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Oxidative Stress and Acclimation Mechanisms in Plants

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INTRODUCTION

One of the most crucial functions of plant cells is their ability to respond to fluctuations in their environment. Understanding the connections between a plant's initial responses and the downstream events that constitute successful adjustment to its altered environment is one of the next grand challenges of plant biology. Oxidative stress from environmental sources and developmental transitions such as seed maturation involves the formation of reactive oxygen species (ROS) in plant cells. The redox-modulated changes that follow are central events in cellular responses. Thiol redox regulation (Figure 1) partially mediated through the redox state of the glutathione pool (GSH/GSSG), regulation of the glutathione biosynthetic pathway, and ROS themselves are each thought to have important roles as environmental sensors and/or modulators of global patterns of gene expression in development and defense. Exposure of green tissue to potentially damaging light intensities involves redox sensing molecular events throughout the plant, originating at the plastoquinone (PQ) pool in the thylakoid membrane. Major defense genes whose expression is affected by the redox state of the PQ pool include both cytosolic and chloroplast ascorbate peroxidases (APX) (Karpinska *et al.* 2000). The superoxide dismutase (SOD) gene families appear to be specialized in function with respect to subcellular location and other as yet unknown factors (Alscher *et al.* 2002). In the case of peroxisomes, the imposition of oxidative stress gives rise to organelle proliferation, thus adding another layer of complexity to stress responses (Lopez-Huertas *et al.* 2000). Defense mechanisms involving molecular chaperones and methionine sulfoxide reductase are becoming recognized as important players in resistance to oxidative stress throughout the cell.

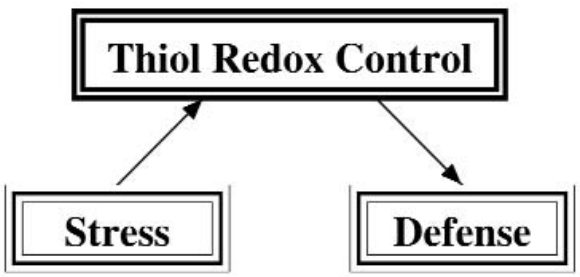

Figure 1. Thiol redox control and stress defense

Intracellular origins of ROS and their multiple damaging effects

Any circumstance in which cellular redox homeostasis is disrupted can lead to oxidative stress or the generation of ROS (Asada 1994). Production of ROS during environmental stress is one of the main causes for decreases in productivity, injury, and death that accompany these stresses in plants. ROS are produced in both unstressed and stressed cells, and in various locations (Halliwell and Gutteridge 1989) (Figure 2). They are generated endogenously during certain developmental transitions such as seed maturation and as a result of normal, unstressed, photosynthetic and respiratory metabolism. An initial oxyradical product, the superoxide radical ($O_2^{-}\cdot$), upon further reaction within the cell, can form more ROS such as hydroxyl radicals and singlet oxygen. Superoxide is a charged molecule and cannot cross biological membranes. Subcellular compartmentation of defense mechanisms is, therefore, crucial for efficient removal of superoxide anions at their sites of generation throughout the cell. Hydrogen peroxide, on the other hand, which is formed as a result of SOD action, is capable of diffusing across membranes and is

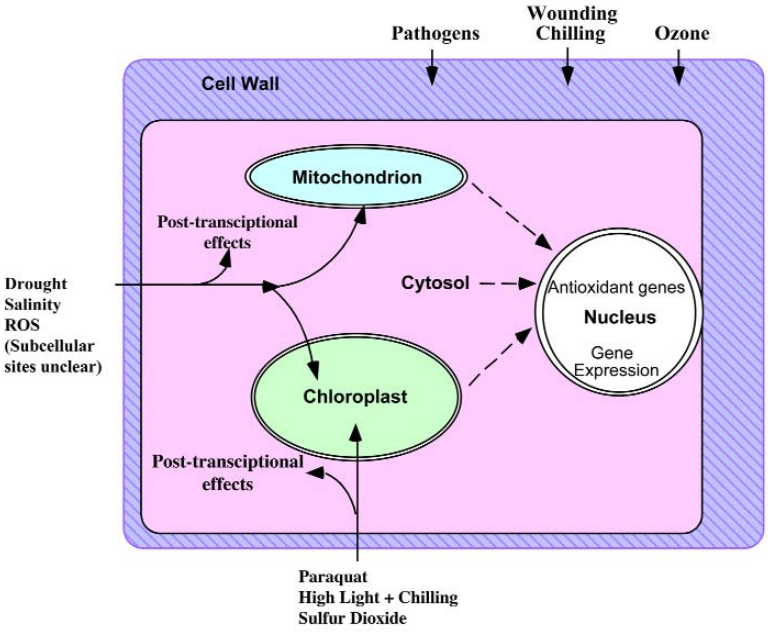

Figure 2. Reactive oxygen species (ROS) arise throughout the cell.

thought to fulfil a signaling function in defense responses (Mullineaux *et al.* 2000).

ROS play an important role in endonuclease activation and consequent DNA damage (Hagar *et al.* 1996). In the presence of metal ions such as Fe or Cu(II), hydroxyl radicals are formed very rapidly. Hydroxyl radicals can cause damage to all classes of biologically important macromolecules, especially nucleic acids. Hydroxyl radicals can also modify proteins so as to make them more susceptible to proteolytic attack. There is evidently considerable specificity associated with this degradative process since proteins have widely differing susceptibilities to attack by ROS (Davies 1987). Once damaged, proteins can be broken down further by specific endopeptidases such as the one found bound to the thylakoid membrane (Casano *et al.* 1994). A multicatalytic proteinase complex has been demonstrated in plant systems, with the capacity to selectively break down oxidatively damaged proteins (Van Nocker *et al.* 1996)

Metabolic defense mechanisms: limiting ROS-mediated damage

Plant cells respond defensively to oxidative stress by removing the ROS and maintaining antioxidant defense compounds at levels that reflect ambient environmental conditions (Scandalios 1997). Metabolic containment mechanisms for ROS involving antioxidant genes and associated processes are likely to have predated or co-evolved with the appearance of aerobiosis and represent fundamental adaptations of aerobic systems to an oxygen dependent metabolism. The mechanisms that act to adjust antioxidant levels to afford protection include changes in antioxidant gene expression (Cushman and Bohnert 2000).

ROS themselves play a role in intracellular redox sensing, activating antioxidant resistance mechanisms, among other adaptive processes (Toledano and Leonard 1991; Karpinski *et al.* 1997; May, *et al.* 1998a). A number of redox sensitive transcription factors have been identified in animal, bacterial, and plant cells (Pastori and Foyer 2001).

Functional roles of these responses include the protection of redox-sensitive enzymatic processes, the preservation of membrane integrity, and the protection of DNA and proteins (Scandalios 1997). Redox-sensitive regulatory enzymes such as fructose-1,6-bisphosphatase (FbPase) can be protected from oxidation/inactivation by the action of antioxidants such as glutathione. Under unstressed conditions, the formation and removal of O_2 are in balance. The defense system, when presented with increased ROS formation under stress conditions, can be overwhelmed when it is unable to remove the toxic molecular species with increased enzymatic or non-enzymatic antioxidant processes.

Organelles such as the peroxisome and the chloroplast, where ROS are being produced at a relatively high rate, are especially at risk. In the case of the chloroplast, changes in light intensity and temperature or limitations in the substrates of photosynthesis occur frequently,

resulting in increased production of ROS (Alscher *et al.* 1997; Karpinska and Karpinski 2000). ROS are produced at high levels in peroxisomes. Hydrogen peroxide is produced in the peroxisomal respiratory pathway by flavin oxidase. Fatty acid beta oxidation and glycolate oxidase action are other sources of hydrogen peroxide production in the peroxisome. Developmental transitions such as seed maturation, in which peroxisomes play an important role, also involve oxidative stress (Leprince *et al.* 1990; Walters 1998).

Antioxidant defense molecules have several roles

Ascorbic acid, glutathione, and α-tocopherol have each been shown to act as antioxidants in the detoxification of ROS. These compounds have central and interrelated roles, acting both non-enzymatically and as substrates in enzyme-catalyzed detoxification reactions (Foyer 1993; Hess 1993; Hausladen and Alscher 1994; Winkler *et al.* 1994; Chaudiere and Ferrari-Iliou 1999). An anti-ROS response includes the induction of genes that belong to ROS scavenging mechanisms.

Metabolic cycles located within the aqueous phase of the peroxisome, chloroplast, cytosol, and the mitochondrion successively oxidize and re-reduce glutathione and ascorbate, using NAD (P) H as the ultimate electron donor. Ascorbate, reduced glutathione (GSH), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), and monodehydroascorbate reductase (MDHAR) are involved in several contexts in antioxidant regeneration throughout the plant cell. The enzymes involved are hydrophilic in nature, although in some instances they are known to be loosely associated with the membranes where the ROS are generated. The version that is found in the chloroplast is shown in Figure 3 including a depiction of the role of the hydrophobic antioxidant α-tocopherol. Different isoforms of the antioxidant enzymes are located in different subcellular compartments (see below). Evidence to date suggests a coordinated response to ROS among different members of the different SOD gene families.

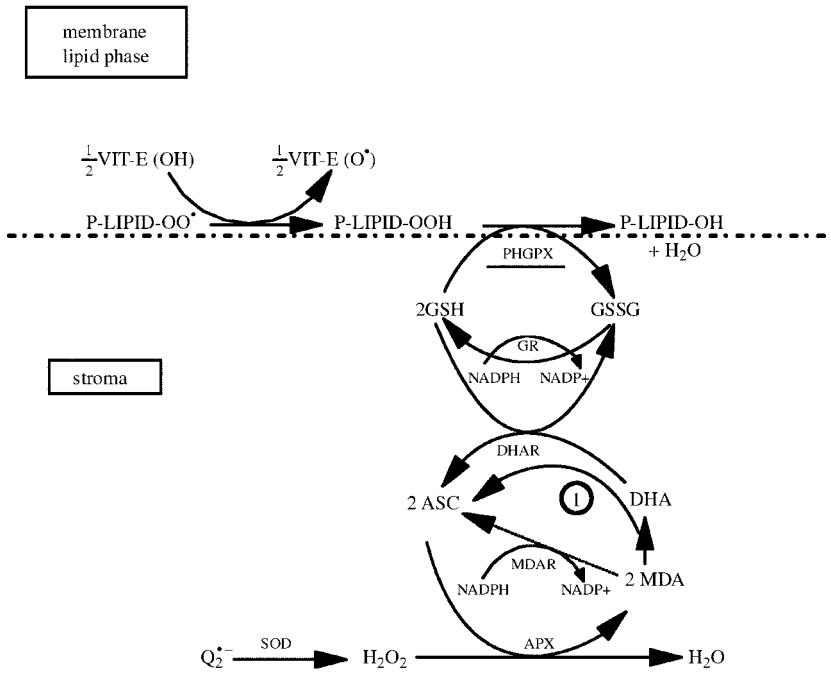

Figure 3. The scavenging of active oxygen species in the chloroplast in both the lipid membrane phase and the stroma, linked to redox cycles for ascorbate and glutathione and the oxidation of α-tocopherol (Vitamin E). Abbreviations are as follows: P-LIPID -OO, phospholipid peroxy radical; P-LIPID-OOH, phospholipid peroxide; P-LIPID-OH, phospholipid alcohol; VIT-E(OH), α-tocopherol (vitamin E); VIT-E (O*), α-chromanoxyl radical; PHGPX, phospholipid hydroperoxide-dependent glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; DHAR, dehydroascorbate reductase; ASC, ascorbic acid; DHA, dehydroascorbate; MDA, monodehydroascorbate free radical; MDAR, monodehydroascorbate free radical reductase; APX, ascorbate reductase; SOD, superoxide dismutase; O_2-, superoxide ion. Reaction 1 is the non-enzyme-catalyzed spontaneous dismutation of two MDA molecules to one ASC and one DHA, respectively. From Mullineaux *et al.* (2000).

SOD and antioxidant defenses

Within a cell, the superoxide dismutases (SODs) constitute the first line of defense against ROS. O_2^- is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell (Elstner 1991), including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol. This being the case, it is not surprising to find that SODs are present in all these subcellular locations (Figure 4). While all compartments of the cell are possible sites for O_2^- formation, chloroplasts, mitochondria, and peroxisomes are thought to be the most important generators of ROS (Fridovich 1986).

Takahashi and Asada (1983) showed that phospholipid membranes are impermeable to charged O_2^- molecules. Therefore, it is crucial that SODs are present for the removal of O_2^- in the compartments where O_2^- radicals are formed (Takahashi and Asada 1983). Based on the metal co-factor used by the enzyme, SODs are classified into three groups: iron SOD (Fe SOD), manganese SOD (Mn SOD), and copper-zinc SOD (Cu-Zn SOD). Fe SODs are located in the chloroplast, Mn SODs in the mitochondrion and the peroxisome, and Cu-Zn SODs in the chloroplast, the cytosol, and possibly the extracellular space (Figure 4). Comparison of deduced amino acid sequences from these three different types of SODs suggest that Mn and Fe SODs are more ancient types of SODs (Figure 5), and these enzymes most probably have arisen from the same ancestral enzyme, whereas Cu-Zn SODs have no sequence similarity to Mn and Fe SODs and probably have evolved separately in eukaryotes (Kanematsu and Asada 1990; Smith and Doolittle 1992). The evolutionary reason for the separation of SODs with different metal requirements is probably related to the different availability of soluble transition metal compounds in the biosphere in relation to the O_2 content of the atmosphere in different geological eras (Bannister *et al.* 1991). We now visit each SOD group in turn.

Iron SODs. The group of Fe SODs probably constitutes the most ancient SOD group. Bannister *et al.* (1991) suggest that iron was probably the first metal used as a metal cofactor at the active site of the first SOD because of an abundance of iron in soluble Fe (II) form at the time. As the levels of O_2 in the environment increased, the mineral components of the environment were oxidized. The decrease in available Fe (II) in the environment caused a shift to the use of a more available metal, Mn (III).

Fe SOD is found both in prokaryotes and in eukaryotes. In eukaryotes it has been isolated from *Euglena gracilis* (Kanematsu and Asada 1979) and higher plants. Fe SOD is inactivated by H_2O_2 and is resistant to KCN inhibition. In all plant species examined to date, it is inferred to be located in the chloroplast.

Kliebenstein *et al.* (1998) report three Fe SODs in Arabidopsis. The absence of Fe SOD in animals has given rise to the proposal that the Fe SOD gene originated in the plastid and moved to the nuclear genome during evolution. Support of this theory comes from the existence of several conserved regions that are present in plant and cyanobacterial Fe SOD sequences, but absent in non-photosynthetic bacteria (Bowler *et al.* 1994). All three Fe SOD

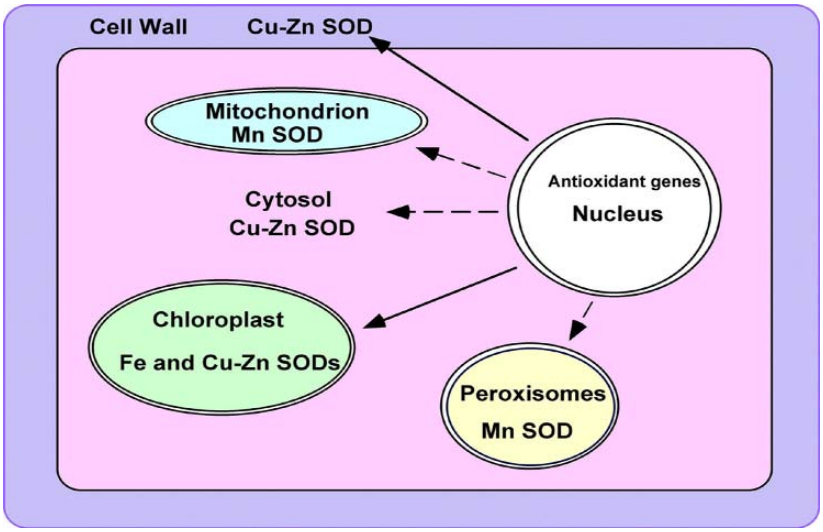

Figure 4. Cellular locations of superoxide dismutase (SOD).

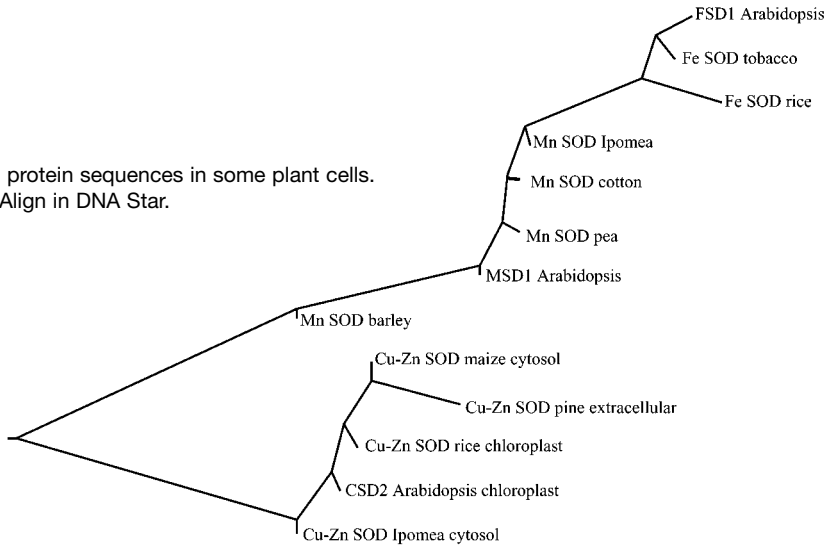

Figure 5. Relatedness of SOD protein sequences in some plant cells. Tree was obtained using MegAlign in DNA Star.

plant sequences encode a unique tripeptide (SRL for *N. plumbaginifolia* and *G. max* and ARL for *A. thaliana*) close to the carboxyl terminus of the enzyme. Although this sequence has shown to direct the proteins to peroxisomes in other proteins, it has yet to be determined whether this is a functional sequence or not. The conserved SRL/ARL sequence is not present in the prokaryotic Fe SOD proteins showing that it is not obligatory for enzyme function (Van Camp *et al.* 1994).

Manganese SODs. As the levels of O_2 in the environment increased, the amount of available Fe (II) in the environment decreased, causing a shift to the more available metal, Mn (III). As a consequence, Mn SODs are likely to be second only to Fe SODs in antiquity and certainly evolved from the ancestral Fe SODs, perhaps by way of the cambialistic SODs. Mn SODs occur in mitochondria and peroxisomes. Mn SODs carry only one metal atom per subunit. These enzymes cannot function without the Mn atom present at the active site. Even though Mn and Fe SODs have a high similarity in their primary, secondary, and tertiary structure, these enzymes have diverged sufficiently that Fe (II) could not restore the activity of Mn SOD and vice versa (Fridovich 1986). Catalysis by Mn SODs is through the attraction of negatively charged O_2^- molecules to a site formed from positively charged amino acids present at the active site of the enzyme. The metal present in the active site then donates an electron directly to the O_2^-, reducing one O_2^- molecule, which in turn forms H_2O_2 by reacting with a proton (Asada 1994; Bowler *et al.* 1994). Plant Mn SODs have approximately 65% sequence similarity to one another, and these enzymes also have high similarities to bacterial Mn SODs (Bowler *et al.* 1994).

Although Mn SOD is known as the mitochondrial enzyme of eukaryotes, an Mn-containing SOD has also been located in the peroxisomes. del Rio *et al.* (1992) showed the presence of one peroxisomal and one mitochondrial Mn SOD by using immunolocalization assays in watermelon. Four genes that encode Mn SOD were reported in maize (*Zea mays*) (Zhu *et al.* 1999). Deduced amino acid sequences from these four isoenzymes have a mitochondrial targeting sequence, indicating that all are located in the mitochondria. In *Nicotiana plumbaginifolis,* two nuclear-encoded Mn SOD genes were isolated and the tissue-specific expression Mn SOD was shown by analyzing promoter fusion with β-glucuronidase (GUS) in transgenic plants (Van Camp *et al.* 1996).

Copper-Zinc SODs. When the atmosphere was completely replenished with oxygen, Fe (II) was almost completely unavailable in the atmosphere and insoluble Cu (I) was converted into soluble Cu (II). At this stage, Cu (II) began to be used as the metal cofactor at the active sites of SODs. Since Fe and Mn have similar electrical properties, the transition from the use of iron to the use of manganese required little change in SOD protein structure. Thus, Mn and Fe SODs are structurally very similar. The existence of archaic Mn/Fe[1]-containing SODs supports this theory. However the electrical properties of Cu-Zn[2] SODs differ greatly from those of Fe and Mn SODs.

[1] Every molecule of Mn and Fe SOD contains either an atom of manganese or an atom of iron depending on the species or the availability of the metal in archaic SODs. The potential use of either Fe or Mn is denoted as such by a slash, Mn/Fe.

[2] Every molecule of the Cu-Zn SOD enzyme contains both an atom of copper and an atom of zinc, as denoted by the hyphen.

Therefore, a major change in the structure of the protein occurred after Cu became a metal cofactor (Bannister *et al.* 1991).

There are at least two forms of Cu-Zn SOD in plant cells, chloroplastic and cytosolic. Deduced amino acid sequences of these two isoforms show approximately 68% similarity, whereas there is approximately 90% similarity among the chloroplastic Cu-Zn SODs (Cu-Zn SOD_{chl}) and 80-90% similarity among the cytosolic Cu-Zn SODs (Cu-Zn SOD_{cyt}) (Figure 6). Cu-Zn SOD_{chl} is a soluble enzyme and is localized in the stroma (Asada and Kiso 1973; Asada *et al.* 1973). Localization studies performed with an immunogold-labeled antibody raised against Cu-Zn SOD_{chl} from spinach leaves showed that this soluble enzyme is not uniformly distributed in the chloroplast but rather is localized mainly on the stromal face of thylakoid membranes (Ogawa *et al.* 1995) where photosystem I (PSI) is located. The two other Cu-Zn SODs are considered "cytoplasmic" because they have not been detected in intact chloroplasts (Ogawa *et al.* 1996). However, when immunogold-labeled antibodies raised against "cytosolic" Cu-Zn SOD were used in localization experiments, it was shown that these enzymes were located in the nucleus and apoplast (Ogawa *et al.* 1996). More than 40% of the immunogold particles were found in the apoplast and approximately 25% was found in the nucleus (Ogawa *et al.* 1996). Researchers proposed that Cu-Zn SOD in the apoplast functions in lignification and that in the nucleus it protects the cell against fatal mutations caused by O_2^- molecules (Ogawa *et al.* 1996; Ogawa *et al.* 1997).

SOD expression and activity under oxidative stress. Kliebenstein *et al.* (1998) subjected Arabidopsis to a series of oxidative stresses and observed changes in the seven SODs -- three Fe SODs denoted FSD1, FSD2, and FSD3; three Cu-Zn SODs denoted CSD1, CSD2, and CSD3; and one Mn SOD denoted MSD1 -- that are present in that plant, both at the mRNA and the protein level. They reported increases in FSD2 in response to UV irradiation, and to high light at the mRNA level, but no response of FSD2 mRNA to ozone exposure. They found that FSD1 is under the control of a circadian clock at the mRNA level. Kliebenstein *et al.* (1998) did not report enzyme activities. However, it is of interest to note that they report FSD2 responding to specific stresses. Thus, it appears that

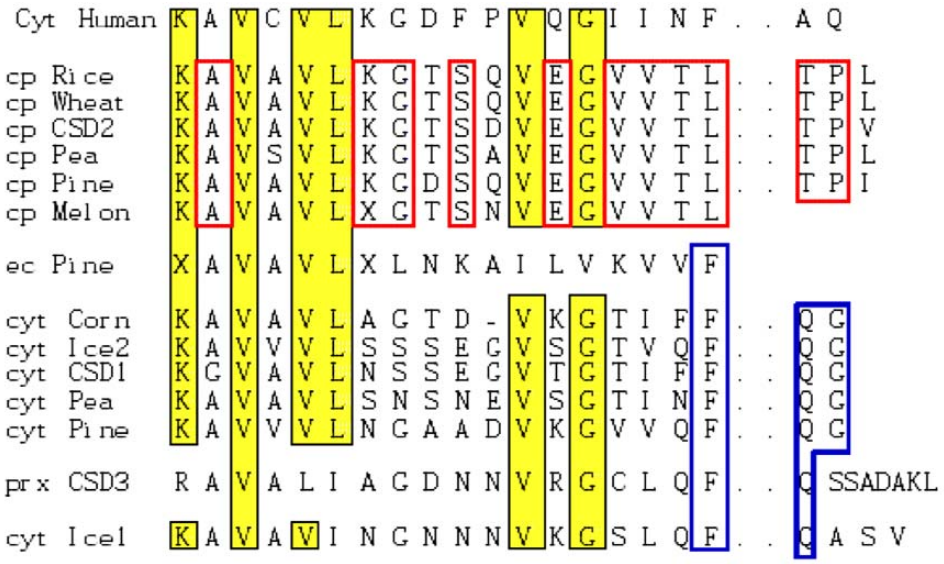

Figure 6. A comparison of cytosolic, chloroplast, and peroxisomal Cu-Zn SODs from higher plants. Abbreviations are as follows: cyt, cytosolic; cp, chloroplast; prx, peroxisomal; ec, extracellular. From Kliebenstein et al. (1998).

members of the Fe SOD family are specialized for specific responses to stress.

Kliebenstein *et al.* (1998) report no effect of their series of oxidative stress treatments on Mn SOD in Arabidopsis. In contrast, Mn SOD was reported to respond positively to salt stress (Hernandez *et al.* 1993; Hernandez *et al.* 1995; Gomez *et al.* 1999), manganese toxicity (Gonzalez *et al.* 1998), chilling stress (Lee *et al.* 1999; Lee and Lee 2000), and drought (Wu *et al.* 1999). An explanation for the differences in responses between the Mn and Fe SODs in plants can be correlated with their disparate subcellular locations and the sites of action of the various oxidative stresses that were used. The stresses that did not affect Mn SOD may all have their site of action in the chloroplast.

Overexpression of SODs can lead to protection against specific stresses. The importance of subcellular location. Plants overexpressing SODs have been engineered with the goal of increasing stress tolerance. Both successful and unsuccessful results have been obtained from attempts to create resistant plants (Tepperman and Dunsmuir 1990; Perl *et al.* 1993; Slooten *et al.* 1995; Van Camp *et al.* 1996).

The site of action for ozone has been suggested to be in the apoplast, but when Mn SOD was overexpressed in the chloroplasts of tobacco plants, less damage was observed in the leaves. When the overproduction of the enzyme was in the mitochondria, less protection was observed. Also, it was shown that high levels of chloroplastic Mn SOD activity protected the plant from visible injury caused by ozone, suggesting that ozone may cause oxidative stress in the chloroplast, as well as the apoplast (Van Camp *et al.* 1994). When an *A. thaliana* Fe SOD gene was targeted into *N. tabacum* cv Petit Havana SR1 chloroplasts, an increased protection against O_2 generated both in the plasmalemma and photosystem II (PSII) was observed (Van Camp *et al.* 1996). Arisi *et al.* (1998) overexpressed an Arabidopsis Fe SOD in poplar and observed a protective effect on PSII under limiting carbon dioxide conditions. Van Camp *et al.* (1994) overexpressed Fe SOD in the chloroplast and found that induction of Cu-Zn SODs was suppressed, lending some credence to the hypothesis that a cross-family signaling pathway for SOD induction exists in the plant cell. In contrast, when mitochondrial Mn SOD was targeted to the chloroplasts of tobacco plants, protection was only observed against stress generated in the plasmalemma. An increase in the activity of APX, DHAR, and MDHAR, other scavenging pathway enzymes, was also observed. It was concluded that the protection provided by overproduction of Mn SOD was dependent on whether the other enzymes — DHAR and MDAR — were or were not present in elevated levels

Bowler *et al.* (1994) targeted *N. plumbaginiafolis* mitochondrial Mn SOD sequence into *N. tabacum* mitochondria and chloroplasts. When the transformed plants were treated with methyl viologen, it was observed that the plants that had Mn SOD expressed in their chloroplast had remarkable protection, accompanied by an increased SOD activity against methyl viologen stress compared to control plants in light. This protective effect was less observable in plants that were kept in the dark, since the H_2O_2 scavenging system is not activated without light (Foyer and Halliwell 1976; Nakano and Asada 1980). Even though Mn SOD is not inactivated by H_2O_2, the balance between generated O_2^- and H_2O_2 is disrupted in these transgenic plants, which may increase the formation of OH·. Although increased protection was observed in plants that had foreign Mn SOD targeted into the mitochondria, this effect was not as remarkable compared to plants that had Mn SOD targeted into the chloroplasts. This may be due to the majority of the O_2 being generated in the chloroplast in plants exposed to methyl viologen in light (Bowler *et al.* 1991).

Taken together, these results suggest that regulation of expression of plant Fe and Mn SODs may differ. The subcellular location of the protein also appears to play a role. In some instances, both chloroplastic and cytosolic Cu-Zn SODs afford protection against the same stresses as Fe SOD. There appears, also, to be specialization within the Fe SOD family. We have compared the upstream sequences of all Arabidopsis SOD genes in order to determine similarities or differences among their respective regulatory regions.

Comparing the information in SOD genomic sequences. With the complete sequencing of the Arabidopsis genome, it is a propitious time to ask what the appropriate application of computation to that genome might reveal about the evolution and regulation of SODs in plants. The upstream regions of the seven Arabidopsis SOD open reading frames were extracted with the intention of mining them for promoter sequences related to the regulation of gene expression under different stresses. We developed bioinformatics tools for searching an upstream region for particular promoter sequences that bind to known transcription factors (Table 1). The area of computational tools for analyzing promoter sequences is in its infancy (Prestridge 2000), and existing tools are limited in their abilities to address our questions and to analyze an entire genome. The ABA responsive element (ABRE) appears to be associated with genes responding to osmotic stress (high osmoticum, salt, desiccation, and cold) and binds to several similar sequences of eight nucleotides (Guan and Scandalios 1998; Choi *et al.* 2000); we used the consensus sequence YACGTGGC. NF-$\kappa\beta$ is a transcription factor that activates immunoglobulin-k genes; we utilized the consensus sequence GGRNNYYCC (Smith *et al.* 2000). The heat shock protein gene promoter consensus sequence is the palindromic sequence TTCN-NGAA (Santos *et al.* 1996). Finally, the Y-box motif has

consensus sequence GATTGG and mediates redox-dependent transcription activation (Guan and Scandalios 1998). The seven promoter regions were examined for these four consensus sequences. Diamonds (♦) in Table 1 summarize the exact or close matches found at plausible upstream locations (within 1000 nucleotides of the ATG where transcription begins). The different patterns of consensus sequences found suggest that the phenomenon of differential expression of the SOD genes under different stresses can be explained, at least in part, through promoter sequence analysis. Further work will include extracting the upstream sequences for all known or putative Arabidopsis genes and comparing these sequences on a genome-wide basis with new computational tools we are developing. Employing also information concerning co-regulated genes it will become possible to identify what promoter sequence combinations correspond to what kind of regulation.

Multiple defense roles for glutathione as antioxidant and redox sensor. Upon the imposition of oxidative stress, the existing pool of reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) and glutathione biosynthesis is stimulated (May and Leaver 1993; Madamanchi *et al.* 1994). The rate-limiting step for glutathione synthesis is thought to be gamma-glutamylcysteine synthetase, which is feedback regulated by GSH and is controlled primarily by the level of available L-cysteine (May *et al.*1998b). Increasing glutathione biosynthetic capacity has been shown to enhance resistance to oxidative stress (Arisi *et al.* 1998; Zhu *et al.* 1999). Glutathione reductase (GR) activities increase as the glutathione pool increases through a multi-level control mechanism, which includes coordinate activation of genes encoding glutathione biosynthetic enzymes and GR (Xiang and Oliver 1998).

Glutathione acts as a redox sensor of environmental cues and forms part of the multiple regulatory circuitry coordinating defense gene expression. There are two known genes encoding GR (Madamanchi *et al.* 1992; Creissen *et al.* 1995a; Creissen *et al.* 1995b). The redox state of the GSH/GSSG couple may act as a direct link between environmental cues and crucial molecular adaptive responses of plant cells (Hausladen and Alscher 1993; Broadbent *et al.* 1995; Roxas *et al.* 1997; May *et al.* 1998a; May *et al.* 1998b; Baginsky *et al.* 1999). Glutathione has been reported to regulate rates of cell division (Sanchez-Fernandez *et al.* 1997) and the induction of antioxidant defenses, as exemplified by the induction of Cu-Zn SOD (Herouart *et al.* 1993). Glutathione has been suggested as an intermediary in a redox sensing signaling pathway in plants involving the ROS-mediated oxidation of membrane lipids to oxylipins as the initial step (Ball *et al.* 2001). It is also thought to act specifically as a regulator of the transcription of chloroplast genes (Baginsky *et al.* 1999). The antioxidant genes known to date are all nuclear-encoded, however.

Previous biochemical data demonstrated the existence of multiple isoforms of GR, which have been assigned to various organelles, including the peroxisome, the chloroplast, the mitochondrion and the peroxisome (Creissen *et al.* 1991). Only two Arabidopsis GR genes have been characterized, however (Stevens *et al.* 2000). A peroxisomal GR gene has not been characterized to date, although a peroxisomal GR protein has been described (Corpas *et al.* 2001). Consequently, it is very likely that other members of the GR gene family exist.

Oxidative stress through impaired mitochondrial electron transport in developing seeds. Role of glutathione.

Seeds develop through a series of stages that may be tracked at morphological, physiological, and molecular levels. *Arabidopsis* seeds are orthodox (desiccation tolerant) and their ontogeny consists of some 20 distinct developmental stages. Embryogenesis in *Arabidopsis* is completed in approximately 9 days at 25°C. During the final stage of embryogenesis, seeds must acquire desiccation tolerance to survive. As both the fruit (silique) and seeds dry, metabolism declines rapidly as water is lost. As respiratory activity slows during desiccation, electrons leak and react with oxygen to generate reduced oxygen species, so antioxidants are necessary to protect mitochondria from damage (Vertucci and Farrant 1995). Desiccation tolerance is one of the unusual properties of seeds that, despite intensive investigation, remains rather poorly understood. Free radical scavengers may provide additional protection

Transcription Factors	CSD1	CSD2	CSD3	FSD1	FSD2	FSD3	MSD1
ABRE	♦					♦	
NF-κβ	♦	♦	♦			♦	♦
Heat shock element			♦		♦		♦
Y-box			♦				♦

Table 1. A comparison of the upstream regions of the seven known Arabidopsis SODs using the identification of sequences known to bind to four transcription factors. (From Alscher *et al.* 2002.)

during desiccation because the development of desiccation tolerance, which involves a period of water stress, coincides with an increase in free radical scavengers in seeds (Vertucci and Farrant 1995; Haslekas *et al.* 1998). Water stress involves the production of ROS and their containment by antioxidants (Bohnert and Jensen 1996; Bohnert and Shevelava 1998). Thus, the availability of antioxidants such as GSH may be essential for seed maturation. Since GR is a major determinant in the maintenance of reduced glutathione levels, it is not surprising to find that seed aging has been linked to decreased GR activity (De Vos *et al.* 1994; Bailly *et al.* 1996; DePaula *et al.* 1996). Lipid peroxidation and autoxidation are oxidative reactions associated with seed deterioration and death (Walters 1998) that may be mitigated by radical scavenging systems.

Transgenic *Arabidopsis*, antisense with respect to either one of the two known GR genes (GR1 and GR2), have been created to examine the protective role of these antioxidant gene products against ROS throughout development. We have characterized transgenic antisense *Arabidopsis thaliana* (L) Heynh. plants that are depleted in the expression of one or other of the two known Arabidopsis glutathione reductase genes (*GR1,* organellar and *GR2,* cytosolic), (Grene, unpublished). Our working hypothesis is that activity levels of GR play a pivotal role in redox sensing and adjustment processes as well as a direct role in the maintenance of reduced glutathione. Abnormal seed morphologies and/or embryo abortions first appeared in antisense *GR2* plants (AN*GR2)* 8 days after flowering in at least four transformant lines. Seeds of antisense *GR1*(AN*GR1*) lines showed 33% abnormalities at maturity. An altered phenotype expressed as a change in seedling growth habit was observed in AN*GR2*, but not in antisense AN*GR1* lines. AN*GR2* lines showed an increased time to flowering. GR genes may play important individual roles in seed development, and subsequent seedling development related to the levels of glutathione and effects of ROS. Since the peroxisome contains GR, its ROS sensing mechanism(s) of peroxisomes are likely to respond to alterations in glutathione metabolism. GR1 is present in the chloroplast where it protects photosynthesis, while GR2 is thought to be cytosolic. However, GR1 may reside in the mitochondrion as well (Creissen *et al.* 1995b). Our data to date suggest the importance of both genes for seed viability and for seedling development.

Increasing glutathione biosynthetic capacity has been shown to enhance resistance to oxidative stress (Arisi *et al.* 1998; Zhu *et al.* 1999). The ability of the plant cell to maintain and increase reduced glutathione levels is an important factor in protecting photosynthesis against sulfur dioxide (Alscher *et al.* 1987), which depends at least in part on GR. Transgenic tobacco overexpressing (3x) an *E. coli* gene (*gor*) encoding GR targeted to the plastid was more resistant to paraquat and sulfur dioxide than were nontransformed plants (Aono *et al.* 1993). Over-expression of *gor* in the chloroplasts of poplar led to increases in both total glutathione pool sizes and in the ratio of GSH to GSSG. Transformed plants showed enhanced protection against chloroplast-localized oxidative stress (Foyer *et al.* 1995). Transgenic tobacco plants with increased levels of GSSG were found to grow better under salinity and chilling stress than their nontransformed counterparts, suggesting that resistance/adaptive pathways were stimulated by GSSG (Roxas *et al.* 1997).

Ascorbate and APX : Multiple roles in antioxidant defenses

APX exists as a multigene family in Arabidopsis. APX 1 and 2 are both cytosolic enzymes. A membrane associated APX has been described in the peroxisome, and also in the chloroplast (Mullen and Trelease 2000). The chloroplast contains two distinct APX enzymes as well, one which is free in the stroma, and one which is associated with the thylakoids. The expression of all of the known members of the APX gene family are affected by events originating in the chloroplast (Karpinski *et al.* 1997) . When photosynthetic tissue is shifted from low to high light (EEE), ROS are produced, in particular hydrogen peroxide. Reduced glutathione is oxidized to GSSG, and cytosolic APX2 mRNA becomes detectable. APX2 mRNA is induced within seven minutes of exposure to EEE. The induction of both APX1 and APX2 is causally related to the redox state of the plastoquinone pool (Pfannschmidt *et al.* 1999).

Sen Gupta *et al.* (1993) reported that 3-fold increases in cytosolic APX activity and APX mRNA occur in transgenic tobacco plants that overexpress chloroplast Cu-Zn SOD. This induction could be mimicked in nontransformed tobacco leaf discs with the addition of H_2O_2. Van Camp *et al.* (1996) demonstrated the existence of three separate APX isozymes in *Arabiodopsis*, two plastidic and one cytosolic, on activity gels. The data of Van Camp et al. (1996) showed that chloroplast APX1 activity was higher in transgenic plants overexpressing Fe SOD. These results suggest that increasing the level of gene product in one part of the pathway can affect other enzymes in the same pathway. This type of co-regulation may be critical in interpreting results using antisense plants where the cytosolic Cu-Zn SOD is suppressed. These data also suggest that H_2O_2 plays a role in a stress-responsive signal transduction pathway, since the presence of active Fe SOD enzyme in the

overexpressors could lead to increased levels of cellular H_2O_2. Prasad *et al.* (1994) present evidence that is compatible with a signaling role for hydrogen peroxide in the acclimation of maize seedlings to chilling stress. Recently a gene which acts to regulate APX gene expression has been found, in which glutathione levels are lower, but the glutathione biosynthetic pathway itself is unaffected (Mullineaux *et al.* 2000).

Stress-mediated changes in the abundance of a particular transcript do not always correlate with corresponding changes in antioxidant protein level and/or enzyme activities (Williamson and Scandalios 1992; Mittler and Zilinskas 1994). Paraquat-mediated increases in APX mRNA levels in pea leaves were not reflected in correspondingly large increases in APX enzyme activities (Donahue *et al.* 1997). The data of Mittler and Zilinskas (1994) for APX responses to drought also suggest the existence of stress-mediated post-transcriptional processes.

In addition to its role in the scavenging cycle, ascorbate acts as a reductant in the regeneration of α-tocopherol and in the zeaxanthin cycle (Foyer 1993) (Figure 3). A third role of ascorbate is at the thylakoid surface within the chloroplast where it acts as reductant in the APX-mediated scavenging of H_2O_2. Ascorbate is reduced to the monodehydroascorbate radical as a result of this thylakoid-associated process (Grace *et al.* 1995). Ascorbic acid is regenerated by a light-dependent process at the thylakoid that can utilize ferredoxin as the source of reductant. In cases where fully oxidized dehydroascorbic acid (DHA) is produced, reduced glutathione (GSH) is the source of reductant for the regeneration of ascorbic acid. Since APX appears to play such an essential role in the scavenging process, the processes within the chloroplast associated with oxygen uptake and APX function and the reduction of molecular oxygen have been named "Mehler-ascorbate peroxidase photorespiration" (Mullineaux *et al.* 2000) (Figure 7).

Molecular chaperones

Four distinct functions have been assigned to molecular chaperones. They can act as repair proteins, they can remove proteins that are irretrievably damaged, and they can facilitate the import of newly synthesized proteins into the interior of organelles such as the peroxisome. The fourth function is as antioxidant molecules themselves in conjunction with protein methionine-sulfoxide reductase.

Molecular chaperones interact to protect against heat and water stress through the repair of denatured proteins. Evidence is accumulating to suggest an important role for heat shock proteins/molecular chaperones in stress resistance in plant and animal systems (Gustavsson *et al.* 1999; Wehmeyer and Vierling 2000; Harndahl *et al.* 2001). Increased expression of HSPs of the 70, 101 and sHSP classes were observed in drought acclimated rooted cut-

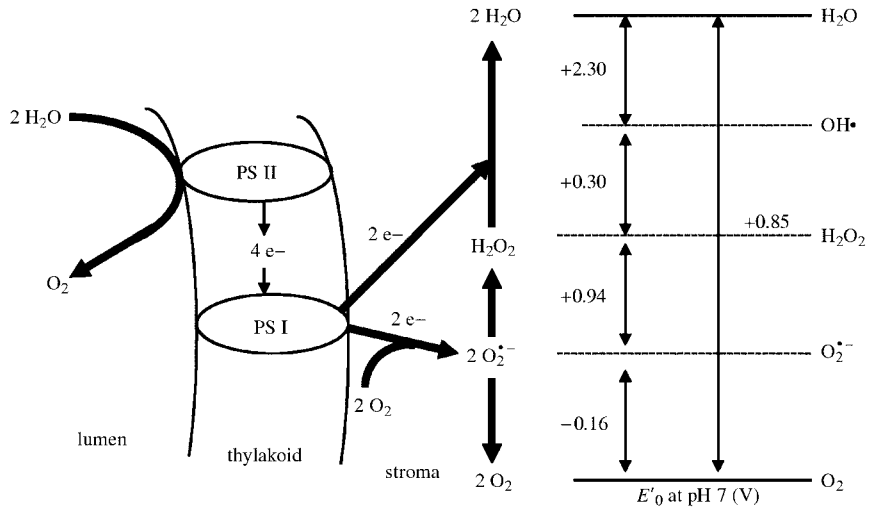

Figure 7. The Mehler peroxidase reaction (from Mullineaux *et al.* 2000). The APX-catalyzed reaction is the reduction of H_2O_2 to water using ascorbate as the electron donor. The oxidized ascorbate, monodehydroascorbate free radical or its disproportionation product dehydroascorbate, are reduced back to ascorbate using two electrons ultimately derived from PS II.

tings of loblolly pine. HSP70 is known to occur in glyoxysomes; in fact, the glyoxysomal protein is encoded by the same gene as the chloroplast form of the protein (Wimmer *et al.* 1997). The small heat shock proteins that are localized to the cytosol appear to respond to specific developmental signals associated with the acquisition of desiccation tolerance that occur during seed development (Wehmeyer and Vierling 2000). A small HSP (HSP18.1) has been shown to interact with HSP70 to reactivate heat-denatured luciferase (Lee and Vierling 2000). HSP90 was ineffective in their reactivation system, and was also found to be unresponsive to drought stress in loblolly pine (Heath *et al.* 2002). The cytosolic HSPs prevent heat-mediated and water-stress-mediated aggregation of proteins. These HSPs may prevent loss of conformation in low-water conditions and may be important in peroxisomes. Denatured substrate proteins are bound to the sHSP oligomers *in vitro* presumably by hydrophobic regions (Lee and Vierling 1998; Harndahl *et al.* 2001). It is thought that the sHSPs act to bind denatured proteins and to maintain them in a state that allows for ATP-dependent refolding by larger HSPs/molecular chaperones (Lee and Vierling 2000). The small heat shock proteins that are localized to the cytosol appear to respond to specific developmental signals associated with the acquisition of desiccation tolerance that occur during seed development (Wehmeyer and Vierling 2000). No small heat shock proteins have been specifically associated with peroxisomes or glyoxysomes to date. However, since the HSP70 present in those organelles is so similar to the corresponding chloroplast HSP70, it is possible that just such an interaction between sHSPs and the larger molecules may in fact exist. A protein of the DnaK (J) class was found to be essential for the HSP70-mediated refolding/repair mechanism (Lee and Vierling 2000). A DnaJ protein has been found in association with glyoxysomes (Diefenbach and Kindl 2000), albeit at the membrane surface. This protein reacts specifically with a particular cytosolic isoform of HSP70, and not with other forms of HSP70. Another DnaJ protein has been identified as a peroxin in a yeast system (Hettema *et al.* 1998).

Molecular chaperones and methionine sulfoxidation. Surface methionine residues are preferentially oxidized in proteins. Methionine residues act as an antioxidant protein reservoir [Figure 8 adapted from Hoshi and

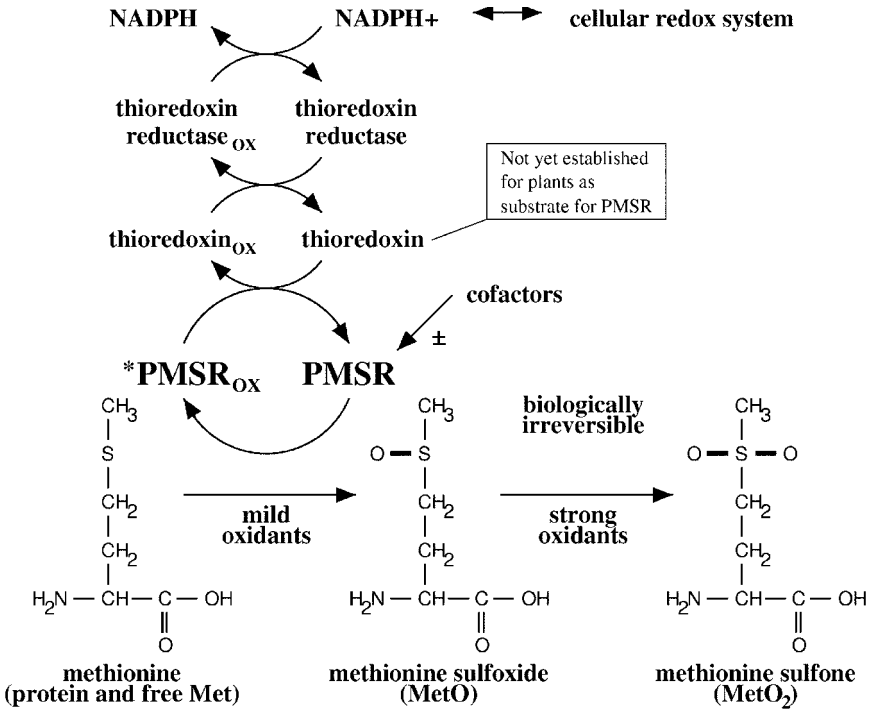

Figure 8. Oxidation and reduction of methionine residues as an antioxidant reservoir. (Adapted from Hoshi and Heinemann, 2001.) *Cytosolic and chloroplast forms of PMSR are known in plants. The thioredoxin activation system in the chloroplast is well known as a source of reductant for redox regulated stromal enzymes.

Heinemann (2001)]. Amino acid residues in proteins are one of the major targets of ROS attack. The side chains of methionine and cysteine are more sensitive to oxidation than the side chains of other amino acids. This differential sensitivity has been exploited by nature to create a protective mechanism against ROS-mediated protein damage. Cysteine residues are maintained in a reduced state through the action of glutathione. Surface-exposed methionine residues are available for oxidation by molecules such as hydrogen peroxide, thus effectively lowering the degree of the threat. In the case of glutamine synthetase from *E. coli*, it was found that 8 of the 16 methionine residues present in the protein could be oxidized with little effect in overall enzyme activity (Levine *et al.* 1999). They are re-reduced through the action of protein methionine sulfoxide reductase (PMSR), using thioredoxin as a source of reductant (Lowther *et al.* 2000; Hoshi and Heinemann 2001). PMSR is a highly conserved protein from *E.coli* to human (Brot and Weissbach 2000). Combined with the reductive action of PMSR, which restores the methionines to their original state, a larger mechanism within the cell is now able to maintain a stable configuration of protein state. It is thought that the degree to which methionine oxidation occurs may be underestimated due to imprecision of assay methods (Squier and Bigelow 2000). A proposed overall scheme for redox regulation of cellular defense processes involving thiol redox control is represented in Figure 1. sHSPs contain a unique methionine rich domain at its N terminus, which exists as an amphipathic alpha helix. Some of these met residues exist at the surface of the protein, and are oxidized very readily, with resultant loss of chaperone activity (Harndahl *et al.* 2001). The chloroplast HSP21 is easily and reversibly oxidized by the same concentration of hydrogen peroxide as that which brings about HSP21 oxidation *in vivo* in Arabidopsis leaves (Harndahl *et al.* 1999). The oxidation of surface methionine residues, which is mediated by the hydroxyl radical, ozone, and poeroxynitrile, as well as hydrogen peroxide, has been proposed to function in an antioxidant capacity in animal and yeast systems (Moskovitz *et al.* 1997; Preville *et al.* 1999).

Molecular chaperones can interact with glutathione to protect against oxidative stress. Mehlen *et al.* (1996) and Garrido *et al.* (1998) present evidence of an interaction between sHSPs and glutathione in mammalian cells that resulted in increased resistance to cell death induced by tumor necrosis factor or by hydrogen peroxide. Resistance was dependent both on increases in reduced glutathione and on increases in expression of sHSPs, and was shown to decrease the levels of cellular ROS. Preville *et al.* (1999) demonstrated that sHSPS can protect against oxidative stress in L929 cells. This interaction has not yet been investigated in plant systems. However, much evidence points to the importance of glutathione biosynthesis in protection against ROS damage (Alscher 1989; Noctor *et al.* 1998; Noctor and Foyer 1998; May *et al.* 1998a).

Redox sensing and photosynthetic function

Studies of the responses of foliar tissue to oxidative stress have focused primarily on the photosynthetic machinery. Within the chloroplast, the rapid loss of oxygen evolution activity in the presence of Cu(II) was found to be mainly due to the formation of OH· radicals from superoxide ion via a Cu(II)-catalyzed Haber-Weiss mechanism (Yruela *et al.* 1996). Hydrogen peroxide and lipid hydroperoxides, other potentially toxic ROS, are also generated. Hydrogen peroxide can cause DNA breakage, as described above, and also can inactivate thiol-containing enzymes such as the thioredoxin-modulated enzymes of the chloroplast stroma (Hagar *et al.* 1996). An imbalance in which the redox steady state of the cell is altered in the direction of pro-oxidants can result in the potentially dangerous univalent reduction of molecular oxygen to the superoxide anion radical described above. In the case of photosynthetic electron transport, oxygen uptake associated with the photoreduction of oxygen to superoxide is called the Mehler reaction, in honor of its initial discoverer (Figure 7). Changing environmental conditions such as vicissitudes of temperature, humidity, water availability, salt stress, or light intensity can lead to increased production of ROS within the chloroplast. Damage of leaves due to air pollutants such as sulfur dioxide and ozone or photodynamic herbicides such as paraquat is also mediated through the production of ROS (Mehlhorn *et al.* 1990; Foyer and Mullineaux 1994; Okpodu *et al.* 1996). The site of action of sulfur dioxide, photodynamic herbicides, and high light is the chloroplast, whereas ozone and most pathogens act in the extracellular space. Species, biotypes, or cultivars often show cross-resistance to two or more oxidative stresses (e.g. paraquat, high light, sulfur dioxide). These observations have been used to propose a common basis for resistance against ROS (Gressel and Galun 1994). However, ozone tolerance was not correlated with resistance to paraquat in tobacco. A first hypothesis might be that cross-resistance occurs between stresses which originate in the same subcellular compartment (e.g., the chloroplast in the case of paraquat and sulfur dioxide) but not between stresses which have disparate sites of action within the plant leaf (e.g., ozone and paraquat).

Redox sensing plays a central role in the interaction of

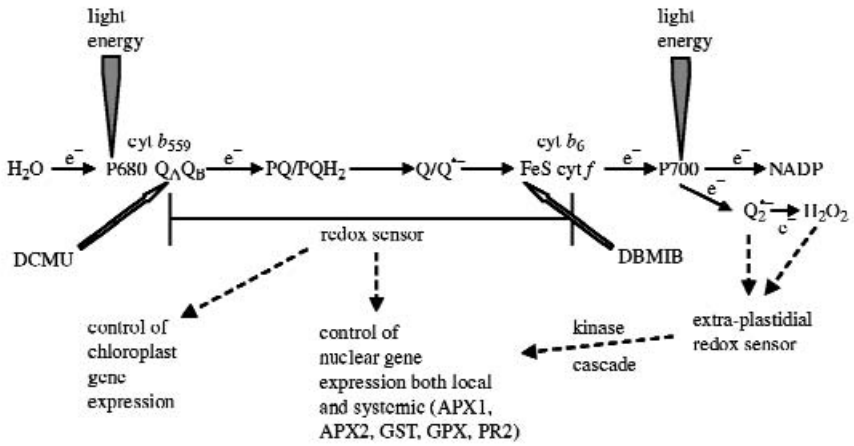

Figure 9. Redox control of gene expression in green cells. The Mehler peroxidase reaction, which generates superoxide from O_2 at PS I, is one possible source of ROS which, along with the PQ-associated redox sensor, regulates antioxidant defense gene expression. (From Mullineaux *et al.* 2000.)

oxidative stress, in particular, excess excitation energy (EEE), with the photosynthetic machinery (Figure 9) (Karpinski *et al.* 1997; Karpinski *et al.* 1999; Pfannschmidt *et al.* 1999; Karpinska *et al.* 2000; Mullineaux *et al.* 2000). Under light saturating conditions, only 10% of absorbed light energy is used to fix carbon (Mullineaux *et al.* 2000). The remaining energy must be dissipated in non-destructive ways. Under these conditions, or under conditions where carbon dioxide or water are limiting, redox changes associated with the plastoquinones are thought to act as environmental sensors, inducing signal transduction pathways that result in the activation of defense genes such as cytosolic APX, among others. Redox sensing via the plastoquinone pool affects both chloroplast and cytosolic gene expression.

Redox sensing: the central role of hydrogen peroxide

Exposure to hydrogen peroxide has been reported to result in the induction of at least 100 genes in Arabidopsis (Neill *et al.* 2001). Hydrogen peroxide accumulation in barley that had been inoculated with powdery mildew was shown to induce glutathione biosynthesis during the hypersensitive response (Vanacker *et al.* 2000). Molecular chaperones, glutathione-S-transferases, various protein kinases, and redox-sensitive transcription factors (Pastori and Foyer 2001) are known ROS-responsive genes. Some of these genes, e.g. the molecular chaperones, are also induced by long term exposure to drought (Heath *et al.* 2002). Hydrogen peroxide is thought to exert its inductive effect through oxidation of cysteine and methionine residues, oxidation of particular membrane lipids which act as oxylipin receptors in a signaling cascade that involves glutathione (Hamberg 1999; Ball *et al.* 2001), and direct influences on protein kinase cascades (Kovtun *et al.* 2000). Kovtun *et al.* (2000) demonstrated the existence of a hydrogen peroxide mediated protein kinase cascade, which signals the activation of defense genes such as HSP18 (small heat shock protein) and glutathione S-transferase.

Hydrogen peroxide has been shown to participate in the systemic acclimation response demonstrated by Karpinski et al. (1999) in which partial exposure of low light adapted Arabidopsis plants to EEE results in acclimation to high light in unexposed leaves (Figure 10). Since more hydrogen peroxide is produced in the peroxisome as photorespiration increases, the peroxisome may contain a redox sensing mechanism in addition to the PQ redox- sensing mechanism in the chloroplast. If EEE-exposed leaves are pre-treated with glutathione or ascorbate, APX1 and 2 induction is less, and the leaves become more susceptible to oxidative stress. The application of catalase, but not SOD, leads to the disappearance of the systemic acclimation phenomenon, confirming the importance of hydrogen peroxide in the signaling pathway. Treatment of leaves with

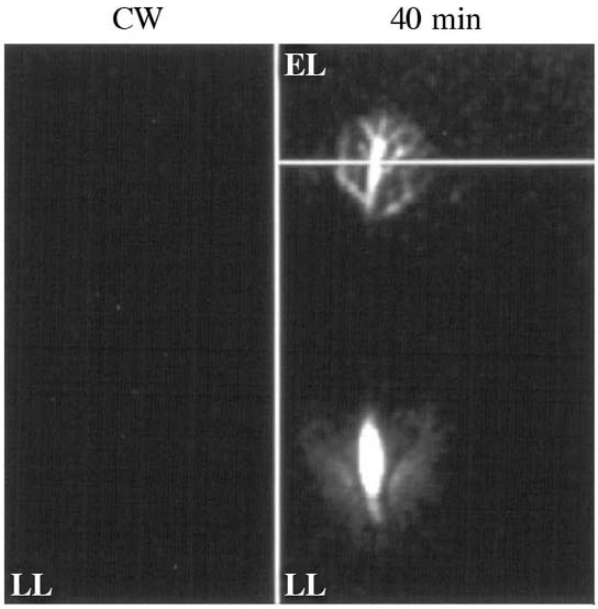

Figure 10. Systemic acquired acclimation. Luciferase expression in a detached wounded leaf from a five-week-old short-day-grown Arabidopsis rosette transgenic for an APX2-promoter-LUC fusion. Leaves were detached from the plant at the petiole and were subjected to a series of parallel slashes in the lowest quarter of the leaf. EL was applied for 40 min to the top one-quarter of the leaf, the remainder being shaded with aluminum foil. At the end of the light stress period, the leaves were sprayed with luciferin and 30 min later an image was recorded using a charged couple device camera. Abbreviations are as follows: CW, control wounded leaf; EL, excess light; LL, low light; LUC, luciferase. (From Karpinski *et al.* 1999.)

hydrogen peroxide prior to their exposure to EEE results in greater resistance to photooxidative damage, presumably because of the induction of defense responses prior to exposure to stress (Karpinska *et al.* 2000).

Hydrogen peroxide mediated peroxisomal biogenesis. Lopez-Huertas *et al.* (2000) reported a stimulatory effect of hydrogen peroxide on peroxisomal biogenesis. Hydrogen peroxide is both a means of induction of stress resistance, and a product of stress imposition in the plant cell. Consequently, it has been widely used as a tool to investigate the mounting of defense mechanisms and the response of defense pathways. In the case of peroxisomes, their response to the presence of hydrogen peroxide consists in an increase in peroxisomal biogenesis. A major component of peroxisomal biogenesis is the import of cytosolic-synthesized proteins into the interior of the organelle. Import of proteins into the peroxisome is known to involve at least one molecular chaperone — an HSP70 (Corpas *et al.* 2001). The HSP70 interacts with a DNA J-like protein at the peroxisomal surface. Since hydrogen peroxide was used as the only representative ROS in the peroxisomal proliferation experiments, there is no information as yet on effects of other ROS on peroxisomal biogenesis. Charged species such as the superoxide anion cannot cross membranes, and thus are not so likely as candidates for mediating signals across bounding membranes. The inhibition of catalase by the superoxide anion would result in increased levels of hydrogen peroxide, however, which could constitute the initiation of a peroxisome-specific signaling cascade. Peroxisomal proliferation also occurs during senescence (Corpas *et al.* 2001) and may result from the well-documented increases in ROS levels that occur with aging.

Prospects for improving stress resistance

The emerging picture of ROS-mediated cellular events and redox control of gene expression is complex (Figure 11). Glutathione remains a prime candidate for engineering increased stress resistance in plant cells, especially in organelles such as the peroxisome where ROS are produced at high levels. Since the ascorbate/glutathione scavenging pathway is present in peroxisomes, chloroplasts, and mitochondria, it is likely that glutathione levels are high in all these subcellular compartments, and that a mechanism for providing GSH to the organelle exists. Transport of glutathione across the peroxisomal and mitochondrial bounding membranes is one possible focus for engineering increased stress resistance. Engineering increased flux through the cytosolic glutathione biosynthetic pathway is another possibility; a strategy that has proved to provide increased protection to photosynthesis in poplar (Foyer *et al.* 1995). HSP70s are known to function in transport of proteins into the interior of the peroxisome, and a member of the HSP70 gene family has been identified in the interior of the organelle. It is not yet known if the peroxisome contains sHSPs as well as HSP70s. Exposure to hydrogen peroxide results in the induction of peroxisomal biogenesis. Kovtun *et al.* (2000) demonstrated the existence of a hydrogen peroxide mediated protein kinase cascade, which signals the activation of defense genes such as HSP18 (small heat shock protein) and glutathione S-transferase. The ROS-sensing mechanism that elicits peroxisomal biogenesis is unknown, but it could also involve sHSPs and glutathione in a manner analogous to the mammalian system.

Although a cytosolic and a plastidic form of PMSR have been described in plants (Sadanandom *et al.* 2000), little information concerning their respective roles in antioxidant defense is yet available. A search for the in *vivo* substrates of PMSR in the peroxisome and the cytosol could yield valuable information. The PMSR mechanism may constitute an important additional antioxidant mechanism. Peroxisomal HSPs containing the methionine rich region present in HSP21 found in plastids are good candidates for PMSR substrates. This mechanism may also form part of the ROS sensing signaling process that gives rise to peroxisomal biogenesis. Taken together, the overall goal would be to improve the interactions shown in Figure 11 so as to increase the speed and efficiency of the signaling pathways that give rise to the mobilization of antioxidant defense mechanisms. Thiol redox control is proposed to play an essential and central role in mediating plant cell antioxidant responses to the imposition of oxidative stress.

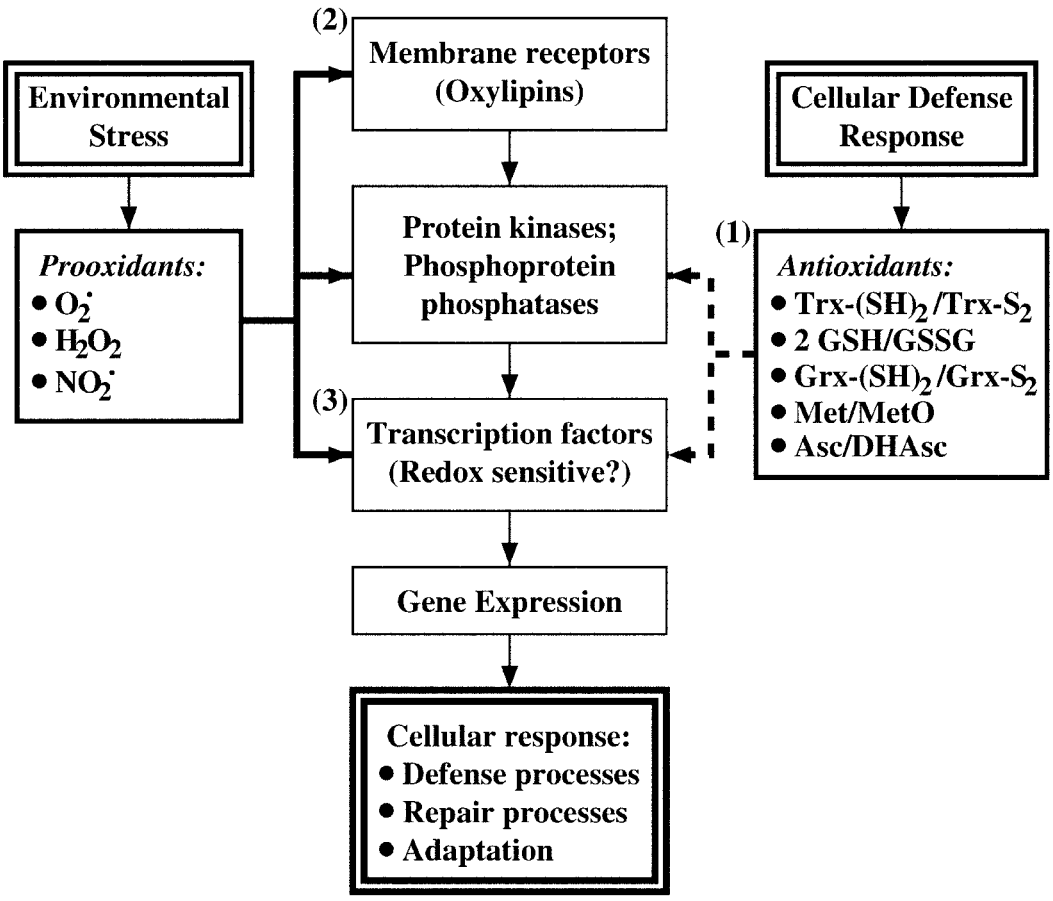

Figure 11. Redox regulation of gene expression. (1), (2) and (3) can function as redox sensors. (Adapted from Hoshi and Heinemann 2001)

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