of obtained incorporation.

4.2.3 Comparison of Thiolation in Other Plants

After discovering that a number of alfalfa proteins from suspension culture cells were thiolated *in-vitro*, it was of interest to determine whether similar pattern of thiolation would be observed using protein preparations from the whole leaves or

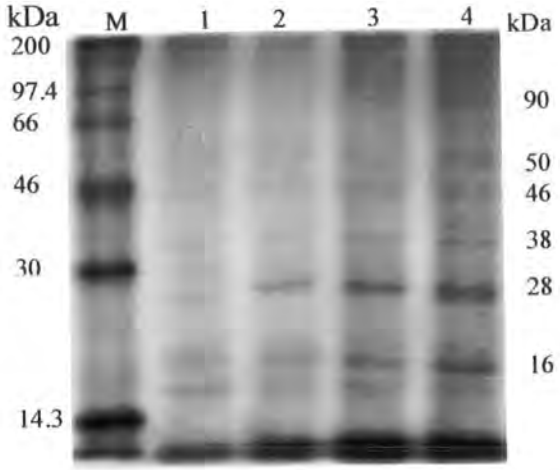

Figure 4.2 SDS-PAGE and fluorographic analysis of alfalfa protein extracts after *in-vitro* thiolation with [^{35}S]-GSH.
Lanes 1 to 4, a time course of 0, 5, 10, and 15 minutes after addition of *t*-HP. Lane M, ^{14}C labelled markers whose approximate molecular masses are shown on the left of the figure. Each lane was loaded with 40 μg protein. The migration of ^{14}C-labelled reference proteins is shown.

chloroplasts (prepared by the method described by Triboush *et al*, 1998) of alfalfa plants. It was also of interest to qualitatively compare the range of polypeptides thiolated in alfalfa with those in other plants similarly derivatised. Protein extracts from wheat, tobacco and alfalfa plants along with purified alfalfa chloroplasts and Arabidopsis cell suspension, were thiolated under the conditions described above and analysed by non-reducing SDS-PAGE (figure 4.3). Although all samples contained multiple polypeptides which became labelled by [^{35}S]-thiolation, there were several distinct differences between the species. Alfalfa leaves contained two labelled polypeptides around 28 kDa (see 1; figure 4.3). Arabidopsis chloroplasts and tobacco had one distinct labelled polypeptide around 26 kDa (see 2; figure 4.3), whereas these polypeptide were not present in the wheat preparations. A major band at 50 kDa (see 3; figure 4.3) was present in all the samples apart from alfalfa leaves, although it was present in the corresponding chloroplast extract. Comparing the alfalfa cell suspension, leaves and chloroplast extracts revealed interesting differences in labelling. Distinct labelled polypeptides at 97 kDa and 40 kDa in the chloroplast were absent from the preparations from alfalfa leaves and the cell cultures. The cell cultures were dark grown and therefore would lack the majority of chloroplastic proteins found in light grown leaves. However, it was surprising that the 97 kDa and 40 kDa thiolated chloroplastic proteins were absent in the whole leaf extract. Presumably these proteins though found in the chloroplast are relatively low in abundance relative to other leaf protein. The alfalfa leaf extract did have distinctive bands at 27 kDa and 28 kDa, which were not seen in the other Arabidopsis tissue types. The plant protein extracts apart from the chloroplast preparation had thiolated proteins in the low molecular weight range (< 20 kDa). These polypeptides in the wheat and Arabidopsis cell cultures extracts appearing to be susceptible to thiolation as determined by the visual intensity of the labelled polypeptides.

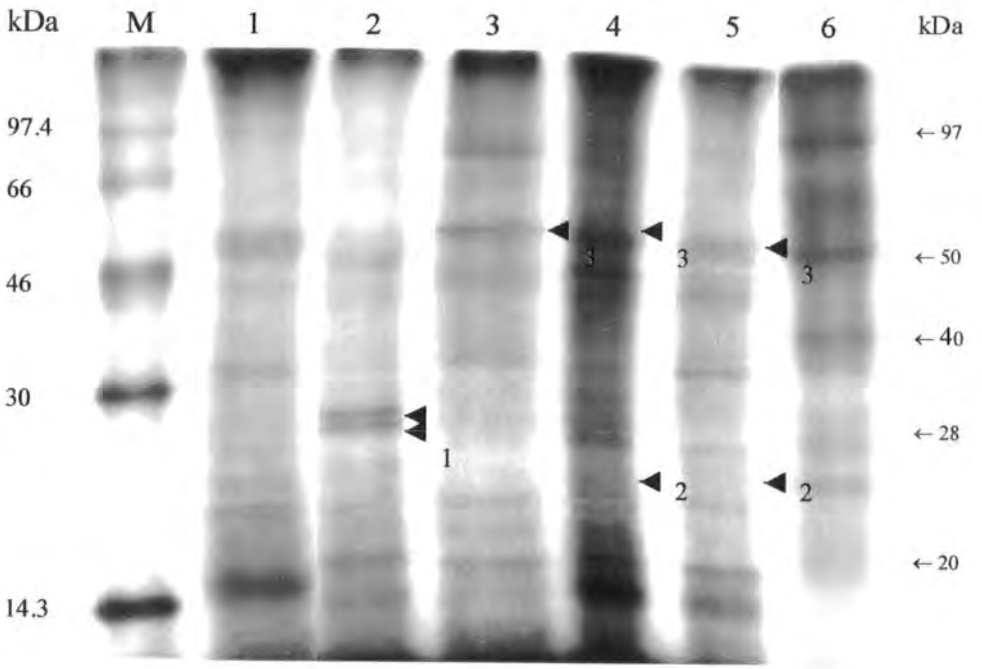

Figure 4.3 *In-vitro* thiolation of a selection of plant protein extracts with [^{35}S]-GSH after incubation with 1 mM *t*-HP for 15 min.
Polypeptides refered to in the text are arrowed and numbered for reference. Lane 1, wheat shoots; lane 2, alfalfa leaf; lane 3, alfalfa cell culture; lane 4, arabidopsis cell culture; lane 5, tobacco leaf; lane 6, alfalfa chloroplast preparation; Lane M, ^{14}C labelled proteins of a known molecular mass. Each lane was loaded with 40 μg protein.

4.2.4 Use of [^{35}S]-GSH and [^{35}S]-hGSH in Thiolation *in-vitro*

To investigate whether the difference in utilisation of GSH and hGSH in protein thiolation observed *in-vivo* (chapter 3) could be replicated *in-vitro*, alfalfa protein extracts were treated with *t*-HP in the presence of identical amounts of either [^{35}S]-GSH or [^{35}S]-hGSH for 15 min and the radiolabelled polypeptides separated by SDS-PAGE. Following exposure of the alfalfa protein extracts to chemically induce oxidative stress, both [^{35}S]-GSH and [^{35}S]-hGSH formed protein mixed disulphides giving very similar patterns of labelling (figure 4.4). This demonstrated that *in-vitro*, both GSH and hGSH are equally active in forming protein disulphides with alfalfa proteins. As it was demonstrated that there was no difference in the reactivity of GSH and hGSH when used for thiolation *in-vitro*, all future experiments used [^{35}S]-GSH for *in-vitro* labelling in all other plant studies.

4.3 Discussion

The labelling of cellular thiols with L-[^{35}S]cysteine to identify proteins susceptible to thiolation *in-vivo* during the elicitation response, was of insufficient sensitivity to visualise individual proteins in alfalfa cell suspensions. This problem was overcome through the development of an *in-vitro* thiolation assay whereby the protein extracts were thiolated using pre-synthesised [^{35}S]-GSH prepared to high specific radioactivity in the presence of a chemical oxidant. This increased specific activity was sufficient to visualise protein mixed disulphide formation with [^{35}S]-GSH.

As discussed previously, alfalfa cell cultures contain both GSH and hGSH in similar concentrations although only GSH appears to be used in protein thiolation. To determine whether there is any difference in reactivity in the two thiols, both [^{35}S]-GSH and [^{35}S]-hGSH were synthesised and included individually in the thiolation assay. These studies clearly demonstrate there was no significant difference in the use of these thiols in the *in-vitro* thiolation experiments. In whole

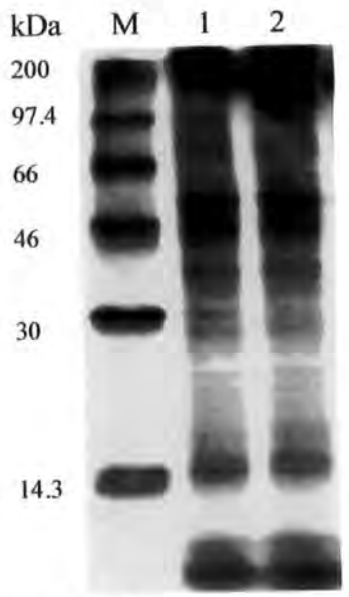

Figure 4.4 *In-vitro* thiolation of alfalfa protein extracts using either [^{35}S]-GSH or [^{35}S]-hGSH in the presence of 1 mM *t*-HP.
Lane M, ^{14}C labelled proteins of a known molecular mass; lane 1, alfalfa protein extracts labelled with 1 μCi [^{35}S]-GSH or lane 2, 1 μCi [^{35}S]-hGSH . Each lane was loaded with 40 μg protein.

plants, either compartmentalisation of the two thiols exists, as has been detected in whole alfalfa plantlets (Frendo *et al*, 1999). It may also be possible that if thiolation *in-vivo* was an enzymatic process, the protein responsible could favour GSH over hGSH, or hGSH may not be available to thiolate proteins due to their physical separation whereas *in-vitro*, non-enzymatic thiolation uses either thiol equally well. In cell free extracts using pre-synthesised L-[^{35}S]cysteine labelled thiols, these considerations are absent.

After optimising the assay conditions for *in-vitro* thiolation, protein extracts from alfalfa cell cultures contained a number of proteins, which became labelled. This was to be expected, as any protein with an exposed cysteine residue is a potential target for thiolation during oxidative stress. In the assay terminated immediately by the addition of NEM, polypeptides of 16 kDa were visible. This was found to be due to the contamination of thiolated *E. coli* polypeptides from [^{35}S]-GSH synthesis. The nature of the 16 kDa contaminant was not further investigated, though its formation in the biosynthesis of [^{35}S]-GSH is interesting. The time dependent increase in the number of thiolated plant proteins and the intensity of these thiolated proteins relative to one another could be due to differences in their abundance or the different reactivity of sulphydryl groups. Some proteins can be thiolated under non-oxidative conditions, for example, carbonic anhydrase III has been shown to modified by thiolation in the absence of oxidative stress *in-vitro* (Cabiscol and Levine, 1996) as has the 20S proteosome of rat liver (Demasi *et al*, 2001). Differences in susceptibility to thiolation are due to the amino-acids surrounding the cysteine, especially where their hydrophobicity regulates the availability of soluble compounds which can oxidise or reduce the protein amino-acid chains (Thomas *et al*, 1995).

When protein extracts from other plant species were thiolated *in-vitro*, a large number of proteins were modified showing that thiolation is a universal reaction during oxidative stress. There were differences between the species with several proteins appearing to be more readily thiolated than others. As protein

composition differs between species, this variation is not surprising. Significantly, within the chloroplast there are a number of proteins which are regulated via a disulphide mechanism (Ruelland and Miginiac-Maslow, 1999). During oxidative stress in plants, the process of identifying thiolation would be further complicated by competing reactions intra-protein disulphide formation and thiolation (Li *et al*, 2001) which could lead to a lower than expected level of protein thiolation. However, the studies in alfalfa clearly show that a large number of polypeptides are thiolated under oxidative conditions.

5.0 Study of GSH Metabolism and *S*-Thiolation in Arabidopsis Suspension Cultures Following Oxidative Stress

5.1 Introduction

The large number of polypeptides thiolated *in-vitro* in alfalfa cell cultures treated with *t*-HP, suggested that protein thiolation had potential as being a widespread modification during oxidative stress. To investigate the identity of the *in-vitro* thiolated proteins, a directed proteomics approach, utilising matrix assisted laser-desorption ionisation-time-of-flight (MALDI-TOF) mass spectrometry was identified as a means of identifying candidate polypeptides. This approach has several advantages over conventional methods of protein identification based on purification to a homogenous preparation. Firstly, separation of thiolated proteins using 2D gel electrophoresis gives high resolution and requires minimal quantities of protein. Secondly, loss of protein throughout the purification steps and loss of the radiolabel due to reduction of the thiol moiety are minimised. The limitation of MALDI-TOF-MS proteome analysis is that extensive databases of putative protein sequences in the plant must be available. Thus, the availability of data for proteomic studies on the model plant species *Arabidopsis thaliana* would be much greater then that available for alfalfa. Therefore, to make efficient use of this technique, protein thiolation studies need to be carried out on Arabidopsis with cell suspension cultures offering a useful system. To ensure the viability of this plant system to study the effect of oxidative stress on thiolation, it was necessary to define changes in thiol metabolism in response to elicitation in Arabidopsis cell cultures.

Arabidopsis cell suspension cultures have been previously used to study the oxidative burst following elicitation with the protein elicitor harpin (Desikan *et al*, 1996). Changes in glutathione synthesis following oxidative stimulation have also been reported in arabidopsis cell cultures (May and Leaver, 1993). The use of

arabidopsis cell cultures significantly simplifies thiol metabolism studies, as GSH is the only major thiol present (May and Leaver, 1993).

5.2 Results

5.2.1 Elicitation of Defence Responses in Arabidopsis Cell Cultures

Initially, experiments were performed to confirm whether or not Arabidopsis cell cultures were suitable for the study of oxidative induced changes in thiol metabolism and protein thiolation. Arabidopsis cell cultures were grown in 100 ml batches in Murashige and Skoog basal salts with minimal organics media. After sub-culturing, the 4 day old cultures were treated either with yeast cell wall elicitor (equivalent to 100 μg of sugar equivalent per ml culture), 1 mM menadione or 1 mM *t*-HP and then harvested after a 4 h period. The PAL activity and GSH content were determined to characterise the elicitation system for future experiments. Table 5.1 shows the PAL activity of the control (water-treated) and elicitor-treated arabidopsis cultures. Treatment with the fungal elicitor did not produce a significant increase in PAL activity compared to the control cultures. Treatment with *t*-HP or menadione resulted in a moderate induction of PAL increasing activity from 7.7 μkats kg^{-1} protein to 13.3 and 11.1 μkats kg^{-1} protein respectively. Although modest, this enhancement was reproducible (data not shown) and showed the cell cultures responded to the oxidative stress by eliciting a defence-like response. Total GSH levels were determined in each of the extracts to confirm changes in thiol metabolism accompanied the induction of the defence response. Total GSH (reduced + oxidised) accumulated to a different extent depending on the elicitation treatment (figure 5.1). The control cells contained approximately 250 nmol of GSH g^{-1} fresh weight (FW). Following treatment with the fungal elicitor, the GSH decreased slightly to approximately 200 nmol g^{-1} FW. When treated with menadione, the GSH content increased slightly, to 300 nmol g^{-1} FW, although the large standard deviation suggested that the increase was hardly

Treatment	PAL activity (μkats kg^{-1} protein)	
	Average	SD
Control	7.7	1.9
Fungal elicitor	7.7	1.4
t-HP	13.3	1.0
Menadione	11.1	0.3

Table 5.1. PAL activity of Arabidopsis cell cultures, following treatment with a fungal elicitor or chemicals which impose oxidative stress.
Arabidopsis cell cultures were treated for 8h with water (control), 100 μg ml^{-1} reducing sugar equivalents yeast cell wall elicitor, 1 mM *t*-butyl hydroperoxide (t-HP) or 1 mM menadione. Data are an average of duplicate cell cultures with the variation in duplicate quoted as standard deviation.

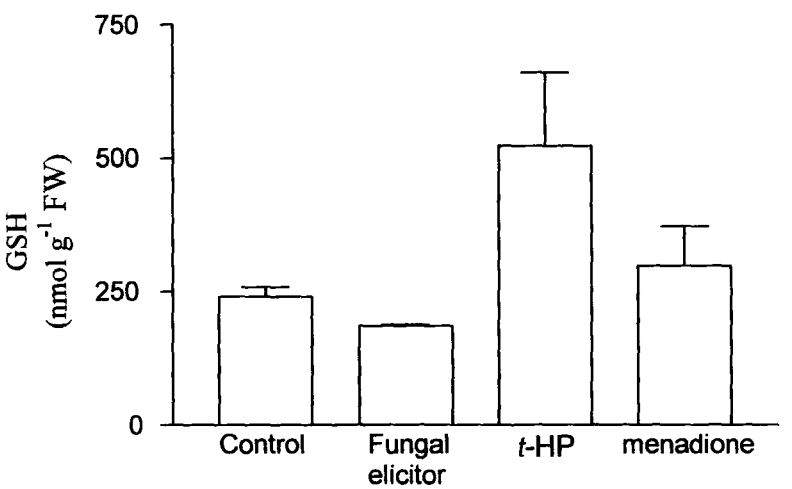

Figure 5.1 Total glutathione content of arabidopsis cell cultures following an 8 h treatment with elicitors.
Arabidopsis cell cultures were treated with water (control), 100 μg^{-1} ml yeast cell wall elicitor (fungal elicitor), 1 mM *t*-butyl hydroperoxide (*t*-HP) or 1 mM menadione. Data are an average of duplicate cell cultures with error bars showing the standard deviations. In the instance where no error bars are visible, the variation lies within the extent of the graphical symbol.

significant. Only when arabidopsis cell cultures were treated with *t*-HP was a significant increase in GSH to 500 nmol g^{-1} FW determined. As treatment with *t*-HP resulted in both an induction of PAL activity and increased GSH content, arabidopsis cell cultures were treated with *t*-HP in the subsequent experiments to elicit oxidative defence responses.

5.2.2 Thiol Metabolism in Arabidopsis Cell Cultures in Response to Oxidative Stress

5.2.2.1 Determination of the Level of Protein Bound GSH following Oxidative Stress

After determining the accumulation of GSH in Arabidopsis cell cultures in response to treatment with *t*-HP, it was then of interest to determine the effects of this treatment on the level of protein bound GSH. Cell cultures (10 ml) were pre-treated with cycloheximide to prevent L-[^{35}S]cysteine incorporation into proteins. The GSH pools were then labelled with 25 μCi L-[^{35}S]cysteine for 4 h prior to treatment with 1 mM *t*-HP. The cell cultures were harvested after 0, 2, 4 and 8 h of *t*-HP treatment and the thiols extracted in buffer in the presence or absence of NEM to allow determination of the levels of both GSH and GSSG. Following derivatisation by mBBr, the thiols were separated by HPLC with the fractions containing GSH collected and the incorporation of L-[^{35}S]cysteine determined by scintillation counting. After determining the specific activity of the L-[^{35}S]cysteine labelled GSH ([^{35}S]-GSH) in the soluble pool, it was possible to quantify protein thiolation. Extracts containing a known amount of protein were acid precipitated on to glass-fibre filter paper and washed three times with acidified cysteine to remove any non-specific radioactivity. The amount of [^{35}S]-GSH bound to the filter papers was then ascertained by scintillation counting. To ascertain levels of L-[^{35}S]cysteine incorporation by thiolation rather than protein synthesis, extracts containing a known amount of protein were treated with or without DTT prior to precipitation and scintillation counting.

The level of protein bound GSH in arabidopsis cells following elicitation with *t*-HP was determined (figure 5.2). In the control cell cultures, the levels of protein bound [^{35}S]-GSH remained low, but increased slightly from 5 to 8 nmol mg^{-1} protein over an 8 h period. This is in contrast to the *t*-HP treated cells where the level of radioactivity associated with protein increased to 15 nmol mg^{-1} protein within 4 h of treatment and to 20 nmol mg^{-1} protein after 8 h. To ensure that the radioactivity in the protein preparations detected by scintillation counting was due to thiolation with [^{35}S]-GSH, duplicated protein extracts from *t*-HP elicited cells were treated with 5 mM DTT for 15 min before precipitation and counting. Treatment of the protein extract with DTT reduced the level of protein bound radioactivity to around 5 nmol mg^{-1} protein. This level remained constant throughout the treatment period and was slightly less than the level detected in the water treated arabidopsis protein extracts. This experiment confirmed that the increase in [^{35}S]-labelling of the proteins observed following *t*-HP treatment was due to mixed disulphide formation rather than incorporation into newly synthesised proteins. Controls carried out whereby the precipitated protein was incubated with DTT to remove the protein bound GSH resulted in a reduction in protein bound GSH to below levels detected in the water treated arabidopsis cell cultures (figure 5.2) suggesting that most of the counts were due to covalently bound [^{35}S]-GSH rather than non-specific L-[^{35}S]cysteine or [^{35}S]-GSH.

5.2.2.2 Changes in Thiol Pools Following Oxidative Stress in Arabidopsis Cell Culture

The treatment used to induce oxidative stress in arabidopsis cell cultures differed from the yeast cell wall elicitor used in alfalfa in that *t*-HP produced an oxidative stress directly rather than relying on the plants natural defences to produce ROS. It was therefore necessary to determine how the thiol distribution, in the form of GSH, GSSG and protein-bound GSH, varied in response to oxidative stress.

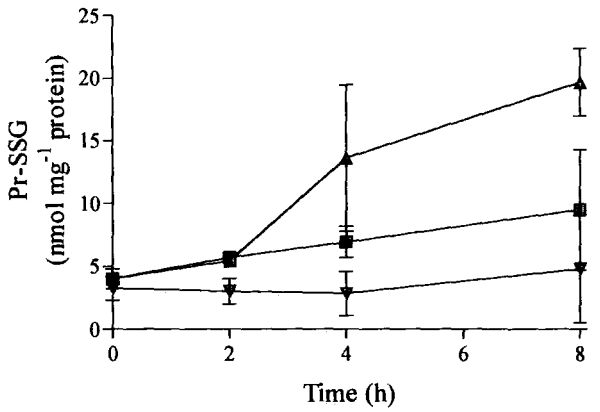

Figure 5.2 Protein thiolation as determined with ^{35}S-labelling in Arabidopsis cell cultures following treatment with 1 mM *t*-HP.
Protein extracts from *t*-HP treatments were radioassayed by precipitation either directly (▲) or after a treatment with DTT to release disulphide bound radiolabel (▼). Radioactivity incorporated into the proteins from control (water) treatment were determined without DTT pre-treatment (■). Data are mean of 2 samples with error bars showing the variation between replicates. In the instance where no error bars are visible, the variation lies within the extent of the graphical symbol.

		Total GSH (nmol g^{-1}FW)		**Percentage of Total GSH Pool**					
Treatment	**Time (h)**	**Ave**	**SD**	**GSH**		**GSSG**		**Pr-SSG**	
				Ave	SD	Ave	SD	Ave	SD
Control	0	222	70	84.0	30.8	14.5	0.7	1.4	1.1
	2	182	0.2	87.8	1.7	10.9	1.8	1.3	0.1
	4	232	6	86.5	0.3	11.1	2.4	2.4	1.6
	8	252	19	86.3	5.4	12.3	2.0	1.3	0.9
t-HP	0	222	70	84.0	30.8	14.5	0.7	1.4	1.1
	2	185	6	76.1	1.0	20.5	2.0	3.4	0.8
	4	325	92	75.5	26.2	19.8	2.2	4.7	1.1
	8	248	11	64.6	1.2	22.0	3.1	13.5	4.3

Table 5.2 Distribution of GSH in arabidopsis cell cultures following treatment with water (control) or 1 mM *t*-HP.
Arabidopsis cell cultures were labelled with 25 μ Ci L-[^{35}S]cysteine in the presence of cycloheximide for 4 h before elicitation. The GSH content of each pool was determined as described in materials and methods. Values are the average of duplicate experiments.

Arabidopsis cell cultures (10 ml) were labelled with 25 μCi L-[^{35}S]cysteine to allow quantification of the protein bound thiol pool over an 8 h period. The soluble thiols were extracted in 100 mM Tris-HCl, pH 7.4 with or without NEM, derivatised by mBBr and the bimane derivatives separated by HPLC. The fraction corresponding to the bimane derivatives was collected and the level of incorporated L-[^{35}S]cysteine determined through scintillation counting to determine specific activities of labelling. GSH metabolism appeared to be significantly altered during *t*-HP-induced oxidative stress (table 5.2). Following treatment with *t*-HP the concentration of total GSH after 4 h was 325 nmol g^{-1} FW as compared with 232 nmol g^{-1} FW in the water treated arabidopsis culture. However, the total GSH pool in the *t*-HP treated cells then declined after 4 h and within 8 h, the total GSH pool was not significantly greater than that determined in the control cultures. There were, however, differences in the distribution of GSH in the different pools following *t*-HP treatment. The proportion of GSSG was initially quite high, accounting for 14 % of the total GSH pool. The proportion of GSSG decreased to around 10 % when treated with water over the 8 h period. In the presence of *t*-HP, the proportion of GSSG increased to 20 % within 2 h and remained around 20 % over the following 6 h. The proportion of GSH which formed mixed disulphides with protein thiols also increased in the presence of *t*-HP. Initially Pr-SSG accounted for 1.4 % of the total GSH pool but rose to 3.4 % within 2 h and continued to rise, accounting for over 10 % of the GSH pool after 8 h. The level of Pr-SSG in the water treated cell cultures remained around 1 % to 2 % of total GSH throughout. Overall, reduced GSH accounted for around 85 % of the total GSH pool in the water treated cultures and following treatment with *t*-HP, dropped to 65 % with the decline due equally to a repartitioning into the GSSG and Pr-SSG pools. Although to ensure a greater level of confidence in the findings, a greater number of replicates should be performed.

5.2.3 Turnover of Protein Bound GSH in Arabidopsis

To determine whether protein bound GSH was turned over during oxidative stress, a pulse-chase experiment was conducted. The GSH pool in Arabidopsis cell cultures was labelled with L-[^{35}S]cysteine in the presence of cycloheximide for 4 h before treating with sterile distilled water or 1 mM *t*-HP for up to 8 h. During the 'chase' experiment, the arabidopsis cell cultures were transferred to fresh media supplemented with 1 mM non-radioactive cysteine to 'chase' out the L-[^{35}S]cysteine incorporated GSH 4 h after treatment with *t*-HP.

Initial labelling of the GSH with L-[^{35}S]cysteine revealed that following treatment with either water or *t*-HP, the level of radioactivity incorporated into soluble GSH increased over time in terms of specific activity of labelling (table 5.3). Following oxidative treatment, the specific activity of incorporation was slightly greater than detected in the control cell cultures, suggesting that GSH may have been turned over at a greater rate during the stress treatment. Upon transfer to fresh media supplemented with 1 mM cysteine, the specific activity of [^{35}S]-GSH reduced (table 5.3), with the added non-radiolabelled cysteine 'chasing out' L-[^{35}S]cysteine during GSH turnover.

As L-[^{35}S]cysteine was 'chased out' by the non-labelled cysteine it was also possible to determine the turnover rate of protein bound GSH. In the cell cultures treated with water, protein thiolation with [^{35}S]-GSH was found at 5 nmol mg^{-1} protein, increasing to 8 nmol mg^{-1} protein after 8 h (figure 5.3). Following transfer to fresh media supplemented with cysteine, the level of L-[^{35}S]cysteine labelled protein bound GSH decreased by half over the 4 h chase period. This suggested that GSH bound to protein was turned over with a half life of around 4 h under control conditions. By calculating from the specific activity of the protein bound GSH at 4 h it was possible to calculate a tentative turnover rate. It was estimated that in water treated cell cultures, between 1 and 2 nmol of GSH mg^{-1} protein h^{-1} underwent dethiolation through turnover. When treated with *t*-HP,

Continuous labelling of GSH pool with L-[^{35}S]cysteine			Pulse-Chase Labelling		
Time (h)	Control (dpm nmol^{-1})	*t*-HP (dpm nmol^{-1})	Time (h)	Control (dpm nmol^{-1})	*t*-HP (dpm nmol^{-1})
0	323	323	4	876	729
2	201	359	5	624	571
4	242	304	6	244	368
8	581	945	8	176	330

Table 5.3 Specific activity of L-[^{35}S]cysteine incorporated into GSH in Arabidopsis cell cultures:

Arabidopsis cell cultures labelled with 25 µCi L-[^{35}S]cysteine in the presence of cycloheximide were treated with water (control) or 1 mM *t*-HP (*t*-HP). Cells were harvested at the specified time point (= continuous labelling) with one set of arabidopsis cell cultures transferred to fresh media containing 1 mM cysteine after 4 h of treatment with either water or *t*-HP and harvested at the specified time points (= pulse labelling). Data are a mean of duplicate cell cultures.

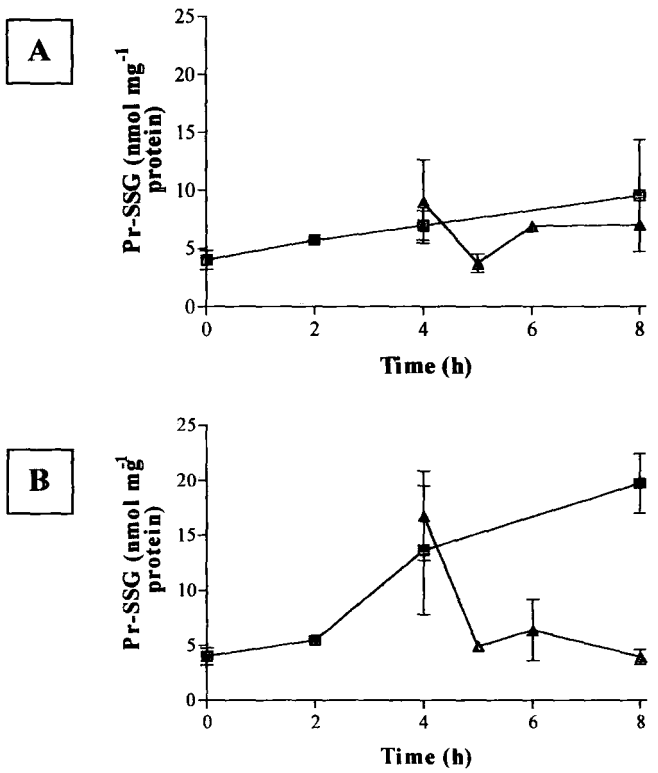

Figure 5.3. Pulse-chase experiment to determine the turnover of protein bound [^{35}S]-GSH in Arabidopsis cell cultures.

Arabidopsis cell cultures were incubated with 25 μCi L-[^{35}S]cysteine for 4 h prior to treatment (t = 0) with A, water, or B, 1 mM *t*-HP. Cell cultures were then incubated for up to a 8 h in the presence of the radiolabel (= continuous labelling; ■) or a set of arabidopsis cell cultures were labelled for 4 h and transferred to fresh media supplemented with 1 mM unlabelled cysteine (= pulse-chase labelling; ▲) to remove the L-[^{35}S]cysteine from the endogenous GSH pool. Error bars refer to the standard deviation of the values.

protein thiolation corresponded to 13.7 nmol GSH mg^{-1} protein within 4 h, increasing to nearly 20 nmol mg^{-1} protein after 8 h. When transferred to fresh media containing cysteine, the level of thiolation detected decreased down to 5 nmol mg^{-1} protein within 1 h (figure 5.3), a protein bound GSH turnover rate of 15 nmol mg^{-1} h^{-1}. The level of protein bound [^{35}S]-GSH in the arabidopsis cell cultures incubated with cysteine almost returned to the basal level (5 nmol GSH mg^{-1} protein) within 1 h. Subsequent levels of protein bound [^{35}S]-GSH incubated with cold cysteine were maintained around 5 nmol GSH mg^{-1} protein. This showed that during oxidative stress, the turnover rate proceeded at a rate fast enough to turnover most protein bound [^{35}S]-GSH within 1 h.

5.2.4 *In-vivo* Thiolation in Arabidopsis Cell Cultures

To identify thiolated proteins in Arabidopsis it was necessary to identify individual polypeptides which formed disulphides with [^{35}S]-GSH. Arabidopsis cell cultures (10 ml) were labelled with 25 μCi L-[^{35}S]cysteine for 4 h in the presence of cycloheximide and treated with *t*-HP for 8 h. The *in-vivo* labelled protein extracts thiolated with [^{35}S]-GSH were then separated by SDS-PAGE and fluorographed on X-ray film. As was found in alfalfa cell cultures, *in-vivo* labelling of the thiol pools with L-[^{35}S]cysteine resulted in low incorporation of L-[^{35}S]cysteine into the [^{35}S]-GSH which had formed mixed disulphides with protein. The resulting fluorograph did not identify any specific proteins (data not shown) suggesting an alternative approach was required.

5.2.5 *In-vitro* Thiolation of Arabidopsis Protein Extracts with [^{35}S]-Glutathione

In order to visualise thiolated proteins, the specific activity of the [^{35}S]-GSH needed to be increased. This was possible by synthesising [^{35}S]-GSH using endogenous *E. coli* GSH synthetase as described in the previous chapter.

Synthesised [^{35}S]-GSH could then be incubated directly with the crude Arabidopsis protein extract in the presence or absence of the oxidative stress *in-vitro* to thiolate susceptible proteins.

To ensure that the assay conditions devised for *in-vitro* thiolation of alfalfa protein extracts were also suitable for arabidopsis protein extracts, the conditions for *in-vitro* thiolation were optimised using protein extracts from 4 day old cell cultures was completed.

Protein extracts the exposed to *t*-HP for 0, 5, 10 or 15 min in the presence of [^{35}S]-GSH. For controls *t*-HP was omitted. In all cases, NEM was added to the extracts at the end time-point to prevent artefactual thiolation. To confirm that radiolabelling was due to thiolation, protein mixed-disulphides were reduced by adding DTT to the [^{35}S]-GSH labelled protein extracts. In the presence of *t*-HP, protein thiolation *in-vitro* proceeded in a time-dependent manner (figure 5.4). In the preparations where thiolation was prevented by addition of NEM at time zero, a 15 kDa radiolabelled polypeptide also observed along with a faint band around 40 kDa. These polypeptides were recalcitrant to dethiolation by DTT and it was concluded that the basal labelling of proteins due to the presence of radiolabelled proteins from derived the biosynthesis of the [^{35}S]-GSH used to carry out the labelling. In the absence of *t*-HP induced oxidative stress, very few proteins were thiolated after 15 min, although labelled polypeptides of 15 kDa, 37 kDa and 39 kDa were visible. In part, these proteins were labelled due to the presence of contaminating [^{35}S]-labelled polypeptides from the treatment preparation. However, it is also reasonable to assume that several Arabidopsis proteins will contain reactive cysteinyl groups which will readily undergo thiolation under basal conditions. Within 5 min of the oxidative stress, proteins with molecular masses of 30 kDa, 37 kDa, 39 kDa, 55 kDa and 80 kDa were selectively thiolated. Within 10 min, the continual exposure to the oxidative stress led to the bands that had been detected after 5 min becoming increasingly intensely labelled with several new reactive polypeptides appearing. These included the low molecular weight

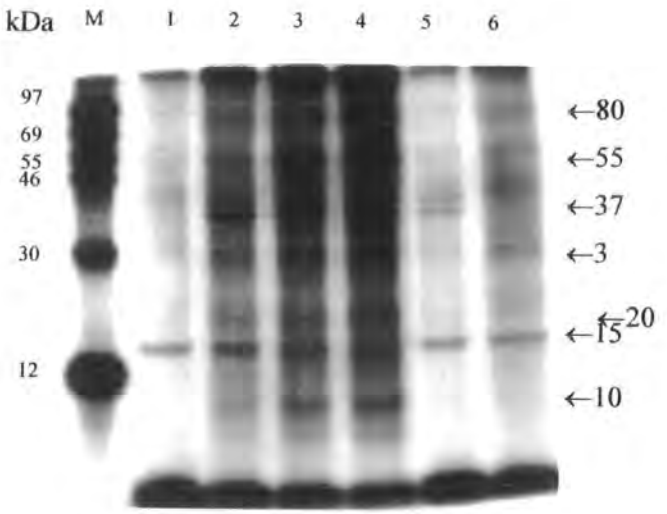

Figure 5.4. *In-vitro* thiolation of a crude Arabidopsis protein extract treated with [^{35}S]-GSH in the presence of 1 mM *t*-HP.

Radiolabelled polypeptides were separated by non-reducing SDS-PAGE and identified by fluorography using X-ray film. Lane 1, reaction terminated immediately; lane 2, terminated after 5 min; lane 3, terminated after 10 min; lane 4, terminated after 15 min; lane 5, labelling obtained in the presence of [^{35}S]-GSH but *t*-HP omitted over 15 min; lane 6, thiolated for 15 min in the presence of *t*-HP followed by incubation with 5 mM DTT for 10 min. Each lane was loaded with 30 μg protein.

polypeptides at 10 kDa and 20 kDa. After 15 min of *t*-HP treatment, the intensity of labelling of multiple proteins further increased. After 15 min of treatment the intensity of labelling of multiple polypeptides was further increased, though the resolution of individual polypeptides was not improved. By labelling a number of proteins in the Arabidopsis cell-free extract *in-vitro*, it was shown that thiolation was a major response to direct oxidative stress in arabidopsis cell free extracts.

5.2.6 Analysis of *In-vitro* Thiolation of Arabidopsis Proteins with [^{35}S]-Glutathione by 2-Dimensional Gel Electrophoresis

The use of Arabidopsis cell cultures to study protein thiolation was adopted because of the potential advantages of this system for proteomic studies. The proteomic approach using the MALDI-TOF mass spectrometer would allow individual proteins thiolated in response to oxidative stress to be tryptically digested and the mass of the peptide fragments matched to a coding sequence. In order for this technique to be used, individual proteins need to be resolved to homogeneity to minimise protein contamination. To achieve this, Arabidopsis protein extracts were separated on the basis of both their pI by iso-electric focusing (IEF) gel electrophoresis, as well as by their molecular masses by SDS-PAGE.

Crude protein extracts from arabidopsis cell cultures were thiolated with [^{35}S]-GSH for 15 min *in-vitro* as described previously. Proteins were then acetone precipitated and washed twice in 40 % (v/v) acetone to reduce the number of compounds present in the protein extraction buffer that would interfere with the iso-electric focusing resulting in decreased resolution. The acetone precipitated protein extracts were resuspended in a non-reducing loading buffer and incubated overnight on an iso-electric focusing gel strip in the presence of ampholytes. The protein extracts immobilised in the IEF gel were then separated on the basis of pI by IEF-gel electrophoresis. The focused IEF strips were then transferred to a SDS-PAGE apparatus and separated on the basis of their molecular masses. The

polypeptides were visualised with coomassie-blue and [^{35}S]-GSH thiolated polypeptides, identified by fluorography following exposure to X-ray film for 3-5 days. The identity of radiolabelled polypeptides could then be cross-referenced to the coomassie stained protein.

Separation of arabidopsis total protein extract following *in-vitro* thiolation with non-radioactive GSH demonstrated the greater polypeptide resolution obtainable following 2D gel electrophoresis (figure 5.5 A). Over 100 polypeptides were visualised following coomassie staining with the majority of polypeptides in the acidic pI range pH 4 to pH 7. After optimising the 2D electrophoresis system with the GSH thiolated crude protein extracts, arabidopsis crude protein extracts were then thiolated *in-vitro* with [^{35}S]-GSH in the presence of *t*-HP. By comparison of polypeptides labelled with [^{35}S]-GSH in the gel with the polypeptide location in the coomassie stained gel it was possible to identify proteins which had undergone thiolation.

Two techniques were used to visualise thiolated proteins after labelling with [^{35}S]-GSH. Following separation by electrophoresis, the gels were fixed with acid, washed in fluorographic reagent and dried, allowing the radioactive labelling to be visualised following exposure to X-ray film. Alternatively proteins were transferred to a nitrocellulose membrane and protein bound [^{35}S]-GSH detected using a phosphorimager.

Unfortunately, when transferred to a nitrocellulose membrane and visualised under the phosphorimager, no spots attributable to thiolated proteins could be visualised (data not shown), possibly due to the low levels of transferred radiolabelled protein. Instead the protein extract was separated on the 2D gels and the [^{35}S]-GSH thiolated proteins visualised following exposure of the dried gel to X-ray film. When developed, the exposed film had many spots determining the location of thiolated polypeptides (figure 5.5 B).

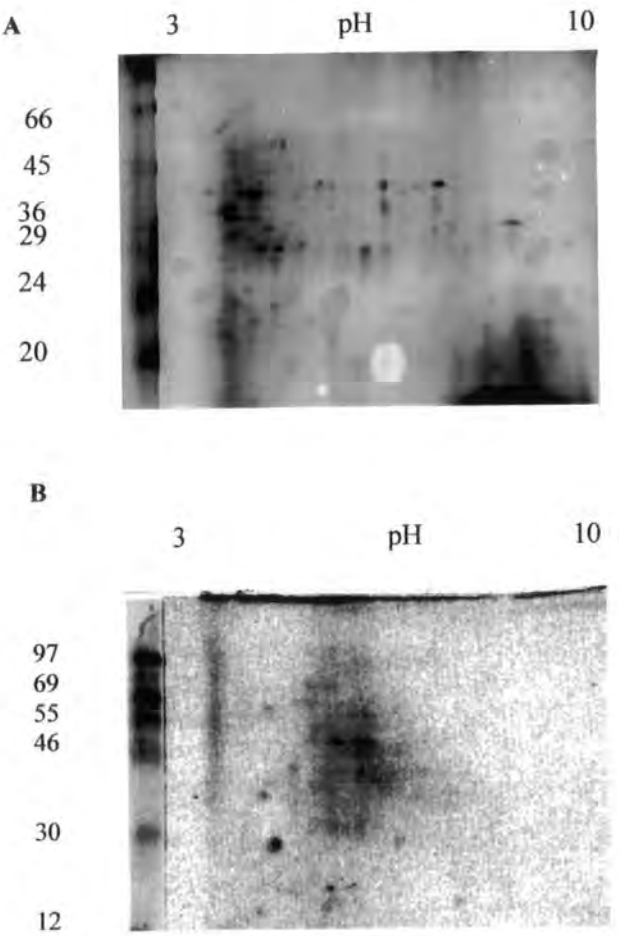

Figure 5.5. Separation of *in-vitro* thiolated Arabidopsis protein extract by 2D electrophoresis.
A, Protein extract thiolated by non-radioactive GSH in the presence of *t*-HP, visualised by silver staining. B. Arabidopsis protein extract thiolated with [^{35}S]-GSH in the presence of *t*-HP. Radioactive polypeptides were visualised by fluorography.

In order to visualise the [^{35}S]-GSH thiolated polypeptides, the gels were initially fixed in acetic acid and methanol and the gels then equilibrated in fluorographing reagent and dried prior to exposure to X-ray film. The changes in gel size on drying made it difficult to align radioactive thiolated protein with specific stained polypeptides. This was found to be a major limitation to using MALDI-TOF analysis of proteins resolved by 2D gel electrophoresis. It would have been most desirable to identify polypeptides directly from the [^{35}S]-GSH labelled gel, however, the fluorgraphy would interfere with tryptic digestion and MALDI-TOF analysis.

Although the technique of sequence analysis following 2D gel electrophoresis has the potential for identification of thiolated proteins, time constraints meant that only preliminary studies were performed using thiolated proteins resolved on 2D gels (figure 5.5B).

5.2.7 Thiolation of Specific Sub-sets of Proteins

The potential for thiolation of a specific family of proteins in arabidopsis was investigated. Glutathione *S*-transferases (GSTs) were selected as they are induced during oxidative stress (Polidoros and Scandalios, 1999) and many contain cysteine residues within and around to the active site region (Thom *et al*, 2001). Also, the activity of one member of the GST family in rat has been shown to be regulated through the formation and reduction of mixed disulphides (Dafre *et al*, 1996).

5.2.8 Purification of GSTs from Arabidopsis

Purification of constitutively expressed GSTs from arabidopsis was achieved by GSH-affinity chromatography as described in section 2.8.1.2. Arabidopsis cell cultures were grown for 4 days following sub-culturing and then harvested. Crude

protein preparations were clarified by centrifugation and applied directly to a GSH-Sepharose column. After washing the non-binding proteins from the column with 20 mM Tris-HCl, pH 7.4, the affinity bound proteins were eluted with 5 mM GSH and assayed for GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) (figure 5.6). Aliquots of the collected fractions were then analysed by SDS-PAGE (figure 5.7). All of the first 4 fractions eluted in the presence of GSH contained a 26 kDa polypeptide which is consistent with the molecular mass of many GST subunits (Dixon *et al*, 1997). The fractions were then combined and desalted by gel filtration chromatography prior to further analysis.

To determine whether or not GSTs were thiolated, the affinity-purified Arabidopsis GSTs were [^{35}S]-GSH thiolated *in-vitro* using the conditions previously described and then analysed by SDS-PAGE and fluorography (figure 5.8). A radiolabelled polypeptide with a molecular mass of 26 kDa was observed since the affinity purified preparation was likely to contain multiple GSTs of similar molecular mass it was of interest to analyse how many polypeptides had become thiolated.

To resolve the GSTs present in the affinity purified preparation the GST extract was thiolated *in-vitro*, initially with non-radioactive GSH, then separated by 2D gel electrophoresis and the polypeptides present visualised by silver-staining. The silver stained gel revealed 5 arabidopsis GST polypeptides with pI between pH 5.5 to pH 7 (figure 5.9).

The affinity-purified preparation was then thiolated *in-vitro* with [^{35}S]-GSH and re-analysed by 2D SDS-PAGE. Of the 5 polypeptides visualised by silver-staining, only 2 subunits of pI around pH 7 were thiolated during oxidative stress (figure 5.10).

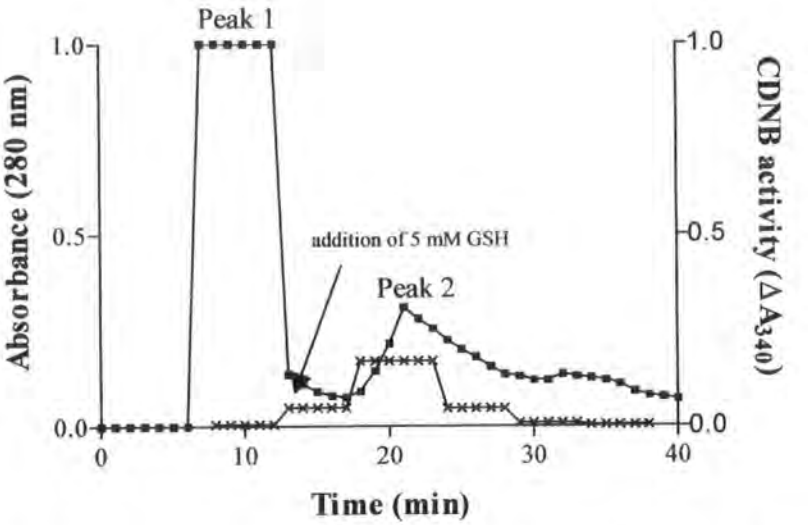

Figure 5.6. GSH affinity chromatography of crude protein extracts from Arabidopsis cell cultures.
Protein extracts in 20 mM Tris-HCl pH 7.4, were loaded on to GSH-Sepharose column at a flow rate of 1 ml min^{-1}. Proteins were eluted initially with 20 mM Tris-HCl, pH 7.4 followed by 20 mM Tris-HCl, pH 7.4 containing 5 mM GSH as indicated. Protein elution was monitored by continuous measurement of the eluate at 280 nm (Fractions of the eluate (5 ml), were collected and assayed for GST activity toward CDNB (-x-).

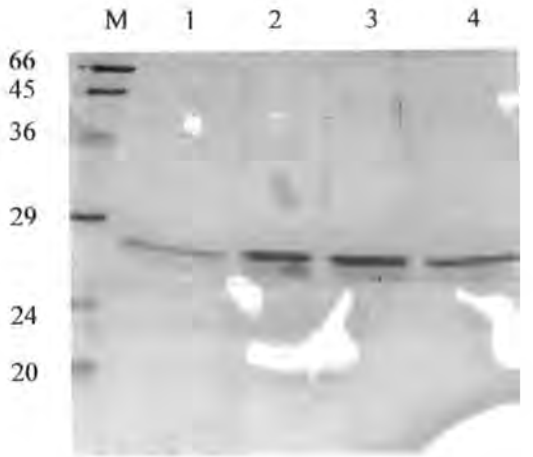

Figure 5.7. SDS-PAGE analysis of affinity purified GSTs from Arabidopsis.
Sequential 5 ml fractions (lanes 1 to 4) were collected following the addition of 5 mM GSH to the elution buffer and a 10 µl sample separated by SDS-PAGE on a 12.5 % gel. Polypeptides present were visualised by staining with GelCode Blue. For reference, lane M shows polypeptides of known molecular mass.

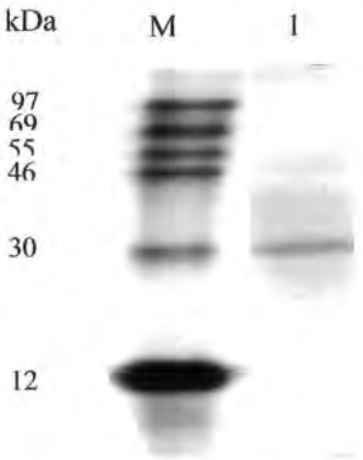

Figure 5.8 *In-vitro* thiolation by [^{35}S]-GSH and *t*-HP of affinity purified Arabidopsis GSTs.
The labelled protein (lane 1) as resolved by non-reducing SDS-PAGE on 12.5 % acrylamide gels with Lane M showing ^{14}C labelled proteins of known molecular mass.

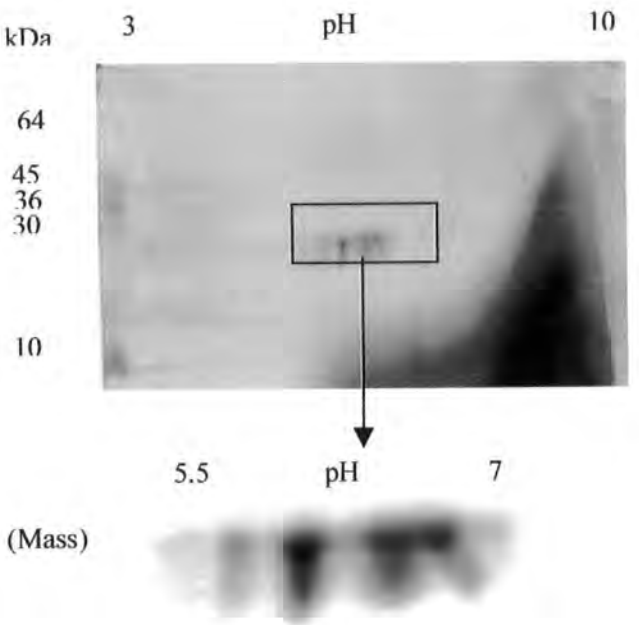

Figure 5.9. Two-dimensional SDS-PAGE analysis of polypeptides recovered following GSH affinity chromatography of crude protein extracts from Arabidopsis cell cultures.
Polypeptides were visualised by silver staining. For clarity, the resolved polypeptides shown in the insert square have been magnified in the image below.

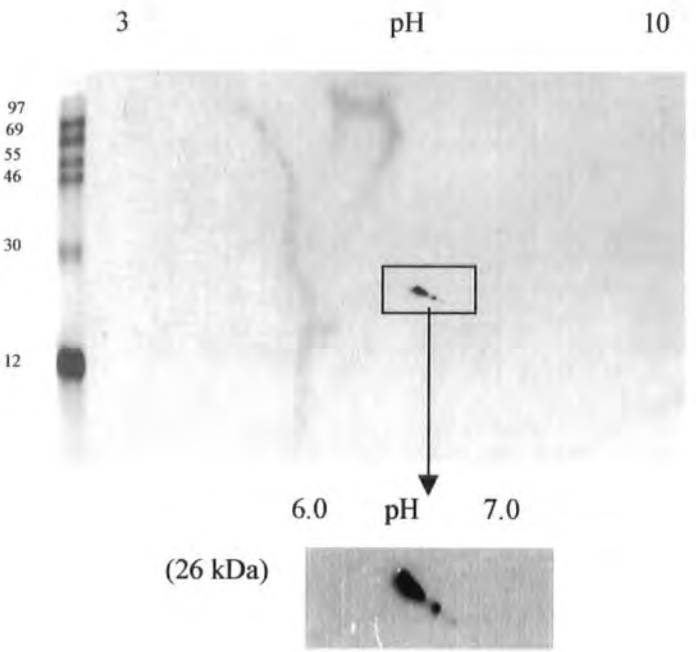

Figure 5.10 Two-dimensional SDS-PAGE analysis of [^{35}S]-GSH labelled Arabidopsis proteins purified by GSH affinity chromatography.
For clarity, the resolved polypeptides shown in the insert square have been magnified in the image below.

5.3 Discussion

Compared with alfalfa cell cultures, the study of thiol metabolism in arabidopsis cell cultures is simplified in several ways. Arabidopsis only contain the one major thiol, GSH, leading to a simplification of the response to oxidative stress. Also, arabidopsis is a model plant species whose genome has been completely sequenced; therefore in the future there is greater amount of data available for protein identification using a proteomic approach. A variety of biotic and chemical elicitors of the defence response were used to assess the defence responses of arabidopsis cell cultures to oxidative stress. After determining that *t*-HP treatment induced PAL activity, a known defence response in plants (Dixon *et al*, 1995) as well as enhancing GSH accumulation, the effect of treatment on protein thiolation was monitored. Following elicitation with *t*-HP, the level of protein bound GSH increased after 2 h and remained high for the following 6 h, accounting for 13 % of the total GSH pool. The difference in the level of protein bound GSH detected in the alfalfa and arabidopsis cell cultures could be due to the different elicitation systems employed. Direct addition of the chemical oxidant to the arabidopsis cell culture may have lead to a more intense oxidative burst then the fungal elicitor in alfalfa cell cultures. An increased level of stress would increase the number of polypeptides susceptible to oxidation leading to thiolation. The levels of protein bound GSH in arabidopsis were similar to those detected in rat hepatocytes treated with diamide (Jung and Thomas, 1996), showing that the thiolation response is an important response in plant and mammalian systems. Indeed, protein sulphydryls have been proposed as an important antioxidant pool, serving as a buffer for the GSSG/2GSH pool as well as having regulatory functions (Schafer and Buettner, 2001; DiSimplico *et al*, 1998).

Unlike previous studies (May and Leaver, 1993) whereby the level of GSH increased 4 fold and remained elevated, following treatment with *t*-HP, the total GSH level decreased relative to the control cultures. However, May and Leaver

(1993) induced mild oxidative stress by the addition of the catalase inhibitor aminotriazole to allow accumulation of H_2O_2 in the cytosol. The mild treatment may induce a different response from the direct application of *t*-HP.

Protein thiolation accounted for a significant proportion of the GSH pool, even though it was not possible to visualise thiolated proteins labelled *in-vivo* following SDS-PAGE separation. To overcome this problem, *in-vitro* thiolation assays were performed on arabidopsis cell free extracts using pre-synthesised [^{35}S]-GSH of high specific radioactivity. Separation of the polypeptides on the basis of molecular mass alone showed it was possible to see that many arabidopsis proteins were thiolated in the presence of oxidative stress. Similar to the observations using alfalfa cell culture protein extracts, thiolation of arabidopsis cell culture protein extracts was dependent on the exposure time to oxidative stress. The difference between proteins in their susceptibility to thiolation was apparent during the time-course. Several proteins were thiolated within 5 min of oxidative stress whereas others were only thiolated 10 or 15 min after exposure to oxidative stress. The differing sulphydryl susceptibility to oxidative stress leading to thiolation could relate to the local environment surrounding the protein sulphydryl group and sulphydryl accessibility (DiSimplico *et al*, 1998). In rat blood haemoglobin, one of the three sulphydryl groups available for *S*-thiolation was 20 times more reactive than the other two cysteines as determined by DTNB titration. Although the pK_a of the sulphydryl influences the susceptibility to oxidation, the location of the sulphydryl group is important. The reactive sulphydryl has also been shown to be thiolated during oxidative stress, thus steric factors influence the susceptibility towards oxidation (DiSimplico *et al*, 1998). These conclusions can help to explain the time-dependency of thiolation observed during *in-vitro* thiolation of arabidopsis protein extracts.

By changing the plant system to arabidopsis, it was hoped that identification of thiolated proteins would be simplified through the use of MALDI-TOF-MS. Due to time constraints, this approach did not lead to the identification of any of the

thiolated proteins of Arabidopsis, though preliminary results of 2D-gel separation was encouraging. To achieve the level of protein separation required for the automated process of protein detection and analysis by the MALTI-TOF-MS, it would be preferable to separate the polypeptides on large format gels. Alternatively, separation on narrow range i.e. single pH unit IEF strip would increase separation of polypeptides over a certain narrow pH range would facilitate polypeptide extraction and analysis where the proteins have similar pI's.

Instead, a directed proteomics approach was employed whereby the susceptibility of arabidopsis GSTs to thiolation during oxidative stress was investigated. By purifying GSTs from cell suspension cultures and separating by 2D electrophoresis, it was possible to demonstrate that at least 2 GST isoforms were thiolated during oxidative stress. It is still unclear as to the role of protein thiolation of GSTs. Thiolation may protect the GST from oxidative stress (Cotgreave and Gerdes, 1998). Alternatively, thiolation may provide a regulatory mechanism as proposed for rat liver microsomal GST (mGST) in which the protein is only catalytically active when GSH forms a mixed disulphide with the active-site cysteine (Dafre *et al*, 1996). To investigate the role of protein thiolation under conditions of oxidative stress in GSTs further, it was concluded that purified GSTs were required.

6.0 Effect of *S*-Thiolation on Activity of Members of the Glutathione Transferase (GST) Superfamily

6.1 Introduction

The importance of protein thiolation as a response to oxidative stress in plants was suggested by the number of Arabidopsis polypeptides thiolated *in-vitro* by [^{35}S]-GSH in the presence of *t*-HP *in-vitro*.

Specific examples of proteins whose activities are regulated by oxidative stress via thiolation are scarce (Klatt and Lamas, 2000). Modulation of the cellular phosphorylation state protein tyrosine phosphatase-1B (PTP-1B) is believed to be regulated via ROS-induced thiolation of the active site cysteine residue (Barrett *et al*, 1999). Protein thiolation is also believed to be of regulatory significance throughout the cell cycle during normal metabolism (Schaffer *et al*, 2001). Protein thiolation associated with the protection of sulphydryl groups from oxidative modification often results in a loss of associated enzyme activity (Cotgreave and Gerdes, 1998). However, one known exception is the thiolation of a rat liver microsomal glutathione transferase (mGST) which has been shown to be activated upon thiolation (Dafre *et al*, 1996).

The complex interactions between amino-acid residues mean that it is not possible to determine the susceptibility of individual sulphydryl groups to thiolation on the basis of sequence information alone. Since our studies with Arabidopsis cell cultures had shown that GST-like proteins can undergo thiolation, it was decided to assess the susceptibility of these enzymes to thiolation and monitor the effect thiolation had on catalytic activity. Individual recombinant GSTs, cloned from arabidopsis, maize and soybean and expressed in *E. coli* DE3 BL21 cells were purified and then exposed to *in-vitro* thiolation. The proteins were then analysed for evidence of mixed disulphide formation and the effect on enzyme activity determined.

By investigating the effects of thiolation on the purified proteins, it was hoped to determine the role potential of thiolation in regulating GST activity and protecting the protein from oxidative stress.

6.2 Results

6.2.1 Expression and Purification of Recombinant Plant GSTs

GSTs were expressed in *E. coli* BL21 DE3 cells and purified by affinity chromatography. Plasmids containing the GST cDNAs cloned from Arabidopsis, Soybean and Maize were obtained from Dr David Dixon and Dr Mark Skipsey (figure 6.1). The GSTs selected for expression were the GST-like dehydroascorbate reductases (DHARs) from Arabidopsis (*At*DHARI, *At*DHARII and *At*DHARIII), the zeta class Arabidopsis *At*GSTZ1 and *At*GSTZ2, the Arabidopsis phi GST *At*GSTF8 and the Arabidopsis lambda GSTs, *At*GSTL1 and *At*GSTL2. In addition, the phi maize *Zm*GSTF1, the tau maize GSTs *Zm*GSTU1 and *Zm*GSTU2 and the soybean glyoxalase 1 (*Gm*Glyox1) were also expressed. In addition, an Arabidopsis esterase D was expressed in *E. coli* (Kordic *et al*, 2002) and the recombinant protein purified by Dr Ian Cummins. The GSTs were either expressed in *E. coli* DE3 BL21 as native proteins or as C-terminal Histidine-tagged fusion proteins. After confirming that the recombinant protein was expressed in the soluble fraction, the GSTs were purified to homogeneity by affinity chromatography.

A typical purification of recombinant GSTs can be seen with *At*GSTF8 applied to a GSH-affinity column (figure 6.2). Following the elution of unbound protein from the column, *At*GSTF8 was eluted by the addition of GSH to the buffer. Fractions containing the affinity purified protein were collected and assayed for GST activity using CDNB as substrate.

```
                0                                                  50
AtDHARI         MALEICVKAAVGAPDHLGDCPFSQRALLTLEEKSLTYKIHLINLSDKPQW
AtDHARII        MALDICVKVAVGAPDVLGDCPFSQRVLLTLEEKKLPYKTHLINVSDKPQW
AtDHARIII       MATAASPLEICVKASITTPNKLGDCPFCQKVLLTMEEKNVPYDMKMVDLS

                51                                                 100
AtDHARI         FLDISPQGKVPVLKIDDKWVTDSDVIVGILEEKYPDPPLKTPAEFASVGS
AtDHARII        FLDISPEGKVPVVKLDGKWVADSDVIVGLLEEKYPEPSLKTPPEFASVGS
AtDHARIII       NKPEWFLKISPEGKVPVVKFDEKWVPDSDVITQALEEKYPEPPLATPPEK

                101                                                150
AtDHARI         NIFGTFGTFLKSKDSNDGSEHALLVELEALENHLKSHDGPFIAGERVSAV
AtDHARII        KIFGAFVTFLKSKDANDGSEKALVDELGALENHLKTHSGPFVAGEKITAV
AtDHARIII       ASVGSKIFSTFVGFLKSKDSGDGTEQVLLDELTTFNDYIKDNGPFINGEK

                151                                                200
AtDHARI         DLSLAPKLYHLQVALGHFKSWSVPESFPHVHNYMKTLFSLDSFEKTKTEE
AtDHARII        DLSLAPKLYHLEVALGHYKNWSVPESLTSVRNYAKALFSRESFENTKAKK
AtDHARIII       ISAADLSLAPKLYHMKIALGHYKNWSVPDSLPFVKSYMENVFSRESFTNT

                201
AtDHARI         KYVISGWAPKVNPVE
AtDHARII        EIVVAGWESKVNA
AtDHARIII       RAETEDVIAGWRPKVMG


                0                                                  47
ZmGSTF1         APMKLYGAVMSWNLTRCATALEEAGSDYEIVPINFATAEHKSPEHLV
ZmGSTU1         MAEEKKQGLQLLDFWVSPFGQRCRIAMDEKGLAYEYLEQDLGNKSEL
ZmGSTU2         MAAAAEVVLLDFWVSPFGQRCRIALAEKGVAYEYREQDLLDKGELLL
GmGlyox1        MAAEPKESPSNNPGLHTTPDEATKGYIMQQTMFRIKDPKVSLDFYFT

                48                                                 94
ZmGSTF1         RNPFGQVPALQDGDLYLFESRAICKYAARKNKPELLREGNLEEAAMV
ZmGSTU1         LLRANPVHKKIPVLLHDGRPVCESLVIVQYLDEAFPAAAPALLPADP
ZmGSTU2         RSNPIHKKIPVLLHAGRPVCESLVILQYIDEAWPDVAPLLPKDDPYA
GmGlyox1        SRVLGMSLLKRLDFPEMKFSLYFMGYENTAEAPSNPIDKVVWTFSQK

                95                                                 141
ZmGSTF1         DVWIEVEANQYTAALNPILFQVLISPMLGGTTDQKVVDENLEKLKKV
ZmGSTU1         YARAQARFWADYVDKKLYDCGTRLWKLKGDGQAQARAEMVEILRTLE
ZmGSTU2         RAQARFWADYIDKKIYDSQTRLWKFEGEAREQAKKDLVEVLETLEGE
GmGlyox1        ATIELTHNWGTEFTSDPEFKGYHNGNSEPRGFGHIGVTVDDTYKACE

                142                                                188
ZmGSTF1         LEVYEARLTKCKYLAGDFLSLADLNHVSVTLCLFATPYASVLDAYPH
ZmGSTU1         GALGDGPFFGGDALGFVDVALVPFTSWFLAYDRFGGVSVEKECPRLA
ZmGSTU2         LADKPFFGGGALGFVDVALVPFTSWFLAYEKLGGFSVQEHCPRIVAW
GmGlyox1        RFQNLGVEFVKKPEDGKMKGIAFIKDFTPDGYWIEIFDRKTIGNVTQ

                189
ZmGSTF1         VKAWWSGLMERPSVQKVAALMKPSA                      213
ZmGSTU1         AWAKRCAERPSVAKNLYPPEKVYDFVCGMKKRLGIE           224
ZmGSTU2         AARCRERESVAKAMSDPAKVLEFVQFLQSKFGAK             222
GmGlyox1        TAA                                            191
```

```
                1                                                   47
AtGSTZ1         MANSGEEKLKLYSYWRSSCAHRVRIALALKGLDYEYIPVNLLKGDFT
AtGSTT1         MMKLKVYADRMSQPSRAVIIFCKVNGIQFDEVLISLAKRQQLSPEFK
AtGSTF8         MANEVILLDFWPSMFGMRTRIALREKGVEFEYREEDLRNKSPLLLQM
AtGSTL1         MALSPPKIFVEDRQVPLDATSDPPALFDGTTRLYISYTCPFAQRVWI
AtGSTL2         MAVVESSRVPELDSSSEPVQVFDGSTRLYISYTCPFAQRAWIARNYK

                48                                                  94
AtGSTZ1         QFDSDFKKINPMGTVPALVDGDVVINDSFAIIMYLDEKYPEPPLLPR
AtGSTT1         DINPLGKVPAIVDGRLKLFESHAILIYLSSAFPSVADHWYPNDLSKR
AtGSTF8          NPIHKKIPVLIHNGKPVNESIIQVQYIDEVWSHKNPILPSDPYLRAQ
AtGSTL1         TRNLKGLQDEIKLVPIDLPNRPAWLKEKVNPANKVPALEHNGKITGE
AtGSTL2         GLQNKIELVPIDLKNRPAWYKEKVYSANKVPALEHNNRVLGESLDLI

                95                                                  141
AtGSTZ1         DLHKRAVNYQAMFTSIVLSGIQPHQNLAVIRYIEEKINVEEKTAWVN
AtGSTT1         AKIHSVLDWHHTNLRRGAAGYVLNSVLGPALGLPLNPKAAAEAEQLL
AtGSTF8         ARFWADFIDKKLYDAQRKVWATKGEEQEAGKKDFIEILKTLESELGD
AtGSTL1         SLDLIKYVDSNFDGPSLYPEDSAKREFGEELLKYVDETFVKTVFGSF
AtGSTL2         KYIDTNFEGPSLTPDGLEKQVVADELLSYTDSFSKAVRSTLNGTDTN

                142                                                 186
AtGSTZ1         NAITKGFTALEKLLVNCAGKHATGDEFTIYLADLFLAPQIHGAINRF
AtGSTT1         TKSLSTLETFWLKGNAKFLLGSNQPSIADLSLVCELMQLQVLDDKDR
AtGSTF8         KPYFSGDDFGYVDIALIGFYTWFPAYEKFANFSIESEVPKLIAWVKK
AtGSTL1         KGDPVKETASAFDHVENALKKFDDGPFFLGELSLVDIAYIPFIERFQ
AtGSTL2         AADVAFDYIEQALSKFNEGPFFLGQFSLVDVAYAPFIERFRLILSDV

                187                                                 235
AtGSTZ1         QINMEPYPTLAKCYESYNELPAFQNALPEKQPDAPSSTIXX
AtGSTT1         LRLLSTHKKVEQWIENTKKATMPHFDETHEILFKVKEGFQKRREMGT
AtGSTF8         CLQRESVAKSLPDPEKVTEFVSELRKKFVPE
AtGSTL1         VFLDEVFKYEIIIGRPNLAAWIEQMNKMVAYTQTKTDSEYVVNYFKR
AtGSTL2         MNVDITSGRPNLALWIQEMNKIEAYTETRQDPQELVERYKRRVQAEA

AtGSTZ1                                 229
AtGSTT1         LSKPGLQSKI              245
AtGSTF8                                 219
AtGSTL1         FM                      237
AtGSTL2         RL                      237
```

Figure 6.1 Amino-acid sequences of GSTs and glutathione-dependent enzymes from *Arabidopsis thaliana* (*At*), *Zea mays* (*Zm*) and *Glycine max* (*Gm*) used in thiolation studies.

Cysteinyl residues, potentially available for mixed-disulphide formation are highlighted. The sequence correspond to the dehydroascorbate reductases of Arabidopsis, *At*DHARI, *At*DHARII and *At*DHARIII, Arabidopsis phi type *At*GSTF8, Arabidopsis lambda type *At*GSTL1 and *At*GSTL2, maize tau type *Zm*GSTU1 and *Zm*GSTU2, maize phi type *Zm*GSTF1 and soybean glyoxalase 1, *Gm*Glyox1.

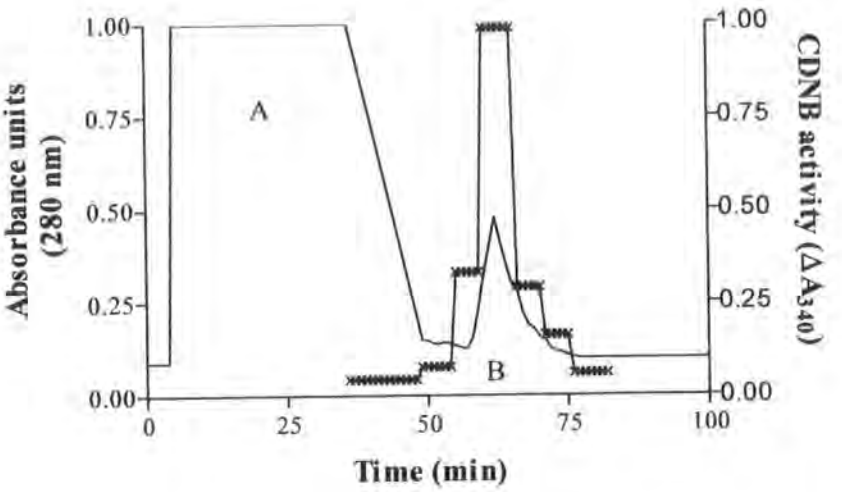

Figure 6.2 Purification of recombinant *At*GSTF8 by affinity chromatography on glutathione-Sepharose.

E. coli DE3 BL21 cells containing *At*GSTF8 plasmid were grown at 37 °C until they reached $O.D._{600} = 0.6$. GST protein expression was then induced by the addition of 1 mM IPTG to the media for 3 h. Following harvesting, cells were disrupted by sonication, the cell free extract precipitated with $(NH4)_2SO_4$. The protein preparation was then desalted, applied to a GSH affinity column and after removing unbound protein (A), the affinity bound protein (B) was recovered by adding GSH to the wash buffer. Elution of protein was monitored at 280 nm (unbroken line) and fraction assayed for GST activity using CDNB as substrate (x-x).

After determining the level of GST activity in the collected fractions, aliquots were analysed by SDS-PAGE, enabling visualisation of the purified protein and confirming the presence of a single polypeptide (figure 6.3). The protein was then precipitated and stored at 4 °C until required. The GSTs were desalted using G25 gel filtration chromatography prior to assaying.

6.2.2. Effect of Oxidative and Alkylating Reagents on Enzyme Activity

To determine the effect of thiolation on enzyme activity, the desalted GSTs were incubated with water (control), nitrosoglutathione (GSNO), *t*-HP alone, GSH alone, *t*-HP and GSH together, NEM or iodoacetamide. After a 5 min incubation, the GSTs from each treatment were assayed for activity (table 6.1).

Of the GSTs assayed, the activity of dehydroascorbate reductase (DHAR) I, II and III was reduced significantly following treatment with the thiol alkylating chemical iodoacetamide and to a lesser extent, with NEM. In particular, the activities of *At*DHARI and *At*DHARII were decreased significantly as compared to the controls. Upon exposure to iodoacetamide, *At*DHARIII activity also decreased compared to the control, although to a lesser extent than either *At*DHARI or *At*DHARII. Treatment of *At*DHARI and *At*DHARIII with NEM also reduced activity compared to the control, although to a lesser extent following treatment with iodoacetamide. *At*DHARII differed in so much as the reduction in activity following treatment with NEM, was as great as the reduction determined following iodoacetamide treatment (table 6.1).

Treatment of *At*DHARI and *At*DHARII with the oxidant *t*-HP, or GSNO led to a reduction in activity compared to the control. However, *At*DHARIII activity was only slightly reduced following treatment with GSNO and was not affected by treatment with *t*-HP. The stability of *At*DHARIII activity in the presence of oxidants was observed following treatment with GSH or *t*-HP and GSH together. *At*DHARI and *At*DHARII activity reduced upon exposure to GSH or *t*-HP and

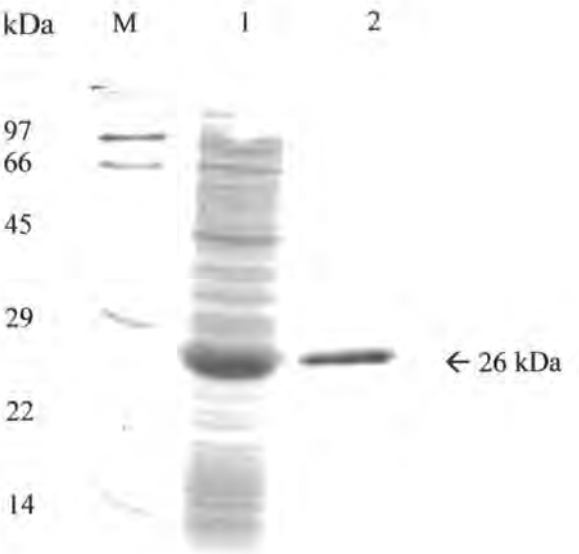

Figure 6.3 SDS-PAGE analysis of recombinant *At*GSTF8.
Lane 1, crude extract from *E. coli* BL21 expressing *At*GSTF8 after induction with 1 mM IPTG for 3 h. Lane 2, *At*GSTF8 following purification using a GSH affinity column. Polypeptides of known molecular mass (kDa) were run in lane M.

		100 % Activity	Activity (% control)						
			Treatment						
Enzyme	Enzyme Activity	nkats mg^{-1} protein	Control	GSNO	*t*-HP	GSH	*t*-HP and GSH	NEM	Iodoacetamide
*Zm*GSTF1	GST	4241	100	124	125	131	93	110	-
*Zm*GSTU1	"	377	100	104	110	117	117	99	-
*Zm*GSTU2	"	1148	100	92	86	99	90	95	-
*At*GSTF8	"	151	100	97	97	103	103	85	-
*At*Theta	"	30	100	81	134	112	101	118	157
*At*Zeta	"	86	100	67	116	109	92	78	82
*Gm*glyox1	glyoxalase	991	100	78	106	104	102	105	-
*At*DHARI	dehydroascorbate reductase	1864	100	71	76	82	85	71	9
*At*DHARIII	"	2415	100	70	71	79	78	10	10
*At*DHARIV	"	1561	100	87	97	102	92	51	43
*At*Theta	GSH peroxidase	186	100	92	87	91	81	92	-
Esterase D	Esterase	87598	100	-	-	-	-	45	5

Table 6.1. Activity of purified recombinant glutathione-dependent enzymes after treatment with sulphydryl modifying agents.
Purified proteins were treated with water (control), oxidant or antioxidant chemicals for 5 min prior to assay. Values are from duplicate assays with the treated protein values represented as a percentage of the control value, (100 %), with the activity of the untreated controls given in nkat mg^{-1} protein. (-) = not determined.

GSH together, although to a lesser extent then determined in the presence of the oxidant compounds *t*-HP or GSNO (table 6.1).

The activity of maize and arabidopsis GSTs was unaffected following treatment with the various oxidising and alkylating reagents. The activities of *Zm*GSTF1 and *Zm*GSTU1 increased slightly upon incubation with GSNO or *t*-HP which suggested that the presence of an oxidant may increase GST activity, however, this increase was also detected when pre-incubated with the antioxidant GSH making it unlikely that the activity increases detected resulted from changes in the cysteinyl redox state. Also the addition of NEM did not result in major changes in activity suggested that reduced cysteinyl residues were not essential for GST activity. The activities of *Zm*GSTU2 and *At*GSTF8 varied very little from the water treated enzyme (table 6.1).

*At*GSTT1 was assayed for GSH peroxidase activity, which was found to be uninhibited by the various chemical treatments. Upon treatment with iodoacetamide or *t*-HP, the GST activity of *At*GSTT1 actually increased by 50 % and 35 % respectively, although the level of catalytic activity detected was very low (29.5 nkats mg^{-1} protein) compared with the other GSTs (table 6.1).

There was no clear effect of incubation with oxidising or reducing reagents with *At*GSTZ1. Following treatment with GSNO, activity decreased by around 30 % and by 20 % when treated with NEM or iodoacetamide. Treatment with *t*-HP, GSH or *t*-HP and GSH had little affect on activity compared to that detected under control conditions (table 6.1).

Glyoxalase I activity remained around the control level (991 nkats mg^{-1} protein) following incubation with the chemicals except when treated with GSNO, which resulted in a loss of approximately 20 % activity. This loss in activity was not mirrored when treated with GSH, *t*-HP or *t*-HP and GSH together, treatments

which would reflect the possible oxidation states of GSH following the decomposition of GSNO (table 6.1).

The esterase D from arabidopsis, which hydrolyses *S*-formyl-glutathione, was found to require a reduced sulphydryl for activity as treatment with iodoacetamide reduced activity by approximately 95 % compared to the control. Treatment with NEM also reduced esterase D activity, although to a lesser extent (table 6.1).

6.2.3 Recovery of Enzyme Activity Following Oxidative Stress

To determine whether thiolation was affording any protection to the protein from oxidative stress, an assay to determine the recovery of enzyme activity was performed. In this instance, *At*DHARI, II and III were each treated with *t*-HP in the presence or absence of GSH. The proteins were then alkyalted with NEM to inactivate any remaining thiol groups. Following treatment with DTT to reduce any bound GSH from the protein, the protein was desalted to remove unreacted NEM and DTT and assayed for DHAR activity. Sulphydryl groups thiolated thiolation treatment (*t*-HP and GSH) should have their activity restored following disulphide reduction by DTT. If however, the sulphydryl residues were not thiolated, the combination of oxidative stress and the addition of NEM, would result in no detectable activity due to the irreversible damage to essential cysteine residues.

Compared to the control protein extracts treated with water, oxidatively stressing the protein in either the presence or absence of GSH reduced *At*DHAR activity in all cases. In the absence of GSH, enzyme activities of *At*DHARI, *At*DHARII and *At*DHARIII reduced to 16 % to 21 % of that observed in the untreated control. However, in the presence of GSH, *At*DHARI and *At*DHARIII activity was only reduced to 40 % and 39 % that determined respectively of the control activity. *At*DHARII was depressed to 32 % of that seen in the control activity, 11 % greater then that determined in the absence of GSH (table 6.2). This assay showed that

Protein	Treatment	Average	Standard Deviation	% control
		Enzyme Activity (nkats mg^{-1} protein)		
*At*DHARI	Control	1745	124	100
	+GSH	700	11	40
	-GSH	307	16	18
*At*DHARII	Control	2250	128	100
	+GSH	713	22	32
	-GSH	473	27	21
*At*DHARIII	Control	1390	72	100
	+GSH	539	25	39
	-GSH	225	10	16

Table 6.2. Loss of DHAR activity following NEM treatment after a pre-incubation with 1 mM *t*-HP in the presence or absence of GSH.
Protein extracts were pre-treated with *t*-HP in the presence (+GSH) or absence (-GSH) of GSH for 15 min prior to the addition of NEM to alkylate any free sulphydryl groups. Excess DTT was then added to reduce protein-mixed disulphides before desalting and assaying for DHAR activity. The values are from duplicate experiments with the standard deviations given.

the presence of GSH gave significant protection of *At*DHAR activity, presumably after thiolating the enzyme.

By comparison, purified Arabidopsis esterase D retained only 40 % of the activity seen in the untreated control when treated with *t*-HP in the absence of GSH and subsequently treated with NEM. However, in the presence of GSH, 94 % of the original activity was recovered (figure 6.4). This suggested the availability of GSH during oxidative stress lends significant protection to enzyme activity presumably by forming mixed disulphides with essential cysteine residues.

6.2.4 *In-vitro* Thiolation of Purified Recombinant GSTs

To monitor the formation of mixed disulphides between the protein sulphydryl and GSH during oxidative stress, purified GSTs were subjected to oxidative stress in the presence of [^{35}S]-GSH *in-vitro* as described previously (chapter 3). Purified proteins (10 μg) were treated *in-vitro* in the presence of 1 μCi [^{35}S]-GSH and *t*-HP and the polypeptides visualised following separation by SDS-PAGE by fluorography. By visualising the formation of radiolabelled polypeptides derived from mixed disulphides it was possible to determine whether or not thiolation had occurred.

Following *in-vitro* oxidative treatment in the presence of [^{35}S]-GSH, the GSTs cloned from *Zea Mays* and soybean (*Glycine max*) were separated by SDS-PAGE. Although the activities of *Zm*GSTF1, *Zm*GSTU1, *Zm*GSTU2 and *Gm*Glyox1 were unaffected by treatment with *t*-HP and GSH, the treatment resulted in the formation of mixed [^{35}S]-GSH-labelled disulphide with *Zm*GSTU1 and *Gm*Glyox1 (figure 6.5). The bands present at 14 kDa and 36 kDa are due to contamination with *E. coli* proteins present in the [^{35}S]-GSH preparation. Analysis of the sequences of *Zm*GSTF1, *Zm*GSTU1 and *Zm*GSTU2 showed they all contained at least 3 cysteine residues within the protein. Following alignment of the GSTs only one cysteine residue was exclusive to *Zm*GSTU1, being 10

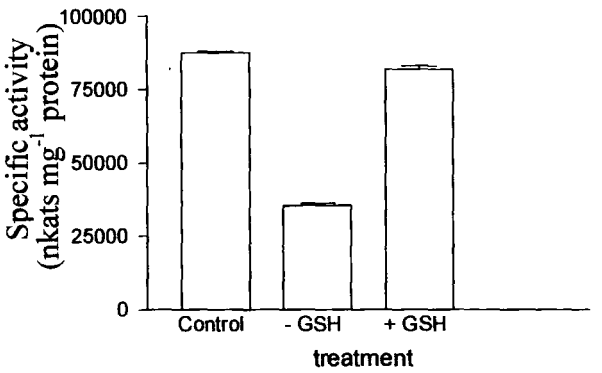

Figure 6.4. Loss of esterase D activity towards *p*-nitrophenol acetate following treatment with 1 mM *t*-HP in the absence or presence of glutathione.
Protein extracts were pre-treated with *t*-HP in the presence (+GSH) or absence (-GSH) of GSH for 15 min prior to the addition of NEM to block any free sulphydryl groups. Excess DTT was then added to reduce the protein-mixed disulphides before desalting and assay. The values are from duplicate experiments with the error bars representing standard deviations.

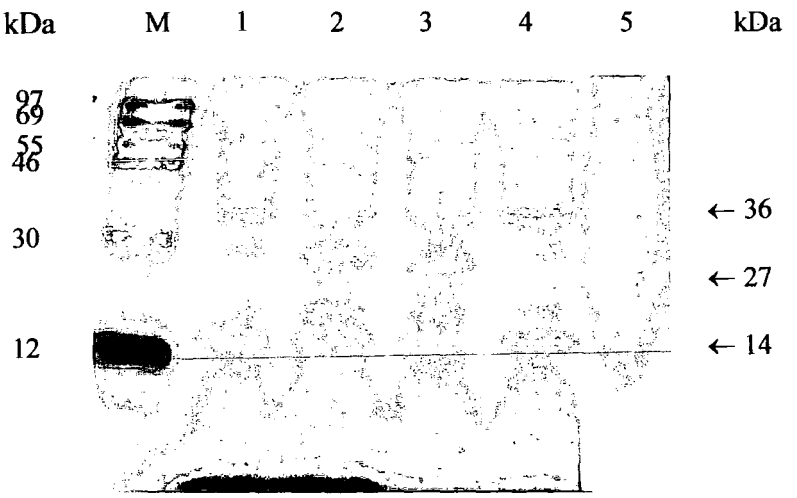

Figure 6.5 Purified recombinant GSTs (10 μg protein) labelled *in-vitro* by treatment with 1 mM *t*-HP in the presence of 1 μCi [^{35}S]-GSH.
Lane 1, *Zm*GSTF1; Lane 2, *Zm*GSTU1, Lane 3, *Zm*GSTU2, Lane 4, *At*GSTT1. lane 5, *Gm*Glyox1. ^{14}C labelled polypeptides of a known molecular mass were run as standards (lane M). The 14 kDa and 36 kDa polypeptides shown correspond to contaminating protein present in the [^{35}S]-GSH preparation used for labelling. The polypeptides indicated around 27 kDa are GST (lane 2) or glyoxalase I (lane 5) subunits.

amino acid residues from the C-terminal end. This C-terminal cysteine may be exposed to thiolation, whereas the other residues are protected or involved in intramolecular disulphides (figure 6.1). *Gm*Glyox1 only contained one cysteine residue which could become thiolated, with the fluorographed SDS-PAGE gel suggesting that *Gm*Glyox1 was susceptible to this derivatisation (figure 6.5).

Following purification, the arabidopsis GSTs, were subjected to *in-vitro* oxidative stress. From the arabidopsis GSTs with known activity, only the *At*DHARs showed any appreciable change in enzyme activity following thiolation treatment (table 6.1). Exposure of the polypeptides to X-ray film revealed that *At*DHARI, *At*DHARII and *At*DHARIII were all thiolated with [^{35}S]-GSH (figure 6.6). *At*GSTL1 and *At*GSTL2 have no known enzyme activity therefore it had not been possible previously to assess whether or not these proteins underwent thiolation. However, *At*GSTL1 and *At*GSTL2 were both thiolated with [^{35}S]-GSH in the presence of *t*-HP, with *At*GSTL2 appearing to be particularly susceptible. The difference in thiolation susceptibility is particularly interesting as *At*GSTL1 and *At*GSTL2 have a high degree of sequence identity and contain only one cysteine residue surrounded by a conserved set of 19 amino acids common to *At*GSTL1 and *At*GSTL2.

*At*GSTF8, *At*GSTT1 and *At*GSTZ1 also underwent thiolation with [^{35}S]-GSH, although to a lesser extent than *At*DHAR or GSTs (figure 6.6). *At*GSTF8 only has one cysteine in its sequence, which may account for its weak thiolation. *At*GSTT1 and *At*GSTZ1 contain 2 and 3 cysteine residues respectively of which one or more may have been thiolated.

Following treatment of the thiolated proteins with DTT, the GST polypeptides were no longer visible following exposure to X-ray film showing that the protein mixed-disulphides with [^{35}S]-GSH formed had been reduced and that the thiolation reaction was freely reversible.

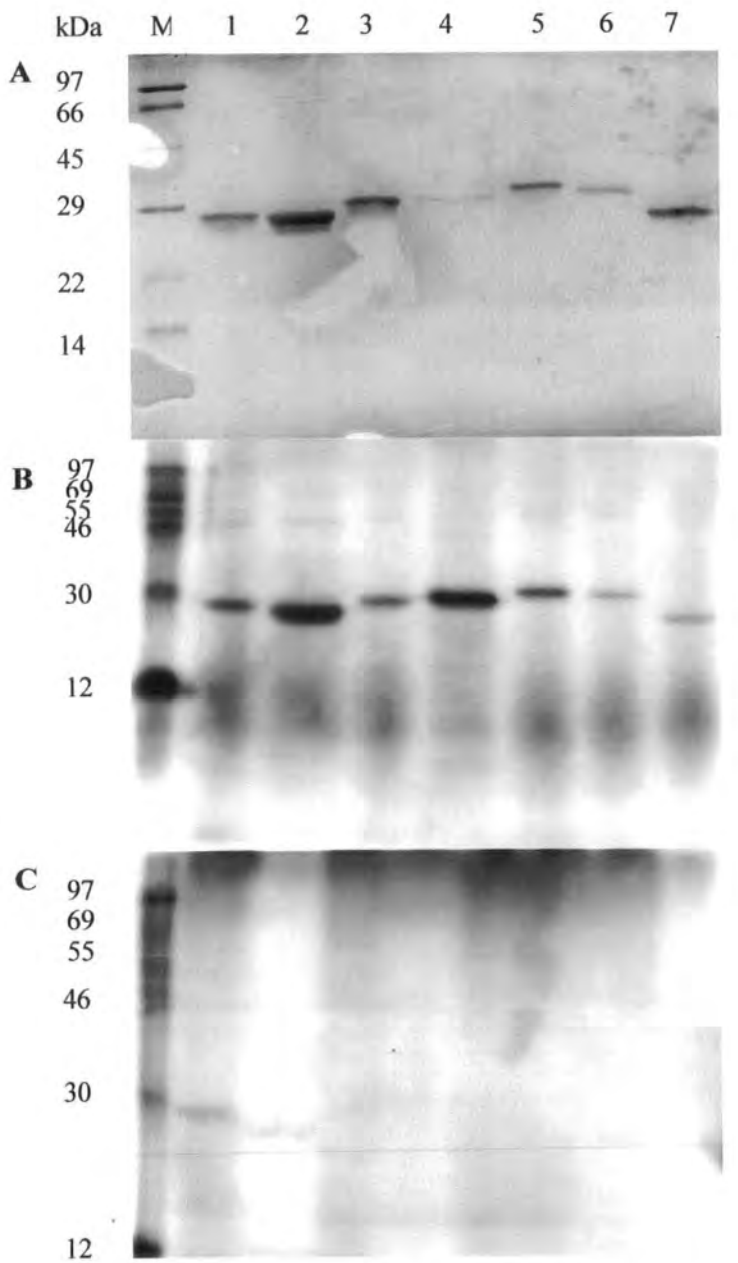

Figure 6.6. SDS-PAGE analysis of purified recombinant Arabidopsis GSTs.
Gel A, Gelcode Blue stained purified proteins. Gel B, purified proteins *in-vitro* thiolated with 1 μCi [^{35}S]-GSH in the presence of 1 mM *t*-HP. Gel C, polypeptides treated as gel B, followed by treatment with 2 mM DTT for 15 min and addition of NEM prior to electrophoresis. Lane 1, *At*DHARI; Lane 2, *At*DHARII; Lane 3, *At*DHARIII; Lane 4, *At*GSTL1; lane 5, *At*GSTL2; lane 6, *At*GSTZ1; lane 7, *At*GSTF8. Molecular mass markers are shown for reference in the left hand lane.

6.2.5. Analysis of Thiolated Proteins by Electro-Spray Ionisation Time-of-Flight Mass-Spectrometry (ESI-TOF-MS)

Several GSTs were identified as being susceptible to thiolation with [^{35}S]-GSH in the presence of *t*-HP by fluorography following SDS-PAGE. However, the number of GSH molecules bound to each of the respective polypeptide molecules was unknown in each case. To determine the number of protein bound GSH molecules, thiolated and non-thiolated proteins were analysed by ESI-TOF-MS.

The protein mass can be accurately determined with ESI-TOF-MS. The sample injected into the ESI-MS forms fine droplets with high charge-densities. The carrier solvent is then evaporated off with the eventual formation of gas phase ions of individual molecules. The quadropole analyser detects the multiply-charged species, in the mass:charge range 500-2000. The data directly obtained is converted (deconvoluted) to the original parent mass using the MaxEntropy software.

Prior to analysis of the GSTs, the ionisation conditions of the mass-spectrometer were determined by analysing the proteins bovine myoglobin and bovine carbonic anhydrase III. This ensured the proteins would be optimally ionised under the set conditions. The mass of myoglobin and carbonic anhydrase III were determined to be 16959 Da and 29029 Da respectively. The mass of the standards obtained showed that the ionisation and mass-spectrometry detection conditions were suitable for the analysis of proteins.

Purified *At*DHARI, *At*DHARII or *At*DHARIII were individually desalted and separated into two samples. One sample was thiolated *in-vitro* by the addition of *t*-HP and GSH whilst the second sample was treated with *t*-HP only, allowing comparison of the native and thiolated molecular masses of the proteins.

ESI-TOF-MS analysis of native *At*DHARI gave a parent mass ion of 24537 Da, 153 Da less than the sequence predicted, suggesting that the N terminal methionine may have been cleaved during post-translational modification in *E. coli*. When *At*DHARI was treated with *t*-HP in the presence of GSH, the difference in mass detected (305 Da) was equivalent to one molecule of GSH binding to *At*DHARI (figure 6.7, A). By comparing the relative signal strength of unmodified *At*DHARI and thiolated protein, it appeared that under the *in-vitro* assay conditions, a large proportion of protein becomes thiolated, assuming both forms were ionised at similar efficiencies.

The molecular mass of native *At*DHARII (24334 Da), also suggested the N-terminal methionine residue was cleaved during processing (figure 6.7, B). The mass increase of 304 Da following thiolation treatment of *At*DHARII demonstrated that one GSH was bound to the protein. Unlike *At*DHARI, the relative signal strength for the native form was greater than the signal strength for the thiolated protein. This may demonstrate a difference in the susceptibility of *At*DHAR cysteinyl residues to oxidative modification.

Similar to *At*DHARI and *At*DHARII, the mass of *At*DHARIII (25128 Da) (figure 6.7, C) suggested that the methionine residue has been cleaved. Following *in-vitro* thiolation, the mass increase (305 Da) was consistent with the binding of one GSH molecule being bound during oxidative stress (25435 Da).

6.2.6 Identification of Thiolated Residues

After quantifying the number of GSH molecules bound to each *At*DHAR during oxidative stress, it was of interest to determine which of the cysteinyl residues were thiolated. *At*DHARI and *At*DHARIII contain 2 cysteinyl residues and *At*DHARIII contains 3 cysteinyl residues (figure 6.1). Thiolated *At*DHARI, *At*DHARII and *At*DHARIII were individually digested with endoproteinase and the resulting peptides fragments analysed by HPLC.

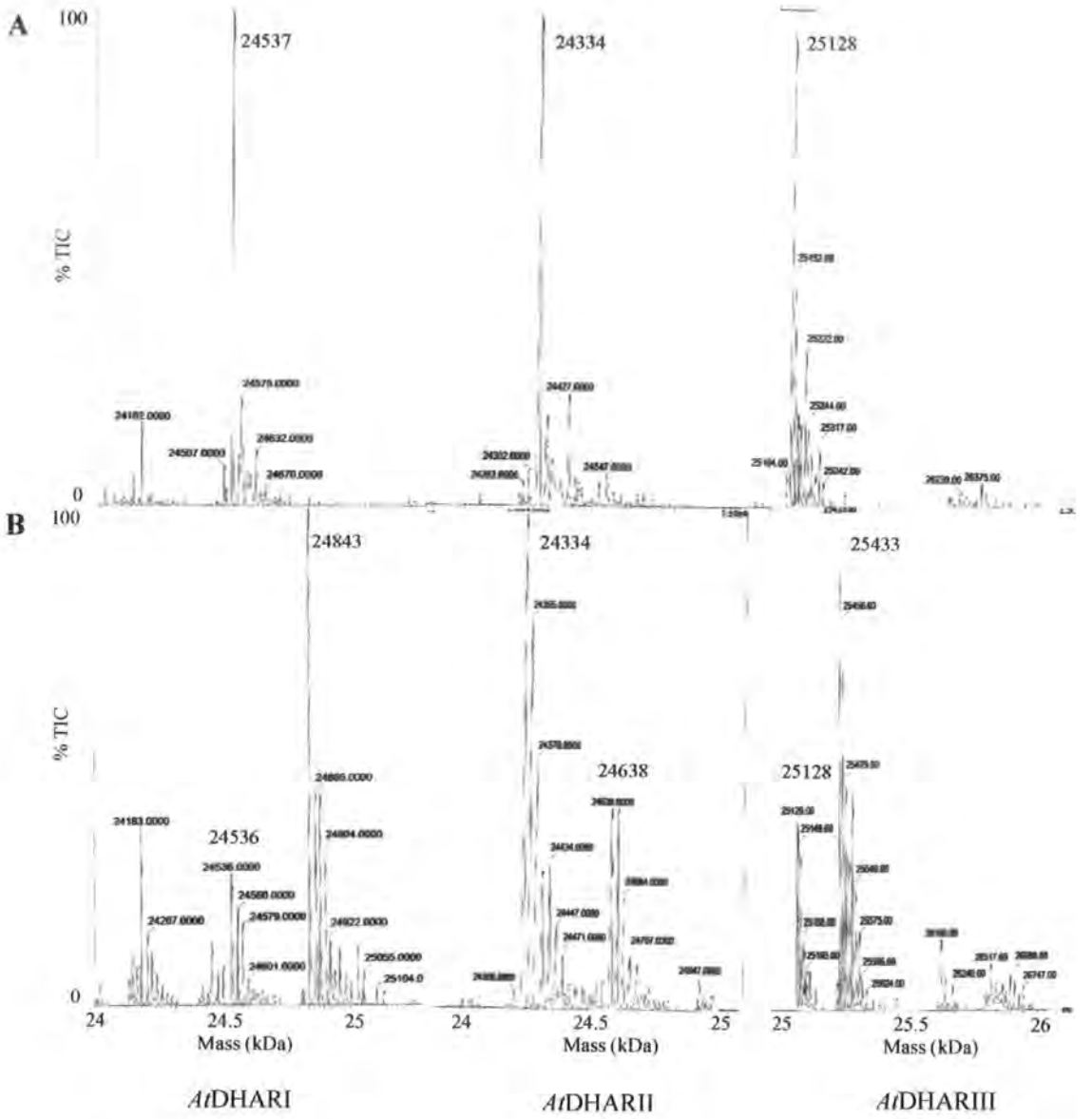

Figure 6.7 ESI-TOF-MS analysis of *At*DHARI, *At*DHARII and *At*DHARIII following treatment with *t*-HP only (A) of with *t*-HP in the presence of GSH.
Protein extracts prepared in acetonitrile and formic acid were analysed directly by ESI-TOF-MS as described in the materials and methods. Data shown represents masses following deconvolution of the multiply charged ion species with the dominant parent mass ion shown in the larger font.

After determining the HPLC run conditions with pure *At*DHARI, *At*DHARII and *At*DHARIII (figure 6.8), the *At*DHARs were denatured by incubation in 5 % acetonitrile at 50 °C for 2 h. Following denaturing, endoproteinase Lys C was added and incubated at 37 °C for 18 h, after which time the reaction was terminated by the addition of 0.1 % TFA. The endoproteinase Lys C (Lys C), cleaves proteins at lysine and minor cleavage at arginine residues (Price, 1996). Cleavage at the lysine and arginine residues digested the protein sufficiently to form fragments predicted to contain only one cysteine residue per fragment in the case of *At*DHARI and *At*DHARII. In contrast, following LysC digestion, Cys_{25} and Cys_{28} in *At*DHARIII were predicted to be present on the same peptide fragment though this would be separated from the peptide containing Cys_{10}.

The protein digests were separated by HPLC using a gradient of TFA and increasing acetonitrile (figure 6.9). The major peak on the elution profiles around 10 min was parent *At*DHARI, *At*DHARII and *At*DHARIII respectively. Separation of the respective peptide digests resulted in approximately 20 UV absorbing peaks (figure 6.9).

Having established conditions for the HPLC resolution of peptide fragments, *At*DHARI, *At*DHARII and *At*DHARIII were thiolated *in-vitro* with [^{35}S]-GSH, prior to Lys C digestion. The [^{35}S]-GSH labelled mixed disulphide was detected by an in-line radioisotope detector following detection of the UV absorbing peaks. After taking into account the delay in radioactivity detection as compared with UV detection, the fragment with the [^{35}S]-GSH bound in *At*DHARI, *At*DHARII and *At*DHARIII can be seen to be eluting as a UV absorbing peak around 13 min (figure 6.9). The resolution of the radioactive peak was far less than that of the peaks detected by UV due to the addition of large volume of scintillation fluid to the eluate required to detect the [^{35}S]-GSH.

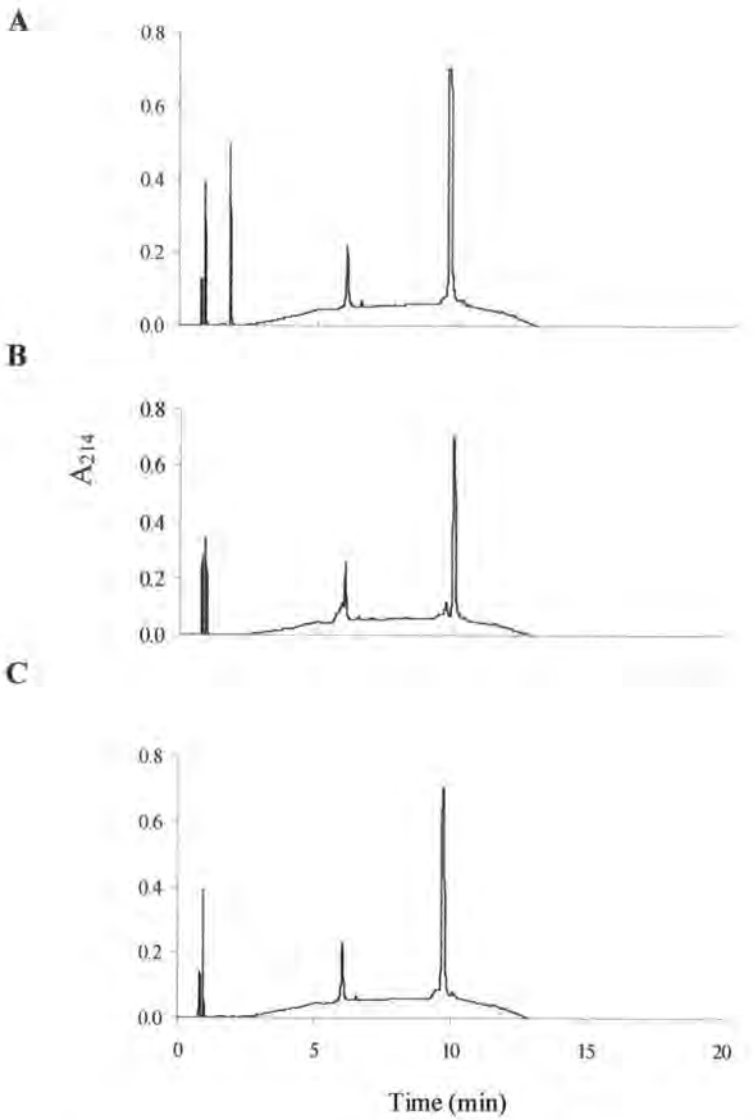

Figure 6.8. Separation of purified *At*DHARI (A); *At*DHARII (B); *At*DHARIII (C) by HPLC.

Protein extracts (10 μg) were injected onto C_{18} reverse-phase HPLC column and eluted with linear increase in acetonitrile concentration. The proteins were detected by monitoring the absorbance at 214 nm.

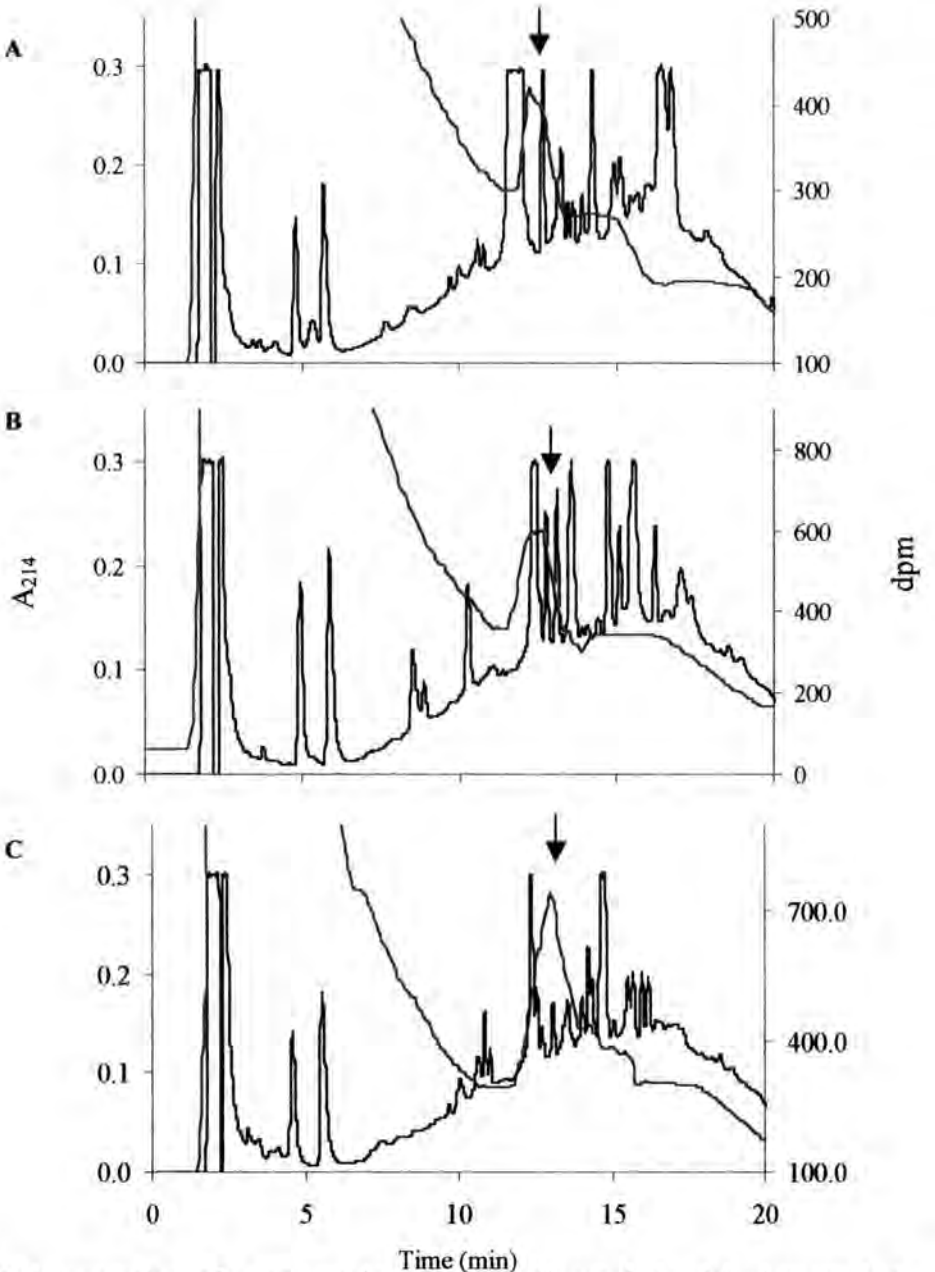

Figure 6.9. HPLC separation of endoproteinase Lys-C digested A, dehydroascorbate reductase (*At*DHAR) I; B, *At*DHARII; C, *At*DHARIII.

Each purified protein was thiolated by [^{35}S]-GSH in the presence of *t*-HP prior to endoproteinase digestion. See materials and methods for conditions. Separation was achieved by reverse-phase HPLC with TFA : acetonitrile gradient. Peptide elution was monitored by the absorbance at 214 nm (shown in bold), with the associated radioactivity monitored using a flow through detector (shown in grey). The radiolabelled UV absorbing peptide is arrowed in each case.

6.2.6. Dethiolation of GSTs

After determining that thiolation was an important response to oxidative stress in arabidopsis protein extracts *in-vitro* it was of interest to identify the reverse reaction i.e. dethiolation.

To identify factors involved in the dethiolation reaction, *At*DHARI was first thiolated with [^{35}S]-GSH *in-vitro*. Crude arabidopsis protein extracts (10 μg of protein) was then added, followed by NEM at timed intervals to alkylate dethiolated cysteinyl residues. In the dethiolation assays, a reductant (ascorbate or cysteine) or co-factor (NADPH) was assayed for its ability to promote the reduction of mixed disulphides. Following termination of the reactions at the appropriate time point, radiolabelled proteins were separated by SDS-PAGE and visualised following exposure to X-ray film.

When analysed immediately after the addition of the Arabidopsis extract (i.e. time = 0), a number of labelled polypeptides were observed in addition to the *At*DHARI, which ran as a 27 kDa polypeptide. These additional labelled proteins appeared to result from the spontaneous thiolation of Arabidopsis proteins by the [^{35}S]-GSH remaining from the DHAR thiolation experiment. Upon the addition of NADPH to the assay, the intensity of [^{35}S]-GSH thiolated *At*DHARI decreased greatly compared with *At*DHARI incubated with arabidopsis extract alone (figure 6.10). The addition of ascorbate to the assay had little affect on dethiolation compared to the addition of arabidopsis extract alone whereas cysteine reduced many of the thiolated arabidopsis proteins. The data suggests that within the arabidopsis protein extract dethiolation may occur via an NADPH-requiring enzyme.

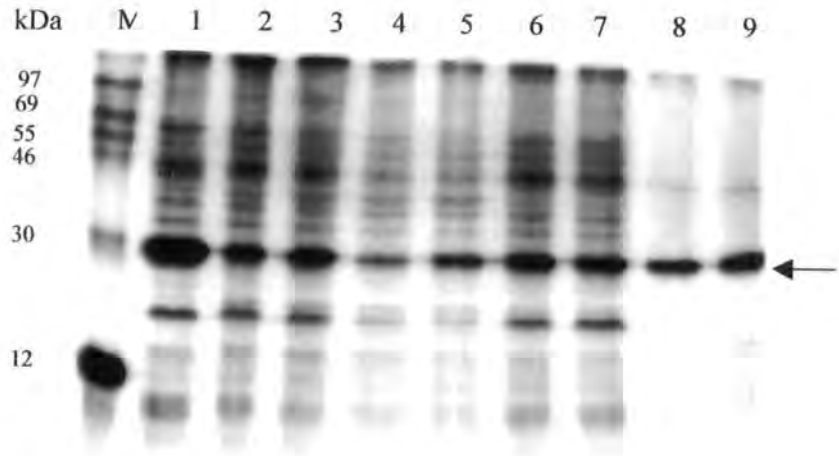

Figure 6.10 Dethiolation of *At*DHARI (arrowed) following incubation with crude Arabidopsis protein extracts.
Lane 1, lane 2 and 3, *At*DHARI incubated with 30 μg crude arabidopsis for 0, 10 and 20 min respectively; lanes 4 and 5, incubated with crude arabidopsis for 10 and 20 min in the presence of 5 mM NADPH; lanes 6 and 7, incubated with 30 μg crude arabidopsis for 10 and 20 min in the presence of 1 mM ascorbate; lanes 8 and 9, incubated with 30 μg crude arabidopsis for 10 and 20 min in the presence of 1 mM cysteine.

6.3 Discussion

Regulation of protein activity is essential during normal cellular metabolism. During periods of stress, oxidation of cysteinyl residues results in loss of activity followed by protein degradation (Cassano *et al*, 1994). Protection of the susceptible protein thiol group though the formation of protein mixed disulphides can result in reduced levels of activity, as is the case with PTP-1B (Barrett *et al*, 1999). However, the formation of mixed disulphides is reversible. Under conditions conducive to the reduction of disulphide bonds, the mixed disulphide is reduced enzymatically by glutaredoxin (Thomas *et al*, 1995), by NADPH-dependent reductases, or non-enzymatically by disulphide exchange. This results in the formation of GSSG and reduced protein sulphydryl. Of the enzymes investigated, treatment with alkylating reagents resulted in a substantial loss of enzyme activity with *At*DHARI, *At*DHARII, *At*DHARIII and esterase D respectively, implicating cysteine residues being involved in each activity. The thiolation protection assays performed with GSH then confirmed that formation of the mixed disulphide was of importance in partially protecting enzyme activity during subsequent alkylation. Although carried out under identical conditions, the protection conferred by thiolation varied between *At*DHARI, *At*DHARII, *At*DHARIII and esterase D.

In contrast, with the *At*DHARs, the enzyme activity of other GSTs was unaffected by oxidative treatment even though these protein were shown to undergo thiolation. In these cases it was possible that the cysteinyl residues thiolated by the oxidative treatment are distanced from the active site and therefore not essential for activity. Interestingly, in the case of the *At*DHARs, mixed disulphide formation between GSH and the active site cysteine may form part of the catalytic mechanism of these enzymes (Dixon *et al*, in press).

Induction of GSTs during periods of oxidative stress in *Phaseolus vulgaris* (Levine, *et al*, 1994) and during tobacco-mosaic virus infection in tobacco (Gullner *et al*, 1999) suggests that GSTs are important in alleviating cellular oxidative stress. As a consequence it might be expected that they are less susceptible to oxidative damage then other proteins. Although Arabidopsis GSTs were shown to be thiolated with [^{35}S]-GSH, the cysteine residues involved in the formation of mixed disulphides were not found to regulate GST activity. However, it is possible that thiolation of non-catalytically essential cysteine residues could protect the protein from proteinase degradation *in-vivo*. In the case of *At*DHARI and *At*DHARII, these proteins contain two cysteinyl residues, both within 15 amino-acid residues of the N-terminal. *At*DHARIII differs from *At*DHARI and *At*DHARII in that a total of 3 cysteinyl residues are present, with 2 cysteine residues in close proximity at position 25 and 28 (figure 6.1) and the third near the N-terminal. From the ESI-TOF-MS data it was possible to show that for all three *At*DHARs that one cysteine underwent thiolation in each case. However, in each case it was unclear which of the cysteine residues formed the mixed disulphide with GSH.

The formation of protein mixed disulphides during oxidative stress plays important roles in protecting the protein against reactive oxygen species. The formation of the bond being fully reversible (dethiolation). Dethiolation mechanisms were investigated using arabidopsis cell culture protein extracts, with a selection of chemical reductants used to dethiolate *At*DHARI labelled with [^{35}S]-GSH. By visualising the level of [^{35}S]-GSH labelled *At*DHARI by SDS-PAGE, it was shown that arabidopsis protein extracts apparently accelerated the dethiolation of *At*DHARI with the addition of NADPH increasing the rate of loss of [^{35}S]-GSH label. Dethiolation following the addition of NADPH suggested that the reaction was catalysed enzymatically. This finding is consistent with an NADPH-dependant glutaredoxin activity. Interestingly, such a glutaredoxin has been identified in arabidopsis plants (Cho *et al*, 2000a).

The use of purified proteins to determine the effect of thiolation on protein activity and the identity of the susceptible sulphydryl groups in proteins with multiple cysteine residues is essential. Protein-protein interactions could alter the environment surrounding the cysteine residue, changing the susceptibility to thiolation. To predict the susceptibility of individual cysteine residues to thiolation requires structural data. The pK_a of cysteine in solution is around 8.5, however, this can be reduced to as low as 3.9 within a protein depending on the surrounding amino acid residues. The decrease in pK_a increases the susceptibility of the residue to oxidation (Schaffer *et al*, 2001). The electrochemical and steric conditions also differ significantly between proteins and even within a family of proteins taken from different species. For example, rat haemoglobin contain 10 cysteine residues, which vary in reactivity over 4 orders of magnitude while human haemoglobin contain 6 cysteine residues, all of which show low reactivity. Such variation makes it difficult to assess susceptibility without examining a protein on an individual basis (DeSimplicico *et al*, 1998).

Redox regulation plays an important role of protein regulation. For example, in the chloroplast, proteins involved in energy conservation e.g. glyceraldehyde 3-phosphate dehydrogenase (GADPH) in the pentose-phosphate pathway are redox-regulated via the ferredoxin-thioredoxin system (Ruelland and Miginiac-Maslow, 1999). Glucose-6-phosphate dehydrogenase (G6PDH), the first and regulatory enzyme in the pentose phosphate pathway (Pandolfi *et al*, 1995) has also been shown to be thiolated during oxidative stress in mammalian cells (Cotgreave and Gerdes, 1998) and is known to be redox-regulated in the chloroplast (Ruelland and Miginiac-Maslow, 1999). The importance of G6PDH and GADPH in the production of NADPH highlights the universal significance of thiolation as a means of protein protection during oxidative stress.

7.0 Discussion

7.1 Overall Summary

In plants, the role of protein thiolation, the process in which cellular thiols form mixed-disulphide bonds with protein thiols, has received relatively little attention. In animals, although early reports suggested the formation of protein mixed disulphides to be of significance during periods of stress in mammalian cells (Grimm *et al*, 1985), relatively few thiolated proteins have been identified nor have the kinetics of this modification been fully characterised (Klatt and Lamas, 2000).

This present study aimed to characterise thiol metabolism in alfalfa and Arabidopsis respectively in response to fungal elicitation or oxidative stress. In the course of the study the thiols involved in the formation of protein-mixed disulphides and the rate at which this reaction occurred have been characteried. As the formation of protein mixed-disulphides could occur with a number a proteins containing free sulphydryl groups, it was also of interest to identify these proteins.

Alfalfa cell cultures were initially chosen to study as they represented a model plant system which accumulates significant quantities of both GSH and hGSH. This differs from the majority of plant and mammalian cells which contain only GSH (Klapheck, 1988). Also, the elicitation system in alfalfa cell cultures, following treatment with a yeast cell wall elicitor preparation, is well characterised in terms of the defence response (Tiller *et al*, 1994).

To determine the optimal fungal elicitor concentration required to induce the defence response in alfalfa cell cultures, these cultures were treated with various concentrations of the fungal elicitor over a period of 24 h. By monitoring the induction of PAL activity and the synthesis of the antimicrobial phytoalexin

medicarpin, the optimal elicitor concentration was determined. Subsequent treatment of alfalfa cell culture with the fungal elicitor revealed that only GSH accumulated; the level of hGSH remaining constant. The accumulation of cellular thiols by alfalfa in response to elicitor induced stress is in agreement with previous studies which investigated thiol levels in response to ozone, high light intensity, drought, air pollution, pathogen attack, or heat stress (May *et al*, 1998). It was of interest to determine the level and identity of any thiols bound to protein in response to elicitation. Previous calculations in mammalian cells estimated the level of available protein sulphydryl groups, i.e. potential protein-mixed disulphide residues, to be as great as the cytosolic GSH concentration (DiSimplicico *et al*, 1998). This suggested the formation of protein mixed-disulphides may form a significant sink for the GSH pool, along with the previously observed increase in the GSSG pool during oxidative stress. Using labelling of the intracellular thiol pool with L-[^{35}S]cysteine it was possible to quantify protein-bound thiols by collecting the fraction corresponding to the elution time and determining the level of incorporated L-[^{35}S]cysteine by scintillation counting. In alfalfa, separation of protein bound thiols identified GSH as the major thiol forming mixed disulphides in response to elicitor treatment. The identification of GSH in mixed-disulphide bond formation reflected the observed increase in total GSH. In contrast, levels of hGSH following elicitor treatment remained constant throughout. Although the total GSH content increased following elicitor treatment, in agreement with previous studies (Edwards *et al*, 1991), the increase in the GSH bound to protein preceded GSH synthesis. This demonstrated the importance which protein sulphydryl groups have in protecting the cell during the elicitation response and the associated oxidative stress. The roles of GSH and hGSH within alfalfa are still not fully understood, however, recent evidence suggests the location of the thiol within the whole alfalfa plant influences the role of the thiol (Frendo *et al*, 1999). For example, GSH is abundant in seeds, suggesting a role in sulphur storage whereas hGSH predominates in the nodules and roots (Frendo *et al*, 1999). The purpose of GSH and hGSH localisation is unknown as many plant species contain

only GSH whilst several leguminous species contain hGSH as the predominant thiol (e.g. soybean) (Klapheck, 1988). Labelling of the cytosolic thiol pools with L-[^{35}S]cysteine revealed several interesting but as yet uncharacterised compounds following derivatisation with FDNB. The two abundant compounds (unknown 1 and unknown 2) were found to be labelled with L-[^{35}S]cysteine. Unfortunately, the identity of these compounds remained unknown although it was thought that they did not contain free cysteine or disulphide bonds as treatment *S*-bimane ± treatment with DTT did not identify any fluorescent bimane conjugates. The levels of unknown 1 and unknown 2 changed markedly upon elicitation; unknown 1 increased, whilst unknown 2 decreased within 2 h of elicitor treatment.

The abundance of protein thiolation was visualised during *in-vitro* assay of alfalfa protein extract, labelled with L-[^{35}S]cysteine containing GSH. During chemical induced oxidative stress, a significant number of proteins were thiolated in a time dependent manner. Contrary to findings *in-vivo*, hGSH readily underwent the formation of protein mixed-disulphides *in-vitro* demonstrating that both thiols were potentially equally active in thiolation.

In-vitro thiolation assays labelled many proteins from a cell-free preparation of alfalfa. It was of interest to identify individual proteins which were susceptible to thiolation and determine the effect of this modification upon enzyme activity. To minimise the number of purification steps, resulting in the dissociation of the [^{35}S]-GSH label and therefore loss of protein, the possibility of protein sequencing directly from polypeptides resolved by gel-electrophoresis was investigated.

In order to use MALDI-TOF-MS to identify proteins resolved by electrophoresis, a database of known protein sequences to compare the mass-ion fragments of tryptic digests is required. To increase the chance of identifying known protein sequences, the system under study was changed from cell-suspension cultures of alfalfa to cell suspension cultures of *Arabidopsis thaliana*.

After characterising the defence response of Arabidopsis cell cultures to *t*-HP, the changes in cellular thiol pools in response to treatment were monitored. The increase in protein bound GSH detected in Arabidopsis cell cultures reflected those determined using alfalfa cell cultures. Arabidopsis protein extracts were thiolated *in-vitro* using [^{35}S]-GSH, revealing a number of polypeptides forming mixed disulphides. This confirmed Arabidopsis to be a suitable system to study protein thiolation. 2D-gel electrophoresis separation of Arabidopsis cell-free extract to visualise thiolated proteins (figure 5.5B) revealed a number of proteins in the pI range pH 4 to pH 7 were thiolated.

An alternative directed proteomic approach, using GSH affinity-chromatography to purify specific protein families for further analysis, was adopted. Glutathione transferases were purified from Arabidopsis cell cultures and then thiolated *in-vitro* with [^{35}S]-GSH revealing several putative GST polypeptides being susceptible to thiolation during oxidative stress.

To further investigate the role of protein thiolation in the GST protein family, a number of cloned GSTs from arabidopsis, maize and soybean were expressed in *E. coli* and purified by liquid chromatography. By obtaining pure protein extracts, it was possible to monitor the effects of thiolation on enzyme activity. Of the proteins investigated, only the activities of *At*DHARI, *At*DHARII and *At*DHARIII were significantly affected by treatment by thiolation or sulphydryl modifying reagents. Treatment of *At*DHARI, *At*DHARII and *At*DHARIII with the sulphydryl alkylating reagents NEM or iodoacetamide demonstrated that cysteinyl residues were essential for catalysis. The role of GSH as a protection mechanism was also shown by thiolating the DHAR prior to treatment with NEM. Following reduction of the mixed disulphide by DTT, activity was restored if the protein was able to form mixed disulphides with GSH prior to the addition of NEM. Protection of essential catalytic residues by thiolation was also shown in the non-GSH-requiring purified arabidopsis esterase D. Activity was reduced

significantly following treatment with NEM or iodoacetamide, however, prior formation of a mixed disulphide led to the recovery of > 90 % esterase D activity.

Analysis of the *in-vitro* thiolated *At*DHARI, *At*DHARII and *At*DHARIII by SDS-PAGE confirmed thiolation of the polypeptide during oxidative stress. Other Arabidopsis GSTs, e.g. *At*GSTF8 were also thiolated, though this had no effect on enzyme activity. *At*DHARI, *At*DHARII and *At*DHARIII contain multiple cysteine residues therefore it was important to determine the number of GSH molecules forming protein mixed-disulphides in response to *t*-HP induced stress. This was achieved using ESI-TOF-MS which determined that only one GSH molecule formed mixed disulphides with *At*DHARI, *At*DHARII and *At*DHARIII respectively. The identity of the cysteinyl residue involved in the formation of the mixed disulphide was further investigated by fragmenting the *in-vitro* [^{35}S]-GSH thiolated protein with endoproteinase LysC. The retention-time of the mixed-disulphide containing peptide was deduced by monitoring the [^{35}S]-GSH with an in-line radioactivity detector.

7.2 Future Work

Despite the importance of protein thiolation in many pathways including transcription factor (c-Jun) regulation (Klatt *et al*, 1999) and the regulation of protein activity (mGST) (Dafre *et al*, 1996) relatively few proteins have been identified which are regulated by thiolation. However, of those which have, several, for example G3PDH, are involved in the production of energy in the form of NADPH. Plant cells are highly susceptible to oxidative stress following the disruption of the GSH cycle (Noctor *et al*, 1998). Together with the catalytic and redox regulated rate of dethiolation (Cho *et al*, 2000), GSH metabolism and protein thiolation perform a significant role in the maintenance and regulation of normal cell growth. The data presented will help to increase the understanding of GSH metabolism, in particular the importance of protein thiolation, during periods of stress.

Tight regulation of protein thiolation can be implied from the fact that not all sulphydryl containing proteins form mixed disulphides (Shenton *et al*, 2002). Evidence of the tight regulation of protein *S*-thiolation in yeast *in-vivo* studies has been reported in studies with the G3DPH isoenzymes Tdh2 and Tdh3, under differing conditions of oxidative stress. These studies reveal the formation of mixed disulphides depended on factors other than the induced oxidative conditions. Both isoenzymes were thiolated when exposed to H_2O_2 induced oxidative stress conditions in cell-free extracts. However, under similar conditions *in-vivo*, only Tdh2 became thiolated, suggesting cellular interactions play an important role in the regulation of protein *S*-thiolation (Shenton *et al*, 2002). The use of reagents such as the thiol specific, lipophilic cation (4-iodobutyl)-triphenylphosphonium (IBTP) in the study of whole organelles such as mitochondria (Lin *et al*, 2002) may aid the identification of proteins which undergo thiolation *in-vivo*, during various stages of the cell cycle or during oxidative stress. Differing responses to oxidative conditions by the formation of protein mixed-disulphides *in-vivo* and in cell-free extracts suggests the interpretation of *in-vitro* data needs careful assessment prior to concluding physiological relevance of changes in the protein activity resulting from protein modifications.

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