

# Hydrogen Peroxide and Plant Stress: A Challenging Relationship

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## ABSTRACT

The relationship between plants and hydrogen peroxide is a challenging one: H<sub>2</sub>O<sub>2</sub> has many essential roles in plant metabolism but at the same time, accumulation related to virtually any environmental stress is potentially damaging. In this review, I consider H<sub>2</sub>O<sub>2</sub> physiology broadly, both as a stress and as a developmentally and physiologically important metabolite, including its sources and mobility, and the vexing question of tissue level concentrations. I then consider problems associated with H<sub>2</sub>O<sub>2</sub> as a signaling molecule, including mechanisms of H<sub>2</sub>O<sub>2</sub> sensing, signaling, and response networks. Finally, I discuss recent advances in transcript network modeling, and complex systems approaches to understanding the interactions between the transcriptome, proteome and metabolome in responses to H<sub>2</sub>O<sub>2</sub>.

**Keywords:** amine oxidase, apoplast, complex systems modeling, NAD(P)H oxidase, peroxidase, transcriptome, photosynthesis, proteome, mitochondria, signaling cascade

**Abbreviations:** APX, ascorbate peroxidase; CAT, catalase; DAO, diamine oxidase; GLP, germin-like protein; HR, hypersensitive response; MAO, monoamine oxidase; MAPK, mitogen-activated protein kinase; PAO, polyamine oxidase; POX, peroxidase; ROS, radical oxygen species; SOD, superoxide dismutase

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## INTRODUCTION

It is now well established that virtually all biotic and abiotic stresses induce or involve oxidative stress to some degree, and the ability of plants to control oxidant levels is highly correlated with stress tolerance. Whether the antioxidant approach to explaining tolerance will, in the long run, be any better as a single factor explanation than, say, the ability to exclude Na from shoots and maintenance of K/Na discrimination ratios has been for salt tolerance, or the ability to accumulate compatible osmotica has been for drought tolerance, remains to be seen. It is now clear, however, that any stress condition or significant change in environment is associated with up- or down-regulation of hundreds of genes, that some proteins important to oxidative metabolism may have high stabilities and low turnover rates, and that even the cell wall, once considered of little biological importance, contains hundreds of proteins and metabolites, many of which may be involved in oxidative

metabolism.

At the same time, it is also well established that oxidative metabolism, and particularly H<sub>2</sub>O<sub>2</sub>, is involved in a wide variety of reactions and signaling cascades necessary for all aspects of plant growth and the integration of activity, ranging from the develop of individual root hairs, to xylem differentiation and lignification, to wall loosening and wall cross-linking, to root/shoot coordination and stomatal control. Thus, while the involvement of H<sub>2</sub>O<sub>2</sub> in stress responses is of particular interest, it really must be considered in the context of, and even as a special case of, H<sub>2</sub>O<sub>2</sub> involvement in “normal” growth and metabolism.

Overall, the current “fashion” in plant stress studies is to grow plants in controlled conditions, apply a stress rather suddenly after a period of unstressed growth, and then compare some aspect or aspects of response – ranging from activity of a single enzyme to whole genome transcript networks – at a fixed time thereafter. Unfortunately for plant biologists, but fortunately for plants, such environmental

perturbations are not typical of the real world, and models built on them may be, as Manfred Eigen put it, “right, but irrelevant” (Eigen 1973). Much more difficult is understanding the small, minute-to-minute or day-to-day variations which preclude the necessity to respond dramatically, and developing relevant models that include them.

My objective for this review is to consider the physiology of  $H_2O_2$  as it relates to plant stress responses, but to do so in a way that recognizes “normal” activities. To do this, I will acknowledge generality more than details of specific responses, and as many of the  $H_2O_2$ -related “tools” that plants have as possible, not just those for which the most detailed genetic models have been derived. This also means that important aspects of stress response that are only indirectly or distantly down-stream related to  $H_2O_2$  will be emphasized less. This effort will, of necessity, leave out references to many reports: there have been more than 2100 journal articles on the topic of peroxide in plants since 2000 alone, and the number is increasing rapidly.

### What is $H_2O_2$ ?

Hydrogen peroxide is the two electron reduction product of  $O_2$ . It is potentially reactive oxygen, but not a free radical (Halliwell *et al.* 2000). By comparison with superoxide,  $O_2^{\cdot-}$ , and certainly by comparison with the hydroxyl radical,  $\cdot OH$ ,  $H_2O_2$  is relatively “safe”: in the absence of transition metals, it is stable and unreactive, even at concentrations much higher than a biological system would ever generate. Functionally, this imparts on it greater mobility within tissues, and potential utility not only as a substrate in a variety of reactions, but as a molecule for ROS-related signaling.

However,  $H_2O_2$  is potentially quite reactive with molecules containing  $Fe^{2+}$  or other transition metals, through the Fenton reaction (Becana *et al.* 1998). The “evil” result of this reaction is the homolysis of  $H_2O_2$  to 2  $\cdot OH$ , and  $H_2O_2$  toxicity is most commonly associated with that action. For example, inhibition of Rubisco by exogenous  $H_2O_2$  results from the fragmentation of the LSU at a glycine in the catalytic site (Ishida *et al.* 1999), dependent on the activation state of the enzyme. An identical fragmentation pattern occurred in intact chloroplasts when oxidant scavenging systems were inhibited (Ishida *et al.* 1999), or when cold-sensitive maize leaves were exposed to low temperatures (Kingston-Smith *et al.* 1999), as a consequence of the fact that chloroplasts contain as much as 80% of the Fe in a plant, and are a good source of radical oxygen species (ROS) generally. Similar Fenton reaction mechanisms have been associated with  $H_2O_2$  (or actually,  $\cdot OH$ ) sensitivity of FeSOD (Bhattacharya *et al.* 2004), and glutamine synthase (Farber and Levine 1986), among other enzymes. By contrast, direct reaction of  $H_2O_2$  with the  $-SH$  groups has been suggested as the mechanism by which  $H_2O_2$  inactivates fructose biphosphatase (Charles and Halliwell 1980, 1981) and sedohepuloose biphosphatase (Wise 1995; Tamoi *et al.* 2006) in chloroplasts, and cytosolic glyceraldehyde 3-phosphate dehydrogenase (Brodie and Reed 1987; Hancock *et al.* 2005).  $H_2O_2$  toxicity is reduced by removing it enzymatically (i.e. by catalase or ascorbate peroxidase), or by complexing Fe(III) and Fe(II) with compounds such as tannic acid and proanthocyanidins, thus preventing  $\cdot OH$  generation (Toda 2005; Andrade *et al.* 2006).

### WHAT ARE THE RELEVANT TISSUE LEVELS OF $H_2O_2$ ?

The basic question here is: how much  $H_2O_2$  is there in plant tissue, and against what background might changes be useful signals? Given that there are so many good methods for assaying  $H_2O_2$  in solutions, some of which are quite specific, it is surprising that there is such a wide range of estimates in plant tissues, spanning nearly seven orders of magnitude, and no apparent consensus concerning how large a stress or treatment related change is physiologically significant. At the low end, Hernández *et al.*

(2001) reported tissue levels ranging from 10 to 150 pmol/gFW in the pea leaf apoplast with the difference (salt induced) being sufficient to cause oxidative lesions. At the other extreme, He *et al.* (2005) reported concentrations in *Poa pratensis* leaves as high as 1.3% of the dry weight, which, based on data in their report, was ca. 60  $\mu mol/gFW$  or 100 mM on a leaf water basis. In maize, Tewari *et al.* (2004) reported concentrations of 20  $\mu mol/gFW$ , rising to 75  $\mu mol/gFW$  with N deficiency. Ben Amor *et al.* (2006), in an interesting study of the coastal halophyte, *Cakile maritima* also reported tissue  $H_2O_2$  contents on the high end, as much as 45  $\mu mol/gFW$ . Veljovik-Jovanovic *et al.* (2002) were the first to recognize possible interferences by plant constituents with  $H_2O_2$  assay protocols, and suggested that leaf levels should generally be less than 0.1  $\mu mol/gFW$ . On the other hand, an analysis of field grown plants, with care to account for potential interferences as well as continued metabolism of  $H_2O_2$  after harvesting, suggested that values in the 1-5  $\mu mol/gFW$  range might be normal (Cheeseman 2006).

Similarly confusing is what it means – in terms of tissue level  $H_2O_2$  concentrations – to have an “oxidative burst”. This issue is undoubtedly complicated by the rapidity of  $H_2O_2$  turnover both *in planta* and after tissue harvesting (Cheeseman 2006). In response to an acute ozone exposure, (200 ppb/ 2 hr), Chen and Gallie (2005) reported tobacco (cv. ‘Xanthi’) leaf  $H_2O_2$  levels had increased ca. 4x (in the 100 nmol/gFW range, using plants grown with as little prior ozone exposure and potential irradiance stress as possible), but increased another four to five-fold after 24 hr recovery. Karpinski *et al.* (1997) on the other hand, reported an oxidative burst in *Arabidopsis* with exposure to excess irradiance - in this case, ten-fold higher than their growth irradiance of 200  $\mu mol\ m^{-2}\ s^{-1}$  - that increased the leaf content from about 5  $\mu mol/gFW$  to less than 7  $\mu mol/gFW$ . The issue here is only partly the order of magnitude and the percent change. The more critical question – which I can not answer – is, what background levels and what sort of changes are needed to support the role of  $H_2O_2$  in signaling, especially if measurements are limited, in practice, to bulk tissue levels?

### WHAT PRODUCES $H_2O_2$ ?

$H_2O_2$ , and ROS generally, are a fundamental fact of life in an aerobic environment (Moller 2001). Understanding the role of  $H_2O_2$  in plant growth or stress responses requires models that accommodate the large number of ways in which it can be formed and degraded at any given time, and that ROS produced by one source may be the drivers or substrates for a second (Allan and Fluhr 1997). Major sources include misfires in the electron transport chains of chloroplasts and mitochondria, the Mehler reaction, a wide variety of limited substrate oxidases, type III peroxidases, and NAD(P)H oxidases (Halliwell and Gutteridge 1999). Some of these produce  $H_2O_2$  directly, and others only via more reactive intermediates (e.g.  $\cdot O$  or  $O_2^{\cdot-}$ ). Broadly, these events are enhanced by stresses (Alscher *et al.* 1997; Bolwell 1999), although they occur as an integral part of many facets of plant development.

### Excess light and other energy imbalances

It is impossible to envision any environmental effect, whether or not we or plants recognize it as a stress, that does not reflect the energy available to respond or imbalances in energy availability, energy transduction and energy metabolism. This necessarily links ROS metabolism with all aspects of plant life. Oxidative stress associated with photosynthesis is a potential problem any time, but especially when the capacity for electron transport exceeds the capacity for recycling the NADPH and ATP which result. This is likely at irradiances above light saturation, but also at lower irradiance when stomates (for example) limit  $CO_2$  supplies. This problem is reduced by activity of the xantho-

phyll cycle, (e.g. Demmig-Adams *et al.* 1999), but the effectiveness is not complete. In that case, the Mehler-Ascorbate Peroxidase, or water-water cycle also contributes to damage prevention (see Allen 1995; Asada 1999; Heber 2002). This has been modeled as operating through the sequential actions of superoxide dismutase (SOD – generating  $H_2O_2$ ), ascorbate peroxidase (APX – reducing  $H_2O_2$  at the expense of ascorbate) and glutathione reductase (GR – regenerating ascorbate at the expense of reduced glutathione). Oxidized glutathione, and the monodehydroascorbate radical can both be re-reduced by NADPH, both allowing the cycle to continue, and reducing the electron pressure for  $O_2$  generation.

That both the xanthophyll cycle and the water-water cycle are important under field conditions has been clearly demonstrated using cultivated (e.g. Logan *et al.* 1998a, 1998b) or wild-grown plants (Streb *et al.* 1997; Logan *et al.* 1998c; Streb *et al.* 1998). Moreover, the interplay between nutrient and  $CO_2$  availability has been demonstrated using FACE (free-air  $CO_2$  enrichment) studies (Polle *et al.* 1997). In the latter case (using three year old *Fagus sylvatica*), “intrinsic oxidative stress” was reduced when photosynthesis was favored over photorespiration at elevated  $CO_2$ , and modulated by relative nutrient resource availability and assimilation.

Intracellular ROS scavenging is both highly efficient and adaptable, and  $H_2O_2$  related stress, or its prevention, resulting from activities in the chloroplasts, mitochondria or other organelles, reflects the integration of all cellular activities. For example, following application of the catalase (CAT) inhibitor, 3-aminotriazole in *Arabidopsis*, oxidant damage was limited by increases in the activities of the pre-existing APX1 and GR1 isoforms (Kang *et al.* 1999) or increased transcription of cytosolic APX (Morita *et al.* 1999). Pea also showed adjustments in the light-independent photosynthetic pathways – net photosynthesis, the RuBP regeneration rate and carboxylation efficiency all declined (Amory *et al.* 1992). Photorespiratory carbon flow was reduced (as indicated by an increase in the formate pool), preventing its return to the Calvin cycle. However, only when the capacity for  $H_2O_2$  reduction was additionally challenged by enhanced photorespiratory conditions, did  $H_2O_2$  concentrations increase. Similar manipulations have also been accomplished using antisense techniques. For example, tobacco was engineered to reduce APX and CAT expression, individually and together (Rizhsky *et al.* 2002). Double antisense plants compensated, preventing oxidative stress, by suppressing photosynthetic activity, up-regulating the pentose phosphate pathway, increasing monodehydroascorbate reductase activity, and inducing a chloroplast homologue of the mitochondrial alternative oxidase. Interestingly, the response network was less complete in plants that lacked only APX or CAT, rendering them more sensitive to oxidative stress.

The emphasis on scavenging may no longer be sufficient, however (Foyer and Noctor 2003). Rather, the involvement of  $H_2O_2$  in signaling demands closer attention: redox cascades in both chloroplast and mitochondrial electron transport chains, and the redox states of compounds including thioredoxins, ascorbate and glutathione, carry information in addition to electrons. Indeed, the cellular redox state may have precedence over ATP production: a *Nicotiana glauca* mutant defective in mitochondrial complex I, for example, compensated through antioxidant crosstalk, a whole network response involving mitochondria and other organelles, maintaining whole cell redox balance (Dutilleul *et al.* 2003). This included markedly increased alternative oxidase (AOX) activity, and enhanced oxidative stress tolerance. Cytosolic APX and glutathione reductase, mitochondrial MnSOD, and two isoforms of CAT also showed substantial increases in transcript levels. Similarly, when low-light-acclimated ( $200 \mu mol m^{-2} s^{-1}$ ) *Arabidopsis* plants were exposed to excess light ( $2000 \mu mol m^{-2} s^{-1}$ ) for 1 hr, inducing reversible photoinhibition, signal transduction reflecting the redox status of the

plastoquinone (PQ) pool, led to elevated expression of two cytosolic ascorbate peroxidases. Preventing the change in the PQ redox poise by supplying reduced glutathione enhanced photoinhibition and prevented the APX transcriptional changes (Karpinski *et al.* 1997).

In mitochondria, the alternative oxidase (AOX) provides an additional way of reducing ROS production which has too often been overlooked in stress response studies (Wagner 1995; Popov *et al.* 1997). Mitochondrial ROS production is particularly associated with electron transfer between the multiple Fe-S centers and cytochromes in Complexes I and III. Under conditions of surplus electron supply or limitations in ATP consumption, AOX and the non-proton-pumping NADH dehydrogenases on the matrix side of the inner membrane function to limit mitochondrial ROS production by keeping the electron transport chain relatively oxidized and minimizing the number of individual electron transfers (e.g. Baxter *et al.* 2007). Antioxidant enzymes in the matrix, together with small antioxidants such as glutathione, help remove ROS that are formed. The antioxidants are kept in a reduced state by matrix NADPH produced by NADP-isocitrate dehydrogenase and non-proton-pumping transhydrogenase activities (e.g. Purvis and Shewfelt 1993; Popov *et al.* 1997; Braidot *et al.* 1999; Maxwell *et al.* 1999; Casolo *et al.* 2000). AOX is induced by a number of stresses (e.g. Farrar and Rayns 1987; Parsons *et al.* 1999; Xie and Chen 1999). If these defenses are overwhelmed, as can occur during both biotic and abiotic stress, the mitochondria may be damaged. This can be induced, for example, by inhibition of AOX with salicyl hydroxamic acid (SHAM) or propyl gallate, stimulating  $H_2O_2$  production with the same substrate dependence as inhibition of CN-insensitive respiration (Popov *et al.* 1997). Antisense suppression of AOX also leads to significantly higher ROS production, while AOX over-expression has the opposite effect (Maxwell *et al.* 1999; Parsons *et al.* 1999).

As important as chloroplast and mitochondrial electron transfer are in generation of ROS, they are not the only sources. Indeed, multiple sources may be involved in many, if not all, stress reactions, and different sources may be important in different species (e.g. Allan and Fluhr 1997; Bolwell *et al.* 1998).

### Limited substrate oxidases

Limited-substrate oxidases such as glycolate oxidase in peroxisomes, and xanthine oxidase and urate oxidase in glyoxisomes, are flavin-containing enzymes which directly produce  $H_2O_2$  (as opposed to indirect production via  $O_2$ ) (Delrio *et al.* 1992). Recently, a  $H_2O_2$ -producing sulfite oxidase has also been identified, localized to peroxisomes (Hansch *et al.* 2006).  $H_2O_2$  produced in these organelles is usually quickly consumed by catalase, although isoforms of ascorbate peroxidase (APX3) localized to the organelles may also contribute to its control (Wang *et al.* 1999). Other flavin-containing oxidases important in specific compartments or tissues include a variety of monoamine (MAO) and polyamine (PAO) oxidases, and germin-like proteins. For example, in maize, flavin-containing polyamine oxidases have been identified especially in cell types destined for lignification (Cona *et al.* 2005; Paschalidis and Roubelakis-Angelakis 2005; Cona *et al.* 2006a, 2006b). Production of polyamines under water or low-temperature stress has also been correlated with protection against oxidative stress (e.g. in chickpea – Nayyar and Chander 2004). The maize PAO is, like many flavin-containing enzymes, DPI sensitive, but its activity can be differentiated from others sensitive to the inhibitor (such as NAD(P)H oxidases) by the fact that  $H_2O_2$  is released on supply of spermidine or other polyamines, and that it is sensitive to phosphatase inhibitors. Diamine (copper-containing) oxidases, (DAO) using putrescine and cadaverine (diamines) or spermidine (triamine) in the apoplast as their substrates, are also important in  $H_2O_2$  production for lignification, as well as being induced in response to fungal elicitors and wounding and in

cells destined for programmed cell death (Angelini *et al.* 1996; Moller and McPherson 1998; Laurenzi *et al.* 2001; Langebartels *et al.* 2002).

In tobacco (*Nicotiana tabacum* cv. 'Xanthi'), ornithine decarboxylase, genes involved in polyamine biosynthesis, and polyamine oxidase activities were up-regulated in response to tobacco mosaic virus infection. These were quantitatively related to the magnitude and size of the hypersensitive response (HR) and HR-like cell death (Yoda *et al.* 2003). Inhibiting polyamine biosynthesis with  $\alpha$ -difluoromethyl-ornithine, or apoplast-localized PAO synthesis by RNAi, suppressed  $H_2O_2$  production and prevented cell death (Yoda *et al.* 2006). Note, however, that amine oxidases are also constitutively present in the apoplast (Liu *et al.* 1995), and different enzymes (e.g. diamine vs. polyamine oxidases) show different patterns of constitutive and pathogen-induced expression (Asthir *et al.* 2004). In *Mesembryanthemum crystallinum*, NaCl shock activated both diamine oxidase and guaiacol peroxidase, as did exogenous cadaverine (Shevyakova *et al.* 2006). Thus, at least in some cases, it appears that synthesis of the amines themselves is the controlling factor in responses, rather than synthesis of the enzymes (Rea *et al.* 2004).

An alternative to polyamines as a substrate for extracellular  $H_2O_2$  production is the organic acid, oxalate. Oxalate oxidase (germin or germin-like protein, GLP) functions in this role, generating  $H_2O_2$  for the purpose of peroxidase-mediated wall cross linking (Caliskan *et al.* 2004), in association with wall formation by protoplasts, and in response to wounding (Bernier and Berna 2001; Le Deunff *et al.* 2004). It also increases resistance to certain pathogens, e.g. the oxalate producing *Sclerotinia sclerotiorum* (Cober *et al.* 2003; Hu *et al.* 2003), and in plants lacking the enzyme, oxalate has been shown to inhibit  $H_2O_2$  production (Cessna *et al.* 2000). In transgenic sunflowers, however, in addition to increased resistance to the fungus, low constitutive expression of oxalate oxidase activated a suite of defense genes and higher expression led to HR-like lesions (Hu *et al.* 2003). Treatment of tobacco (cv. 'Petit Havana SR1') with 100 mM NaCl also led to increased apoplastic accumulation of the protein (Dani *et al.* 2005).

As the name suggests, GLP also plays a role in seed germination. Combining *in vitro* germination experiments with data on emergence potential of sugar beet (*Beta vulgaris*) in the field, de los Reyes and McGrath (2003) screened for germination-enhancing and stress-induced genes. In accessions with superior germination potential, GLP gene expression, oxalate oxidase activity, and  $H_2O_2$  content (but not catalase activity), were induced under flooding, salt, osmotic, or oxalate treatment. In this case,  $H_2O_2$  production promoted germination, and partially compensated for salt or osmotically-related inhibitions. Accessions with poorer rates of germination had correspondingly lower activity of oxalate oxidase.

### Type III peroxidases

Unlike APX which is largely intracellular and involved in the control of cellular  $H_2O_2$  levels (Veitch 2004), type III peroxidases (POX) are more frequently secreted into the apoplast and involved in phenolic metabolism using  $H_2O_2$  as a substrate. Despite their classification in one group, they perform a wide diversity of functions, inspiring their comparison to a Swiss army knife (Passardi *et al.* 2005). In part, this is possible because of their large number. In *Arabidopsis*, for example, there are 73 POX genes and their products are found in the cytosol and vacuole as well as in the apoplast (Mittler *et al.* 2004). Peroxidases show tissue and developmental specificity (Kay and Basile 1987; Perez and Burgos 2004) and vary with respect to substrate specificity and pH optima (Bestwick *et al.* 1998). The presence of multiple peroxidase isoforms with different substrate specificities can affect cell wall composition, cell wall rigidity, the wall redox environment, signaling and defense. In addition, different oxidized substrates differ in

their potential to be re-reduced by ascorbate, and presumably, other reducing agents (Pearse *et al.* 2005). However, other than, perhaps, in its role in lignification, the actual, *in vivo* substrates of peroxidase are unclear (Halliwell and Gutteridge 1999).

In addition to their role in oxidation of phenolics, some forms of POX, especially basic forms, can generate  $H_2O_2$  coupled to oxidation of NADH (Ros Barceló 2000; Koutaniemi *et al.* 2005; Sukalovic *et al.* 2005). In such reactions, peroxidase acts as an oxidase, creating a substrate free radical (XH<sup>•</sup>) which reduces  $O_2$  (Halliwell and Gutteridge 1999). This activity was first demonstrated with NADH using horse radish peroxidase (HRP) by Akazawa and Conn (1958), and 25 years ago, Mäder and Amberg-Fisher, showed that two cell wall peroxidases from tobacco differing in pI and in their ability to polymerize cinnamyl alcohols, could act similarly (Mäder and Amberg-Fisher 1982). It has received considerable attention since then. Details of the reaction and reaction mechanisms have been most intensively studied with respect to NADH, although an analogous mechanism has been postulated for stimulation of  $H_2O_2$  production by salicylic acid (SA) (Kawano and Muto 2000). Whether or not NADH is a potential substrate *in vivo* clearly depends on whether or not it is present in the same compartment as the peroxidase. In the apoplast, this is doubtful (Otter and Polle 1997; Karkonen *et al.* 2002).

Type III peroxidases are also important in the responses of plants to pathogens, with distinct differences between isoenzyme effects: changes in the activity and distribution of the enzyme were examined during the development of a nonhost hypersensitive reaction (HR) to *Pseudomonas syringae* pv. phaseolicola and an hrp mutant of the bacterium in lettuce (Bestwick *et al.* 1998). Inoculation with water or with wild-type or hrp mutant strains of the bacteria caused an initial decline in total POX activity, followed by recovery dependent on the phenolic substrate. In tissues experiencing the HR, guaiacol peroxidase (pH<sub>opt</sub> 6.0) recovered more rapidly, while recovery of tetramethylbenzidine peroxidase (pH<sub>opt</sub> 4.5) was independent of the type of interaction, and chlorogenic acid peroxidase activity (pH<sub>opt</sub> 6.0) was significantly higher in response to the hrp mutant. Direct involvement of wall peroxidases in  $H_2O_2$  production in response to fungal elicitors has been demonstrated in French bean cell cultures (Bolwell *et al.* 1998), *Arabidopsis* (Bolwell *et al.* 2002; Bindschedler *et al.* 2006), cotton (Martinez *et al.* 1998), and other species, but there clearly appear to be species specific differences in this activity (Bolwell *et al.* 1998). The complexity of plant responses and  $H_2O_2$  metabolism is especially clear with respect to pathogens: in the hypersensitive response of *Arabidopsis* responding to *Fusarium*, for example,  $H_2O_2$  production via POX in the apoplast stimulated NAD(P)H oxidase activation, and apoplastic Ca, K, Cl and wall alkalization were intimately associated with this in a signaling cascade (Davies *et al.* 2006).

Apart from the enzyme-substrate relationship between POX and phenolics, phenolic metabolism is part of normal plant growth and responses to environment, and there are very large differences in the extent to which plants accumulate phenolics, including tannins, constitutively or following induction. It is important that phenolic accumulation not be considered indicative of pathology alone; phenolic acids are critical to normal leaf development and senescence (Tamagnone *et al.* 1998), and more broadly, are often critical to successfully integrating overall resource acquisition and allocation. This is reflected in "tissue quality", e.g. toughness and phenolic content, both of which are associated with  $H_2O_2$  metabolism. As noted by Haslam (1985, 1986), when C and energy utilization are limited by lack of other resources, the resulting metabolic imbalance requires diversion of carbon from energy production to energy consuming pathways. This occurs within chloroplasts, in the glycolytic pathway, and within the mitochondria. For example, pyruvate, PEP, acetyl-CoA and 3-phosphogly-

cerate may be shunted into end products which are metabolically harmless, but ecologically useful in defensive roles. While this may reduce the potential for oxidative damage to mitochondria, it will not necessarily eliminate it. The response of oxidative (and anti-oxidative) metabolism to nutrient limitations is, thus, also closely tied to mitochondrial protection (see above). Recently, Baxter *et al.* (2007) demonstrated the extent of both transcriptome and metabolome changes associated with oxidative stress in heterotrophic *Arabidopsis* cells, confirming the extent to which rapid metabolic adjustments can occur.

Both abiotic and biotic stresses can cause shifts in phenolic metabolism. In bean (*Phaseolus vulgaris*) for example, Malusa *et al.* (2002) reported that induction of mild oxidative stress, lipid peroxidation, and an increase in phenolic production reflecting a redirection of carbon metabolism, all occurred under conditions of P-limitation, while Cakmak (1994) reported strong induction with either K or Mg deficiency. Similar induction has also been associated with other nutrient deficiencies, e.g. K (Shin and Schachtman 2004), Mg (Tewari *et al.* 2006), and Fe (Ranieri *et al.* 2001). In response to herbivory, POX responses are critical to reducing tissue palatability by wall cross linking (Brisson *et al.* 1994). It is important to note, however, that shifting phenolic metabolism does not necessarily mean induction of POX genes, at least in the short term. POX enzymes are frequently stable and long-lived, enabling rapid and flexible responses (Pearse *et al.* 2005).

### NAD(P)H oxidases

These membrane proteins oxidize NADPH at the cytosolic surface of the plasmamembrane, and reduce  $O_2$  to  $O_2^{\cdot}$  at the outer surface (Sagi and Fluhr 2006).  $H_2O_2$  is produced indirectly by spontaneous or SOD-mediated dismutation. Plant NAD(P)H oxidases were first identified in 1987 in purified plasmamembrane fractions from cauliflower (Askerlund *et al.* 1987) and the activity was attributed to a membrane bound peroxidase. *Trans*-plasmamembrane electron transport and NAD(P)H dehydrogenase activity were subsequently identified, not associated with peroxidases (Misra 1991; Serrano *et al.* 1994), and insensitive to POX inhibitors but sensitive to DPI, also an inhibitor of neutrophil NADPH oxidase (Murphy and Auh 1996). However, at least some enzymes with these characteristics were found to lack flavin cofactors, suggesting that they were mechanistically different from the mammalian enzyme (Murphy *et al.* 2000). In some cases, e.g. cultured soybean cells, NADH can be oxidized on either side of the plasmamembrane (de Hahn *et al.* 1997). Given that NADH has not been found in the apoplast, an alternative function has been suggested for this enzyme in protein disulfide-thiol interchange (Chueh *et al.* 1997; de Hahn *et al.* 1997). Note, too, that DPI inhibition is far from diagnostic of NAD(P)H oxidases: it also inhibits mitochondrial NADH-ubiquinone reductase, NO synthase, xanthine oxidase, and cytochrome P-450 reductase due to phenylation of a flavin co-factor or a haem (in the case of cytochrome P-450), during enzyme turnover (O'Donnell *et al.* 1993).

Plant homologs to neutrophyl NADPH oxidase make up a gene family identified as respiratory burst oxidase homologs (rboh) of which there are 10 members in *Arabidopsis*. All have significant similarity to one subunit [*gp91(phox)*] of the neutrophil enzyme (Keller *et al.* 1998), but plant transcripts are larger and have a hydrophilic N-terminal domain with binding sites suggesting Ca, and G protein stimulation of  $O_2^{\cdot}$  production. Also unlike the mammalian enzymes, plant forms are not glycosylated. Different rboh family members are constitutively expressed or inducible, and expressed throughout the plant or limited to specific tissues. *AtrbohA*, for example, is constitutively expressed and largely restricted to roots, *atrbohD* is involved in ROS production during the hypersensitive response to pathogens, and *atrbohF* is associated with control of programmed cell death (Torres *et al.* 2002). In to-

bacco (*N. benthamiana*), *nbrbohA*, is expressed constitutively at a low level in leaves, but mere infiltration with buffer increases its expression. *NbrbohB*, on the other hand, was specifically induced by a protein elicitor from *Phytophthora infestans*. Based on virus-induced gene silencing, both are involved in programmed cell death responses (Yoshioka *et al.* 2003). A similar pattern was demonstrated in potato tubers (*strobhA* and *strobhB*) where the proteins were localized to the plasmamembrane by immunolocalization and the  $O_2^{\cdot}$ -generating capacity (sensitivity to DPI, but not azide) was shown (Kobayashi *et al.* 2006). Specificity of function of the enzymes in the absence of pathogen attack is suggested by expression of different homologues in the mesophyll, epidermis and guard cells in leaves and their association with darkness- and ABA-induced stomatal closure (Desikan *et al.* 2004).

The activity of rboh proteins as integrating agents between ROS production and plant responses to stress is suggested by evidence linking them to Ca-dependent signaling associated with such diverse activities as root hair growth (Preuss *et al.* 2004; Shin *et al.* 2005; Carol and Dolan 2006), abscisic acid (ABA) induced Ca-channel activation in guard cells (Kwak *et al.* 2003; Desikan *et al.* 2004; Bright *et al.* 2006; Desikan *et al.* 2006), and activation of a mitogen-activated protein kinase (MAPK) cascade (Desikan *et al.* 1999; Hancock *et al.* 2001; Mittler *et al.* 2004; Zhang *et al.* 2006). Recently, extracellular ATP, which unlike NAD(P)H has been demonstrated to occur, has been added to the list of agents interacting with the proteins as well as stimulating their expression (Song *et al.* 2006). The involvement of NAD(P)H oxidases in  $H_2O_2$  production associated with lignification and the HR was concluded for cells in the xylem of *Zinnia elegans* based on sensitivity to a variety of NADPH oxidase inhibitors (Ros Barceló 1999). Interestingly, ROS, particularly  $O_2^{\cdot}$ , are also required for wall loosening and leaf extension (Rodriguez *et al.* 2002; Liszky *et al.* 2004), and root elongation (Renew *et al.* 2005).

In some, but not all species, NAD(P)H oxidase has been implicated in responses to drought and other abiotic stresses. For example, in maize leaves subjected to a sudden stress by floating them on PEG,  $H_2O_2$  production increased transiently by about 50% over a period of 2 hr (Jiang and Zhang 2002), preceded by an increase in ABA concentration and followed by increased activities of antioxidant enzymes. Pretreatment with non-enzymatic ROS scavengers or DPI prevented the increase, as did suppression of ABA accumulation with tungstate.

### Others and unknowns

In addition to the major enzymatic sources of  $H_2O_2$  discussed above, there are many physiologically interesting, interspecific differences, even differences between accessions of a single species, which are poorly characterized and which might shed light on the ecological breadth of  $H_2O_2$  involvement in stress responses if they were pursued. A few examples will illustrate this: when the response to 100 mM NaCl stress was compared under controlled conditions in *Lycopersicon esculentum* and its wild relative, *L. pennellii*, the changes were substantially opposite (Mittova *et al.* 2003). *L. esculentum* showed oxidative stress in the form of increased lipid peroxidation and  $H_2O_2$  levels while *L. pennellii* did not. The levels of antioxidant enzymes remained the same or decreased in the domesticated species, but increased in the wild relative. Or, consider the coastal halophyte, *Cakile maritime*, which is also adapted to oligotrophic conditions. Ben Amor *et al.* (2006) studied two accessions which, although their growth was different under non-saline conditions, showed no differences at salinities ranging from 100 to 400 mM NaCl. On the other hand, all measures of oxidant/antioxidant activity, from accumulation of ascorbate and  $H_2O_2$ , to lipid peroxidation and electrolyte leakage, to activities of antioxidant enzymes, differed significantly and substantially with increasing sal-

inity: in one accession, the damage related measures were unaffected by salinity while levels of antioxidants and enzyme activities increased. In the other, enzyme activities changed little while H<sub>2</sub>O<sub>2</sub>, electrolyte leakage and lipid peroxidation increased.

Drought tolerance is another area where more information is needed to understand the enzymatic basis for ROS generation and oxidative stress. Drought tolerance has been reported to be directly correlated with increases in antioxidant enzymes and inversely correlated with levels of lipid peroxidation and H<sub>2</sub>O<sub>2</sub> accumulation (Zlatev *et al.* 2006). The potential complexity of the oxidant sources was also indicated in tomato and *Arabidopsis* under water stress (Yesbergenova *et al.* 2005), involving xanthine dehydrogenase (O<sub>2</sub><sup>•</sup>) and ascorbate oxidase (H<sub>2</sub>O<sub>2</sub>), neither of which was associated with the pathogen-induced HR. H<sub>2</sub>O<sub>2</sub> production was insensitive to DPI and both ROS production and transcript levels for the two enzymes were up-regulated by ABA and water stress. Interactions of water stress and factors such as mycorrhizal infection and the enhancement of tolerance associated with it (Gafur *et al.* 2004; Fester and Hause 2005; Wu *et al.* 2006) also deserve further consideration, if possible under field conditions.

### HOW MOBILE IS H<sub>2</sub>O<sub>2</sub>? WHERE IS IT?

Although it was previously hypothesized that H<sub>2</sub>O<sub>2</sub> produced intracellularly diffuses to other cells for use by POX and other defensive enzymes (Takahama and Oniki 1997; Yamasaki *et al.* 1997), it now appears more probable that intracellularly produced H<sub>2</sub>O<sub>2</sub> is consumed quickly and locally, and that extracellular metabolism uses H<sub>2</sub>O<sub>2</sub> produced extracellularly (Bestwick *et al.* 1998). Transmembrane movements of H<sub>2</sub>O<sub>2</sub> (e.g. from the apoplast to the cytosol) probably involve controlled passage through aquaporins (Bienert *et al.* 2007). Expressed in yeast, for example, two *Arabidopsis* aquaporins decreased growth and survival when cells were challenged with H<sub>2</sub>O<sub>2</sub>, and blocking the channels reversed the effect. The effect also interacts with other stresses: low temperature exposure of cucumber roots led to extracellular accumulation of H<sub>2</sub>O<sub>2</sub> in the millimolar range and reduced hydraulic conductivity (Lee *et al.* 2004), consistent with subsequent reports on applied H<sub>2</sub>O<sub>2</sub> effects (Ye and Steudle 2006). On the other hand, at lower levels of H<sub>2</sub>O<sub>2</sub>, transport through aquaporins may be important in eliciting responses intracellularly, such as those reported by Allan and Fluhr (1997). This may be essential to H<sub>2</sub>O<sub>2</sub>-dependent signaling and in toxicity of extracellular oxidants (de Marco and Roubelakis-Angelakis 1996; Bestwick *et al.* 1997; Pellinen *et al.* 1999).

H<sub>2</sub>O<sub>2</sub> diffusion not involving membrane transit is also restricted to short distances, although much longer than movements of other ROS which are even more restricted by their greater reactivity. H<sub>2</sub>O<sub>2</sub> localization within tissues, sometimes to portions of cell walls in root hairs (Carol and Dolan 2006), or in epidermal cells in association with wounding or stomatal movements (Allan and Fluhr 1997), indicates the extent to which plants control their internal environments, as does compartmentation at the tissue level within leaves (Doullis *et al.* 1997; Pastori *et al.* 2000), in vascular tissues (Ogawa *et al.* 1997; Moller and McPherson 1998), and in areas of regeneration (rhizogenesis) (Neves *et al.* 1998). As exemplified by comparative ozone studies, resistance may be determined by the extent to which H<sub>2</sub>O<sub>2</sub> can be kept, first, out of cells, and then, out of chloroplasts (Pellinen *et al.* 1999; Oksanen *et al.* 2004). Localization with respect to lignification and xylem differentiation in "normal" growth and development has been particularly well established (Olson and Varner 1993; Richardson *et al.* 1997; Ros Barceló 1998; Repka 1999; Ros Barceló 1999; Paschalidis and Roubelakis-Angelakis 2005; Ros Barceló 2005), although the actual mechanism of H<sub>2</sub>O<sub>2</sub> production at the cell surface remains unclear; this reflects, in part, differences between species or even within single plants responding to different environmental stimuli

(Ros Barceló and Ferrer 1999).

Using tissue printing techniques in a variety of plants, Schopfer (1994) and Olson and Varner (1993) demonstrated longitudinal and radial gradients during hypocotyl growth, response to ethylene, association with lignification, with light mediated inhibition of elongation and with wounding, while Neves (1998) documented a progression of tissue level changes in H<sub>2</sub>O<sub>2</sub> localization during auxin-induced rooting of grapevine cuttings. K-deprivation led to ROS accumulation in regions of *Arabidopsis* roots which were active in K uptake and transport, and this accumulation was suppressed, independent of the induction of high affinity transporters, by mutation of the *atrbohC* NADPH oxidase gene (Shin and Schachtman 2004). Interestingly, the application of H<sub>2</sub>O<sub>2</sub> induced those transporters even under K-sufficient conditions. The same authors examined the role of ROS in *Arabidopsis* root hair mutants in response to N and P deprivation (Shin *et al.* 2005). The patterns of increased ROS production indicated that root hairs were important in N and K responses, but that P responses were localized in cortical layers. Even with respect to bacterial attack, H<sub>2</sub>O<sub>2</sub> production can be highly localized to bacterial attachment sites in cell walls (Bestwick *et al.* 1998).

### SIGNALING AND RESPONSE NETWORKS

The production of H<sub>2</sub>O<sub>2</sub> is seldom if ever the end of the story; frequently, if not always, it is associated with additional responses and plays a major role in signaling. There is much better understanding of the fact that H<sub>2</sub>O<sub>2</sub> is involved in signaling and for some of the intricacies of downstream processing than there is for what the meaning of the signal is, how a plant decides what the threat is, or how a decoded signal is interpreted for a particular stress or other metabolic need. In part this is due to uncertainty about what actual concentrations of H<sub>2</sub>O<sub>2</sub> are in tissues; as noted above, a reported range of seven orders of magnitude is really too great to fit into any model. In part it is also due to the cellular and tissue-level, spatial restrictions of some responses. And in many ways, considering the concentration of H<sub>2</sub>O<sub>2</sub> in tissue and its relationship to stress or defense is analogous to the situation with calcium. Bulk calcium levels in leaf tissues are much higher than could be tolerated intracellularly and indicate nothing about its activity in signaling and metabolic control. Some of the most exciting advances in integrative plant biology in recent years have been directed at understanding sensing, signaling and response networks, and it is appropriate to conclude this review with a brief consideration of those results. In the end, however, there are many similarities in the response networks to different environmental stimuli and developmental states, crosstalk between them seems certain, and understanding how plants avert problems inherent with that complexity remains a daunting but exciting challenge.

#### Sensing H<sub>2</sub>O<sub>2</sub>

If, for now, we accept the "normal" tissue level concentrations of H<sub>2</sub>O<sub>2</sub> in, for example, leaves, to be between about 0.1 and 5 μmol/gFW (Veljovic-Jovanovic *et al.* 2002; Cheeseman 2006), that H<sub>2</sub>O<sub>2</sub> turnover is rapid, and that it and other ROS have a number of important roles in development and physiology in the absence of stress, then sensing is clearly a complex problem. Instead of simple presence or absence, cells would need to be able to sense change, perhaps even qualitative change (e.g. Spiro *et al.* 1998). Chandra and Low (1995) presented one model for this, involving protein phosphorylation. They reported that kinase inhibitors blocked the oxidative burst in cultured soybean cells, and if added once the burst were underway, terminated it. Phosphatase inhibitors, on the other hand, stimulated it in the absence of other stimuli. They concluded that the kinases involved may be constitutively active and that the burst was signaled when their phosphorylated

forms were stabilized. On the other hand, Hancock *et al.* (2006) recently noted that the small size of the H<sub>2</sub>O<sub>2</sub> molecule made it unlikely that there would be specific receptor proteins involved in its sensing. They presented an alternative suggestion that ROS perception in general was moderated by proteins with other roles, but sharing the characteristic of having active thiol groups as redox targets. That is, sensing was a result of oxidation of the thiol groups by H<sub>2</sub>O<sub>2</sub> and other ROS, including NO, perhaps even involving competition of the two types of oxidants for the same modification sites (see also Foyer *et al.* 1997; Neill *et al.* 2002). One example of this would be the histidine kinase receptor, ETR1, essential for sensing H<sub>2</sub>O<sub>2</sub> leading to stomatal closure (Desikan *et al.* 2005). Interestingly, the kinase domain itself was not required for this, but a single cysteine (Cys65) was. Kolbe *et al.* (2006) have presented results using both genomic and proteomic analysis to support this model.

Yet another integrating hypothesis has come from analysis of heat shock responses, involving interactions between H<sub>2</sub>O<sub>2</sub> and heat shock promoter elements as sensors (Volkov *et al.* 2006). Miller and Mittler (2006) have argued that heat shock transcription factors are the molecular sensors of ROS, and that their complexity, flexibility and specialization allow them to control the expression of a wide range of stress response genes, not only those involving heat shock.

## Signaling

The connection between H<sub>2</sub>O<sub>2</sub> and signaling networks has been extensively documented for a number of stress responses, including to pathogen elicitors, insect feeding, wounding, high temperature and ABA associated stomatal closure (Larkindale and Knight 2002; Apel and Hirt 2004; Peng *et al.* 2004; Mateo *et al.* 2006). These share many common features, including the relationship between H<sub>2</sub>O<sub>2</sub> and Ca, and rather than attempt to review each of them here, my approach will be to focus first on that link, then on initial aspects of the response cascade, and finally on some aspects of the problem which pose the greatest challenges and opportunities.

The link between H<sub>2</sub>O<sub>2</sub>, Ca, and stomatal closure was clearly established by Pei *et al.* (2000) using patch clamp techniques, showing that the well-established signaling cascade connecting ABA to stomatal closure runs through H<sub>2</sub>O<sub>2</sub> and is mediated by calcium channels. Using fluorescent Ca-sensitive dye, the Ca current was shown to lead to increased Ca<sub>cyt</sub>. The sensitivity of ABA-induced stomatal closure to DPI suggested that the increased Ca<sub>cyt</sub> stimulated NAD(P)H oxidase activity, leading to extracellular release of O<sub>2</sub><sup>•</sup>, followed by dismutation to H<sub>2</sub>O<sub>2</sub>. Subsequently, Chen and Gallie (2004) showed that stomatal responsiveness reflects the internal redox state of the guard cells and diurnal variations in ascorbate levels and H<sub>2</sub>O<sub>2</sub> production.

## Response networks

In the last five years, this response network has been repeatedly extended and summarized, not only for stomatal responses but for responses to other biotic and abiotic stresses, and the signaling cascades have been shown to have many similarities (e.g. Desikan *et al.* 1999; Taylor *et al.* 2001; Mittler *et al.* 2004; Baier *et al.* 2005; Kalbina and Strid 2006; Kotchoni and Gachomo 2006; Mishra *et al.* 2006; Suzuki and Mittler 2006; Zhang *et al.* 2006). Commonalities include both early steps involving Ca or phosphatidic acid activated serine/threonine protein kinase (OXI1), a mitogen activated protein kinase (MAPK3/6) cascade, and downstream transcription factors which influence both transcription of scavenging enzymes and NAD(P)H oxidase. As a result, the initial response to H<sub>2</sub>O<sub>2</sub> can both reduce and amplify the oxidative signal, allowing graded or controlled response to particular elicitation events (Suzuki and Mittler 2006).

The availability of much-of-the-genome microarrays, especially for *Arabidopsis*, has led to even greater extension of this response network. In 2004, Mittler *et al.* (2004) annotated 152 genes involved in ROS control in *Arabidopsis*, while Hancock *et al.* (2006) expanded this to at least 400. The network is both redundant and dynamic, as should be expected because of the involvement of ROS in development (which is cell and tissue specific), metabolism and defense, and the need to maintain a steady-state on which critical signals can be registered. Even more complex and complete models, including analysis of gene response clusters, has been possible with shared *Arabidopsis* microarray result databases (e.g. <http://www.arabidopsis.org/info/expression/ATGenExpress.jsp>) (Ma *et al.* 2006; Schreiber and Baumann 2007).

Although relevant studies have included numerous experimental conditions, ozone responses provide a convenient illustration both of the extent of the networks and their dependence on the conditions of the experiments (e.g. Olbrich *et al.* 2005; D'Haese *et al.* 2006; Lee and Yun 2006; Li *et al.* 2006a; Tosti *et al.* 2006). Ozone toxicity has been recognized for more than 50 years, but neither the mechanism of action nor the response of organisms at the molecular level is well understood (Rao *et al.* 2000a). An early role was recognized for ascorbic acid in ozone detoxification (Tanaka *et al.* 1985), and mathematical modeling (Chameldes 1989) as well as studies using ascorbate deficient or enhanced mutants suggested that it could account for a substantial portion of ozone defense (Conklin *et al.* 1996; Chen and Gallie 2005). This model has not, however, gone unchallenged (D'Haese *et al.* 2005).

At the molecular level, initial studies of the response network showed that it had extensive similarities to the networks for other oxidative stresses (Baier *et al.* 2005). A recent study by Li *et al.* (2006a), however, illustrates the extent of differences possible even within one species and with close relatives of differing overall stress tolerance. These authors used microarrays to study the response of *Arabidopsis* and its stress-tolerant relative, *Thellungiella halophila* to ozone. Uniquely, they performed the study in the field. Ozone was manipulated using free air concentration enrichment (FACE) conditions, comparing plants at ambient levels and at a mere 1.2-times ambient (dynamically adjusted). Three *Arabidopsis* ecotypes were included: Columbia-0 (Col-0), Cape Verde Islands (Cvi-0), and Wasilewskija (WS). Even within this small range of genotypes, the responses at both the physiological and transcription levels were significantly different. The number of genes responding (up- or down-regulation) to elevated ozone ranged from 320 in *Thellungiella* to more than 2900 in WS. Among *Arabidopsis* ecotypes, WS was also the most affected, i.e. showed the greatest actual damage to leaves, and transcriptome responses included photosynthetic light reaction genes, genes in the phenylpropanoid pathway, ROS scavenging, photorespiration and the reductive pentose phosphate pathway, and hormone biosynthesis and response (ethylene, jasmonic acid and salicylic acid). Interestingly, but underscoring the importance of conducting field studies rather than relying on highly contrived controlled conditions, the relative ozone resistance of the *Arabidopsis* genotypes was reversed under FACE conditions to what it had been reported with acute exposure (see Rao *et al.* 2000b). The authors emphasized, in addition, the dependence of the results on local weather conditions, time of growth and harvesting, and potential biotic stresses during the experiment. The "take home lesson" from this is that any "definitive" model of a stress response should be accepted very cautiously.

## Complex systems modeling and proteomics

Returning to stomatal responses – this phenomenon provides the basis for models extending in a different direction. Stomatal closure can be mediated by external H<sub>2</sub>O<sub>2</sub> directly, without prior activation of the NAD(P)H oxidase or ABA;

it happens without preceding changes in gene expression. In this case, the H<sub>2</sub>O<sub>2</sub> can be produced by apoplastic POX or amine oxidases (Allan and Fluhr 1997; Kawano *et al.* 2000), the former being stimulated by SA, and involving SA\* as an intermediate (Kawano *et al.* 1998; Kawano and Muto 2000; Mori *et al.* 2001). Alternately, the Ca-dependent changes can be mediated by other extracellular elicitors such as oligogalactouronic acid (Hu *et al.* 2004). As a generalization, whether H<sub>2</sub>O<sub>2</sub> production leads or follows the increase in Ca<sub>cyt</sub> depends on the signal. In the case of ABA, for example, H<sub>2</sub>O<sub>2</sub> follows (Pei *et al.* 2000). In the case of extracellular elicitation, the bulk of the H<sub>2</sub>O<sub>2</sub> production leads (Hu *et al.* 2004) followed by other responses dependent on the type of insult (Orozco-Cárdenas *et al.* 2001; Miles *et al.* 2002; Mur *et al.* 2005). In sum, it includes activities and responses, simultaneously, of the proteome, the metabolome and the transcriptome.

Li *et al.* (2006b) combined more than 40 pathway and physiological components known to be involved in stomatal responses into a single, dynamic model using a complex systems approach. It allowed simulation of stimulus and response, as well as inhibitor effects and gene disruptions. In addition, individual components of physiological pathways could be manipulated – ion fluxes, electrophysiological parameters, signaling cascades or cellular characteristics – and the results were very largely in keeping with experimental observations. This type of modeling essentially says, “if we know what we think we know, then we ought to be able to predict responses to manipulations, or even design novel, new experiments.” Perhaps most significant is the fact that this type of complex systems modeling allows quantitative simulation based on limited quantitative background information. It allows, for example, “manipulation” of the apoplastic proteome and metabolome, otherwise poorly understood, to generate testable predictions.

Direct proteome analysis is much more difficult than transcriptome analysis because of the greater difficulty of protein isolation and sequencing, and the difficulty of extracting the apoplast without contamination by other compartments (Watson *et al.* 2004; Zhu *et al.* 2006), although the tools are developing rapidly. The promise of wall proteome studies was shown by analysis of tobacco leaves and their response to salt stress (Dani *et al.* 2005). Using two-dimensional electrophoresis of apoplastic fluids, they identified 150 polypeptide spots, 20 of which changed in abundance with salt stress, but other than identification of one germin-like protein, the results were still somewhat disappointing. That the activity of apoplastic enzymes can be influenced by biotically and abiotically-induced oxidative stress (Diaz-Vivancos *et al.* 2006), and be modified by stress-related hormones, e.g. methyl jasmonate (Maksymiec and Krupa 2002), is clear. As importantly, some apoplastic enzymes are well known for their stability (e.g. peroxidases), which means a comparative proteomic analysis would not identify them as responding. Moreover, the correlation between enzyme activity and expression of the associated mRNAs is, in other cases, demonstrably poor (e.g. DAO - Angelini *et al.* 1996).

While these problems can be addressed at one level using the systems modeling approach, experimentally, as was the case for many of the other responses discussed in this review, cell cultures have the advantage of simplicity that can provide initial models if not appropriately represent the responses of intact organisms. Chivasa *et al.* (2005), for example, used maize cell cultures to elucidate responses of the wall proteome to pathogen elicitors. The responses included changes in phosphorylation status (extracellular peroxidases), disappearance of some proteins (e.g. a putative extracellular β-N-acetylglucosaminidase), and accumulation of others (a secreted putative xylanase inhibitor), and appearance of some classically cytosolic proteins (e.g. glyceraldehyde-3-phosphate dehydrogenase).

## CONCLUSION

Understanding the integrated responses of plants to their environment throughout their life cycles which enable them to acquire and allocate resources, grow, and reproduce in the face of serious and dynamic environmental constraints is as challenging now as it has been throughout the history of plant biology. In this review, I have focused on one metabolite which is both a constraint and an essential element of physiology, and the tools plants have at their disposal to deal with it. While it would be naïve to think that recent advances in analytical techniques, standardized experimental systems and modeling put us on the threshold of fully understanding the role of H<sub>2</sub>O<sub>2</sub> in plant metabolism, it is certain that they will open new levels of exciting uncertainty.

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