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Peroxide processing in photosynthesis: antioxidant coupling and redox signalling

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Photosynthesis has a high capacity for production of hydrogen peroxide (H_2O_2), but the intracellular levels of this relatively weak oxidant are controlled by the antioxidant system, comprising a network of enzymatic and non-enzymatic components that notably includes reactions linked to the intracellular ascorbate and glutathione pools. Mutants and transformed plants with specific decreases in key components offer the opportunity to dissect the complex system that maintains redox homeostasis. Since H_2O_2 is a signal-transducing molecule relaying information on intracellular redox state, the pool size must be rigorously controlled within each compartment of the cell. This review focuses on compartment-specific differences in the stringency of redox coupling between ascorbate and glutathione, and the significance this may have for the flexibility of the control of gene expression that is linked to photosynthetic H_2O_2 production.

Keywords: hydrogen peroxide; ascorbate; glutathione; catalase; 2-cys peroxiredoxin; ascorbate peroxidase

1. INTRODUCTION: INTERPLAY BETWEEN PHOTOSYNTHETIC HYDROGEN PEROXIDE PRODUCTION AND THE ANTIOXIDANT SYSTEM

Active oxygen species (AOS) are produced in aerobic organisms during the course of metabolism and are processed by the antioxidant system, which comprises enzymes and compounds of low molecular weight (Noctor & Foyer 1998). The accelerated production of AOS is a key feature of physiological stress. While this phenomenon may be largely the unavoidable consequence of life in an atmosphere containing 21% O_2, the once prevalent view that enhanced AOS production has only negative effects on metabolism and growth has been gradually replaced by one that recognizes the importance of these molecules in relaying information on cellular redox status. The interplay between the antioxidant system and processes generating AOS has widespread repercussions for gene expression and the integration of cellular physiology.

The sedentary nature of plants means that they must be able to acclimate to environmental change in an appropriate way. During evolution, plant metabolism has harnessed the potential of AOS and antioxidants as internal monitors of external change. Leaf cells probably contain higher concentrations of O_2, AOS and antioxidants than many other types of living cell. These high contents are only indirectly related to photosynthetic O_2 evolution from water, because the effective diffusion of O_2 through biological membranes means that cellular concentrations are, at most, only slightly above ambient. However, because many leaves present relatively large surface areas in order to capture light and CO_2, O_2 concentrations are probably rarely depressed below ambient levels, contrasting with conditions inside some living cells (e.g. those in actively respiring muscle). The notion that many leaf cells have evolved to tolerate comparatively high AOS concentrations receives circumstantial support from the observation that maize bundle sheath cells are unusually sensitive to oxidative damage (Kingston-Smith & Foyer 2000). In the maize bundle sheath, O_2 concentrations are probably lower than in many other photosynthetic cells due to low rates of photosystem II (PS II)-dependent O_2 evolution and restricted exchange of gases with the atmosphere (Furbank & Foyer 1988).

Although numerous processes in leaves are capable of producing AOS such as superoxide and H_2O_2, those with the highest capacity under most conditions are the Mehler reaction and photorespiration (Foyer & Noctor 2000). The flux through both of these pathways will be variable, generally being favoured by high light and, in the case of photorespiration, by elevated temperatures and low CO_2 availability. In the Mehler reaction, the low potential acceptors of chloroplastic photosystem I (PS I) reduce O_2 to superoxide, from which H_2O_2 is then produced by dismutation or further reduction. In C_3 plants, photorespiration produces H_2O_2 at high rates through the action of peroxisomal glycollate oxidase. While H_2O_2 is relatively unreactive, its univalent reductive cleavage, catalysed by transition metals, yields the hydroxyl radical, an extremely reactive species capable of oxidizing protein groups, mutagenizing DNA and initiating lipid peroxide chain reactions, thereby leading to membrane disruption. In addition, physiological concentrations of H_2O_2 can oxidize regulatory protein

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thiol groups such as those found in several chloroplast enzymes involved in photosynthesis (Kaiser 1979). The cellular titre of H_2O_2 must therefore be subject to rigorous compartment-specific and time-specific controls.

Hydrogen peroxide is processed by catalases, which catalyse its disproportionation, and peroxidases that use reductants to convert it to water. In plants, the most important reductant for H_2O_2-removing peroxidase activity is ascorbate (Foyer & Halliwell 1976; Groden & Beck 1979; Kelly & Latzko 1979; Nakano & Asada 1980), which is oxidized to the monodehydroascorbate radical (MDHA). While ascorbate is the immediate electron donor in this reaction, ascorbate peroxidase (APX) activity can be linked directly to electron transport chains via reduction of MDHA by ferredoxin or cytochromes (Miyake & Asada 1994; Horemans *et al.* 1999), to turnover of NADPH pools due to MDHA reductase activity (Hossain *et al.* 1984), or to glutathione redox cycling through the ascorbate–glutathione cycle (Foyer & Halliwell 1976).

As the most stable of the AOS, H_2O_2 is implicated in signal-transduction pathways that involve modifications in gene expression in response to biotic and abiotic stress (Levine *et al.* 1994; Foyer *et al.* 1997). The interaction between H_2O_2 production and the antioxidant system means that these transduction pathways are likely to be complex and to call into play key antioxidants such as ascorbate and glutathione. The discussion below focuses on the contribution that studies of mutants with altered capacity for specific components of the antioxidative system can make to understanding redox coupling in the antioxidant system and the role of antioxidants in signal transduction linked to photosynthesis.

2. THE CONTRIBUTION OF ASCORBATE PEROXIDASE TO HYDROGEN PEROXIDE PROCESSING IN THE CHLOROPLAST AND CYTOSOL

Ascorbate peroxidase activity was first detected in chloroplasts (Groden & Beck 1979; Kelly & Latzko 1979), but a cytosolic isoform has since been found (Chen & Asada 1989; Mittler & Zilinskas 1991), as well as a form that is associated with the membranes of both glyoxysomes and leaf peroxisomes and whose active site probably faces the cytosol (Yamaguchi *et al.* 1995). The isoforms differ in size, specificity for electron donor and sensitivity to inactivation (Chen & Asada 1989; Yoshimura *et al.* 1998). The chloroplast enzyme, which is found in both stroma soluble and thylakoid bound forms, is rapidly inactivated (half-life 10–20 s) if the concentration of ascorbate is below *ca.* 20 μM (Hossain & Asada 1984). In contrast, the non-chloroplastic isoforms are more stable in the absence of ascorbate (Chen & Asada 1989; Yoshimura *et al.* 1998). As the ascorbate concentrations in the chloroplast and cytosol are probably 100–1000 times higher than that necessary to prevent APX inactivation (Foyer *et al.* 1983; Foyer & Lelandais 1996) the physiological significance of this process is not clear. However, the differential sensitivity to inactivation of the chloroplastic and non-chloroplastic isoforms can be exploited in whole-leaf extracts as an investigative tool to determine the relative extractable activities attributable to these compartments (Amako *et al.* 1994).

In addition to functioning as a substrate for APX, ascorbate is an important non-enzymatic antioxidant and a substrate for violaxanthin de-epoxidase in the xanthophyll cycle. Ascorbate is also implicated in the regulation of cell growth (Noctor & Foyer 1998). Foliar ascorbate contents are markedly dependent on developmental stage and environmental conditions such as growth light intensity (Gillham & Dodge 1987; Grace & Logan 1996; Conklin *et al.* 1996). In view of the multiple roles of ascorbate, changes in its concentration may have important consequences for cellular and metabolic regulation. However, an evaluation of the influence of ascorbate concentration in development and in response to the environment must overcome the difficulty of separating ascorbate-mediated effects from those due to concomitant changes in other factors. A very useful tool was made available by the isolation of an ascorbate-deficient mutant of *Arabidopsis thaliana*, *vtc1* (Conklin *et al.* 1996). Initially identified via its sensitivity to elevated ozone concentrations, this mutant was instrumental in the elucidation of a novel pathway of ascorbate biosynthesis, which is probably the predominant route in plants (Wheeler *et al.* 1998; Conklin *et al.* 1999). The mutant possesses decreased activity of guanosine diphosphate (GDP)-mannose pyrophosphorylase and, as a result, accumulates ascorbate to only about 30% of wild-type levels (Conklin *et al.* 1996, 1999).

We have exploited this mutant to assess the effects of decreased foliar ascorbate concentration on growth under optimal conditions, on energy dissipation at PS II, and on the leaf antioxidant system. The principal findings of this study are summarized in table 1. The mutant was found to display slower shoot growth than the wild-type both in air and at high CO_2, where oxidative stress is minimized. Leaves were generally smaller and shoot fresh weight was lower in the mutant. On the other hand, no significant difference in the irradiance saturation curves for CO_2 assimilation were found in air or at high CO_2, suggesting that the effect on growth was not due to decreased photosynthetic capacity in the mutant. Analysis of chlorophyll fluorescence quenching revealed a slight effect of decreased ascorbate content on non-photochemical energy dissipation. Two conclusions may tentatively be drawn:

(i) 30% of the wild-type foliar content is sufficient to satisfy most of the ascorbate demand of both chloroplastic APX activities and violaxanthin de-epoxidase activity on the lumenal face of the thylakoid membrane;
(ii) decreased growth in the mutant is not due to increased oxidative stress.

The negative effect on growth may reflect a requirement for ascorbate in cell elongation and division (Noctor & Foyer 1998). More likely, perhaps, is that the growth effect is not related to ascorbate concentrations *per se*, but results from insufficient capacity to synthesize cell wall precursors caused by lower rates of GDP-mannose formation in *vtc1*. However, it is worth noting here that the fractional decrease in ascorbate in the mutant is greater than that in the extractable activity of GDP-mannose

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Table 1. *Comparison of parameters in the ascorbate-deficient mutant,* vtc1*, and wild-type* Arabidopsis

(The data show the value in *vtc1* divided by the value in the wild-type. NPQ, non-photochemical quenching of chlorophyll fluorescence; APX, ascorbate peroxidase; GuPX, non-specific (guaiacol-type) peroxidase. Growth was measured as shoot fresh weight. CO_2 assimilation and NPQ were measured on attainment of the steady state at light saturation (900–1000 µmol quanta m^{-2} s^{-1} at the leaf surface).)

growth	CO_2 assimilation	NPQ	ascorbate	foliar H_2O_2	total soluble APX	stromal APX	extra-chloroplast APX	GuPX
0.52	0.94	0.86	0.28	1.04	1.17	0.77	6.56	2.50

pyrophosphorylase (Conklin *et al.* 1999). This suggests that, even if the primary effect on ascorbate contents in *vtc1* is due to a specific point mutation in the gene encoding this enzyme, pleiotropic effects on other enzymes cannot be discounted. For instance, one possibility is that high ascorbate contents are expendable and that these are sacrificed to allow maximal diversion of carbon to cell wall biosynthesis when there is potential competition for substrates. Even if this occurs, it might not be sufficient to compensate for the reduced flux to GDP-mannose, perhaps explaining lower growth in the mutants.

The similar rates of photosynthesis and energy dissipation in the mutant begs the question of why leaves require such high amounts of ascorbate. There was no evidence for significant upregulation of other elements of the antioxidant system to compensate for decreased ascorbate concentrations (Conklin *et al.* 1997). We have confirmed this observation, with respect to total extractable activities of soluble APX (table 1) and other antioxidative enzymes. Similarly, no change in total foliar H_2O_2 contents was observed (table 1). Evidence was found, however, for an effect on the distribution of APX activity between chloroplastic and non-chloroplastic APX activities—using H_2O_2 sensitivity as a means of distinguishing between activities in these compartments (Amako *et al.* 1994), non-chloroplastic APX activity was found to make only a minor contribution to the total APX activity in the wild-type but to be enhanced more than sixfold in the mutant (table 1). Likewise, guaiacol-type peroxidase activity was significantly increased in the mutant. This last observation might be indicative of up-regulation of non-specific peroxidases in the mutant, as is often observed in stressed plant tissues. Alternatively, it could reflect specifically enhanced amounts of the cytosolic APX, which is known to be less specific for ascorbate than chloroplastic forms, and to be able to catalyse H_2O_2-dependent oxidation of substrates such as pyrogallol at a higher rate than oxidation of ascorbate (Chen & Asada 1989; Yoshimura *et al.* 1998).

The significance of increased activities of non-chloroplastic isoforms of APX is not yet clear. We are in the process of determining whether these effects are due to increased amounts of cytosolic APX protein and transcripts in the mutant. If so, the data would implicate ascorbate concentration in the regulation of the compartmentalization of the antioxidant system in *Arabidopsis*. Such an effect is clearly not linked to the ascorbate redox state since this is similar in the wild-type and mutant (Conklin *et al.* 1996; G. Noctor, S. Veljovic-Jovanovic & C. H. Foyer, unpublished data). A key question remaining in the characterization of the mutant relates to the distribution of ascorbate between different cellular compartments. The final step of ascorbate synthesis takes place in the mitochondrial intermembrane space (Bartoli *et al.* 2000), from where ascorbate must be transported to other compartments. Plant cells contain multiple transporters for ascorbate and dehydroascorbate (Horemans *et al.* 1999), but it remains unclear whether, in the mutant, uptake of either or both of these species across the chloroplast envelope (Foyer & Lelandais 1996) is capable of maintaining chloroplastic concentrations at values close to those in the wild-type. This would be one possible explanation of the lack of effect on photosynthesis in the mutant, and would presumably mean that cytosolic ascorbate concentrations would be decreased in the mutant even more severely than is indicated by measurements of total foliar contents.

3. PROCESSING OF PHOTORESPIRATORY HYDROGEN PEROXIDE

The application of genetic and molecular techniques to the exploration of metabolism has often revealed the organization of cellular biochemistry to be more complex than hitherto suspected. One example is the processing of photorespiratory H_2O_2, once considered the exclusive domain of catalase. Detection of APX activities associated with microbodies (Yamaguchi *et al.* 1995; Bunkelmann & Trelease 1996; Jiménez *et al.* 1997) has been confirmed by molecular studies (Yoshimura *et al.* 1998; Zhang *et al.* 1997).

Despite the presence of an alternative route for H_2O_2 processing associated with peroxisomes, it is clear that catalase is required to cope with high photorespiratory flux (Foyer & Noctor 2000). This was first demonstrated by isolation of a catalase-deficient barley mutant (Kendall *et al.* 1983) and has been confirmed by subsequent studies in tobacco in which the major leaf catalase isoform was decreased by antisense technology (Willekens *et al.* 1997; Takahashi *et al.* 1997; Brisson *et al.* 1998). When these plants are placed in conditions favouring high rates of photorespiration (ambient CO_2, high light, warm temperatures), photosynthesis is inhibited, the foliar antioxidant system is perturbed and necrotic lesions appear on the leaves. In the transformed tobacco, induction of defence-related proteins is observed, both locally in the necrotic regions and in leaves that do not suffer necrosis (Willekens *et al.* 1997). These data not only confirm the importance of catalase in photorespiration but also implicate photorespiratory H_2O_2 production in the induction of systemic responses that appear to share some features with the systemic acquired resistance that can follow

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Table 2. *Rapid and sustained changes in antioxidants on transfer of wild-type and catalase-deficient barley from 0.6% CO_2 to air*

(The data are normalized to values at time zero. For total pools, these values were 1.34 μmol mg^{-1} chlorophyll (ascorbate, wild-type), 1.26 μmol mg^{-1} chlorophyll (ascorbate, mutant), 0.135 μmol mg^{-1} chlorophyll (glutathione, wild-type), 0.223 μmol mg^{-1} chlorophyll (glutathione, mutant). The percentage of these pools in the DHA or GSSG form at time zero was 8% (ascorbate, wild-type), 6% (ascorbate, mutant), 6% (glutathione, wild-type) and 11% (glutathione, mutant), GSSG, glutathione disulphide; DHA, dehydroascorbate.)

	hours after transfer	ascorbate		glutathione	
		fold increase in total pool	fold increase in % as DHA	fold increase in total pool	fold increase in % as GSSG
wild-type	0	1.0	1.0	1.0	1.0
	1	1.0	3.1	1.2	5.2
	72	1.6	1.3	1.9	2.1
mutant	0	1.0	1.0	1.0	1.0
	1	1.1	2.8	1.1	4.6
	72	1.7	1.0	3.9	4.4

pathogen attack (Willekens *et al.* 1997; Takahashi *et al.* 1997). Given that catalase activities have been shown to decline under certain stress conditions (Hertwig *et al.* 1992), local and systemic responses to photorespiratory H_2O_2 observed in plants with artificially decreased catalase may be of considerable physiological relevance.

High rates of photorespiration in the plants with decreased catalase induce specific changes in the antioxidant system. It was first shown in the barley mutant that transfer from high CO_2 to air induced marked accumulation of the glutathione pool, accompanied by a dramatic shift in its redox state (Smith *et al.* 1984). These observations were confirmed in the tobacco transformants, where it was shown that the increased oxidation state of glutathione was not accompanied by changes in the redox state of the ascorbate pool (Willekens *et al.* 1997). While these effects implicate glutathione content and redox state in the genetic responses to the increased oxidative load that results from photorespiration, they also beg the question of why increased oxidation state is specific to the glutathione pool.

We have recently re-examined these effects in the barley mutant by monitoring CO_2 assimilation, H_2O_2, ascorbate and glutathione in the leaves of plants transferred to air following growth at 0.6% CO_2 for four weeks. Particular attention was paid to the first few hours after transfer, with the aim of establishing whether any of the changes observed in the mutant might also be observed in the wild-type. Catalase activities decrease under conditions that suppress photorespiration, such as elevated CO_2 (e.g. Azevedo *et al.* 1998), and so significant information might be gleaned during the lag between the rapid resumption of high rates of photorespiration and the slower induction of catalase activity. A sufficiently low irradiance allowed interactions between oxidant generation and the antioxidant system to be explored without a marked inhibition of photosynthesis.

Both in the mutant and the wild-type barley, transfer to air caused a rapid increase of foliar H_2O_2 that was not totally reversed within the duration of the experiment (four days). Concomitant changes in leaf ascorbate and glutathione pools are summarized in table 2. In the mutant, as previously reported (Smith *et al.* 1984), the glutathione pool (> 95% reduced at 0.6% CO_2) showed an increased oxidation state within 1 h of transfer and the glutathione reduction state continued to decline over the duration of the experiment (< 40% reduced after four days in air). Oxidation of the glutathione pool preceded accumulation of total glutathione (table 2), the latter effect beginning on the second day after transfer and continuing until the pool was threefold larger than prior to transfer. As discussed previously (Noctor & Foyer 1998), it is unlikely that such accumulation can be explained simply by alleviation of feedback inhibition of glutathione synthesis; rather, oxidant linked upregulation of enzyme synthesis is necessary, and this has been shown in *Arabidopsis* to occur at the level of translation (Xiang *et al.* 2000). In agreement with the data obtained from tobacco transformants (Willekens *et al.* 1997), sustained perturbation of the ascorbate redox state was not observed in either type of plant (table 2). However, transfer to air caused a transient oxidation of the ascorbate pool in both the mutant and the wild-type. Similarly, in the wild-type, transfer to photorespiratory conditions caused a transient perturbation of the glutathione redox state (table 2), which declined to about 80% after 2 h and then regained its highly reduced state within a similar time-scale. These data demonstrate that photorespiratory activity is capable of perturbing the foliar glutathione and ascorbate pools, but that when catalase activity is sufficient, the redox state of the antioxidant pools recover (even though H_2O_2 contents remain elevated) and upregulation of glutathione synthesis does not occur.

The induction of glutathione synthesis might be viewed as an 'attempt' by the leaf cell to increase the availability of a cycling antioxidant and so to buffer the cell from further increases in H_2O_2. Oxidation of glutathione followed by accumulation is also observed upon fungal infection of resistant barley cultivars and follows precisely the production of H_2O_2 by the underlying mesophyll cells prior to the oxidative burst in the attacked cells that leads to cell death. Since the underlying mesophyll cells do not die, it is suggested that glutathione accumulation at an

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early stage of infection allows cell survival during the oxidative burst (Vanacker *et al.* 1998). Here, we observed little difference in H_2O_2 levels in the catalase mutant and the wild-type, suggesting that the increased oxidative burden in the mutant is borne primarily at the expense of the redox state of the glutathione pool. Although glutathione reductase (GR) activities have been shown to increase under these conditions (Azevedo *et al.* 1998), it appears that the recycling of reduced glutathione cannot keep pace with its oxidation when the mutant is placed in air. As our experiments were carried out under conditions in which only slight effects on overall leaf metabolism (as assessed by rates of CO_2 assimilation) were observed, it is unlikely that the oxidation of the glutathione pool could be explained primarily by a markedly decreased supply of NADPH. A more probable explanation is that GR activities are insufficient to keep pace with the increased redox flux through the glutathione pool. Although sustained oxidation of the ascorbate pool was not observed, it is likely that the immediate reductant for H_2O_2 removal in the mutant is ascorbate, given the high activity of APX in leaves. Thus, although a small contribution of glutathione peroxidase (see § 5) cannot be discounted, the net oxidation of the glutathione pool probably results from electron transfer between reduced glutathione and dehydroascorbate, formed (ultimately) from APX activity. Indeed, accelerated formation of the immediate product of ascorbate peroxidation, MDHA, has been reported on inhibition of catalase in bean leaves (Veljovic-Jovanovic *et al.* 1998). The relative contribution of the different APX isoforms to the removal of H_2O_2 originating in the peroxisome is not known. It has been shown in the barley mutant that the accumulation of glutathione and its net oxidation occur in both chloroplastic and extra-chloroplastic compartments (Smith *et al.* 1985).

In view of the perturbation of cellular homeostasis that accompanies insufficient catalase activity, we might wonder whether peroxisomal APX activity plays a very significant role in the processing of photorespiratory H_2O_2 in wild-type plants. The activity of APX in purified leaf peroxisomes is more than three orders of magnitude lower than catalase (Yamaguchi *et al.* 1995). However, the significance of peroxisomal APX is almost certainly not to be seen in terms of its contribution to the overall flux but rather in terms of its effect on local H_2O_2 concentrations. The low affinity of catalase for H_2O_2 means that maintenance of very low concentrations of this oxidant requires very high amounts of the protein. It may not be possible to pack the peroxisome with sufficient catalase to prevent H_2O_2 accumulation when photorespiration is rapid. Although bounded by only a single membrane, the ultrastructure of the peroxisome may favour metabolite channelling (Heupel & Heldt 1994) and allow H_2O_2 to accumulate internally, thereby favouring rapid catalase activity. The significance of the external location of peroxisomal APX might lie in its acting as a first line of defence to minimize leakage of H_2O_2 into the cytosol (Yamaguchi *et al.* 1995). With respect to cytosolic H_2O_2 concentrations, it is interesting that cytosolic APX has a higher affinity for H_2O_2 than the chloroplastic isoforms (Chen & Asada 1989; Yoshimura *et al.* 1998), even though it is often considered that a key role of the latter is to keep H_2O_2 comparatively low in the chloroplast stroma, in order to prevent inactivation of the thiol-regulated enzymes. However, the effect of APX activities on H_2O_2 concentrations will depend not only on the affinities of the enzyme for its substrates but also on the amounts of the protein present in each compartment. The intracellular distribution of APX can vary between species and tissues (Amako *et al.* 1994) and may reflect the status of components of the anti-oxidative system, as discussed in § 2 for *Arabidopsis*.

4. CONTROL OF ORGANIC PEROXIDES IN THE CHLOROPLAST

Like H_2O_2, organic peroxides are formed during metabolism and might also be involved in signal transduction (Baier & Dietz 1999*a*). The lifetime of these species must be rigorously controlled because chain-type cascade reactions, particularly involving lipid hydroperoxides, can lead to severe disruption of membrane systems. Several plant proteins have been identified that might carry out the important task of reducing these species to the corresponding harmless alcohols. These include glutathione peroxidases (GPX; Eshdat *et al.* 1997), glutathione-S-transferases (Bartling *et al.* 1993), and 2-cys peroxiredoxins (Baier & Dietz 1997). Although limited information is available on the biochemical characteristics of GPX and peroxiredoxins, both differ from other known higher-plant peroxidases in that they do not contain haem (Baier & Dietz 1999*b*). The plant GPX also differs from animal GPX as the selenocysteine residue at the active site of the latter is replaced by cysteine, which diminishes the nucleophilic properties of the enzyme and probably accounts for its much lower activity with H_2O_2. Although both plant GPX and peroxiredoxins can probably reduce H_2O_2 to water, their low activity means that APX is by far the most important H_2O_2-removing peroxidase in plant tissue. The significance of GPX and 2-cys peroxiredoxins may lie in their location. Both cytosolic and chloroplastic isoforms of GPX exist (Eshdat *et al.* 1997; Mullineaux *et al.* 1998) while thus far only a chloroplastic 2-cys peroxiredoxin isoform has been reported (Baier & Dietz 1997). The exact site of either enzyme is not established, though it has been suggested that chloroplastic GPX may be primarily found as a soluble enzyme in the stromal phase, whereas 2-cys peroxiredoxins may be closely associated with the thylakoid membranes (Baier & Dietz 1999*b*). In *E. coli*, 2-cys peroxiredoxins will oxidize NADPH or glutaredoxin, whereas the yeast enzyme uses reduced thioredoxin, as may be the case in plants (Baier & Dietz 1999*b*).

Because peroxiredoxin activity is difficult to measure directly, the significance of this protein has been evaluated by examining the effect of modified expression on photosynthesis and the antioxidant system in *Arabidopsis* (Baier & Dietz 1999*c*; Baier *et al.* 2000). Introduction of an antisense construct caused a significant reduction in protein level in developing *Arabidopsis*, producing an attendant disruption of photosynthesis and induction of non-specific peroxidases (Baier & Dietz 1999*c*). Although the expression of the enzyme was dependent on developmental stage in both untransformed and transformed *Arabidopsis*, transcripts were much more abundant in all cases than those for GPX (Baier *et al.* 2000). The developmental stage associated with low peroxiredoxin activity

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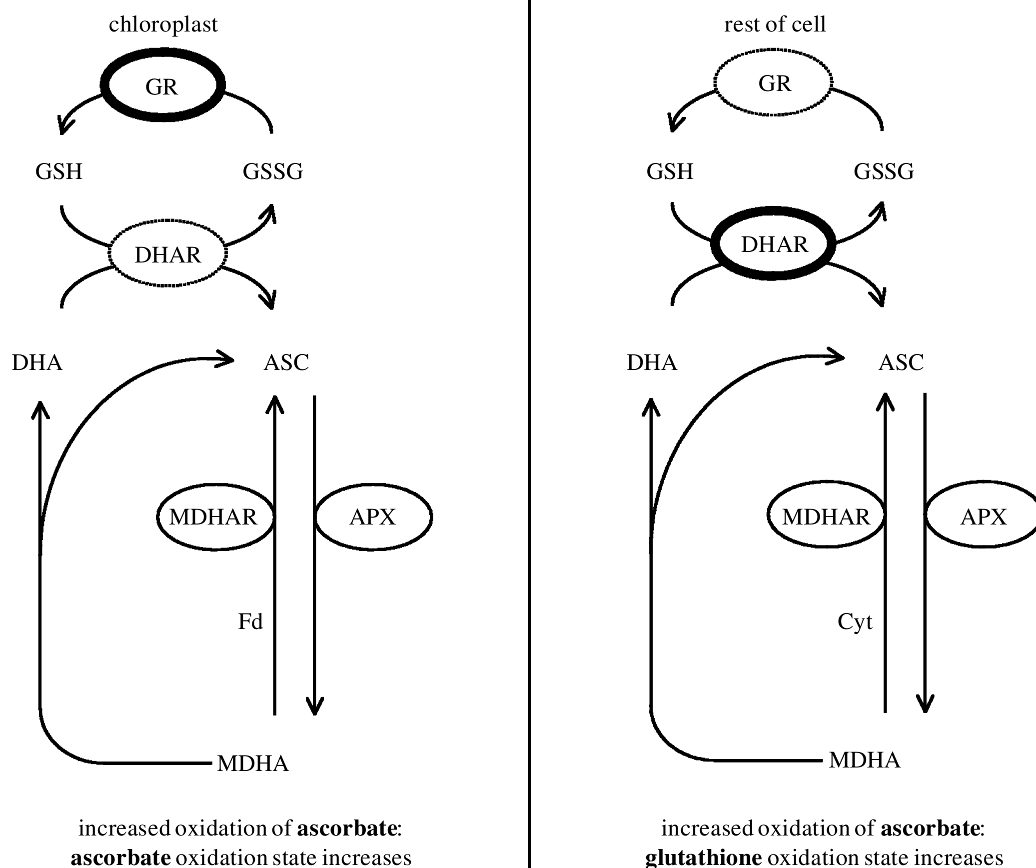

Figure 1. Hypothetical model of ascorbate–glutathione redox coupling in the chloroplast and the rest of the cell. It is proposed that the relatively lower dehydroascorbate reductase (DHAR) activities in the chloroplast allow less strict coupling of the redox states of the ascorbate and glutathione pools in this compartment. In other compartments, such as the cytosol, higher relative activities of enzymes catalysing the reduction of dehydroascorbate (DHA) by reduced glutathione (GSH) ensure that the two antioxidants remain tightly coupled. APX, ascorbate peroxidase; ASC, ascorbate; Cyt, cytochromes; Fd, ferredoxin; GR, glutathione reductase; GSSG, glutathione disulphide; MDHA, monodehydroascorbate.

in the transformants was characterized by specific effects on the established antioxidant system. Enhanced abundance of transcripts for thylakoid APX, stroma-soluble APX and stromal MDHA reductase was found in the transformants, and this was reflected in markedly enhanced activity of the latter enzyme in leaf extracts (Baier *et al.* 2000). Total extractable APX activity was not significantly increased, a further example of the frequent lack of correspondence between APX transcript abundance and extractable activity. These changes in expression were correlated with increased oxidation of foliar ascorbate, but not glutathione (Baier *et al.* 2000). This observation contrasts with the findings in plants with decreased catalase activities and is discussed further in the following sections.

Taken together, the data provide evidence that chloroplastic 2-cys peroxiredoxin is an important constituent of the antioxidative system in *Arabidopsis*, and may be particularly important in protecting the photosynthetic apparatus from the potentially harmful effects of organic hydroperoxides. These species can be produced by reaction of alkyl side-chains with the hydroxyl radical. They may also result from accelerated production of singlet oxygen, through photodynamic energy transfer to ground-state singlet oxygen. Although the thylakoid membrane contains several energy-quenching pathways that minimize the formation of singlet oxygen (Foyer & Harbinson 1999), any that is produced will tend to favour generation of organic hydroperoxyl radicals and hydroperoxides—2-cys peroxiredoxin activity could be crucial in ensuring the ongoing reduction of these species. If thioredoxin is the regenerating reductant of the peroxiredoxin active site, this would constitute a system that allows the elimination of disruptive oxidants in the thylakoid membrane by diversion of reducing power from the electron transport chain.

5. REDOX COUPLING BETWEEN ASCORBATE AND GLUTATHIONE

The earliest published notion of an ascorbate–glutathione cycle in plants probably dates from the 1930s, when these compounds were proposed to be intermediates in respiratory electron transport to O_2, with ascorbate

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oxidase being the terminal enzyme (see references in Mapson 1958). It was also considered possible that peroxidation of ascorbate via flavonoid peroxidases might be linked to glutathione cycling (Mapson 1958). In the 1970s it was suggested that glutathione redox cycling in the chloroplast is a necessary part of H_2O_2 removal by ascorbate peroxidation (Foyer & Halliwell 1976) and shortly afterwards the presence of a highly active chloroplastic APX was reported (Groden & Beck 1979; Kelly & Latzko 1979). Redox coupling between ascorbate and glutathione is probably a universal phenomenon in cells that contain both compounds—it represents an instance of a biochemical trait whose discovery in plants preceded its recognition in animals, though this is rarely acknowledged explicitly (Meister 1994).

The relative redox potentials of the two antioxidant couples favour net electron flow from reduced glutathione to dehydroascorbate (Foyer & Noctor 2000) and this reaction can occur at significant rates even in the absence of an enzyme, particularly at alkaline pH (Winkler 1992). In conditions of high APX activity, however, dehydroascorbate reductase activity could be necessary to ensure effective maintenance of the reduced form of ascorbate. In animals, this activity can be catalysed by proteins such as glutaredoxins and protein disulphide isomerases (Wells *et al.* 1990). These proteins, along with certain types of trypsin inhibitor (Trumper *et al.* 1994), might also catalyse the reaction in plants, and dehydroascorbate reductase activity has been independently purified from several plant tissues (Foyer & Halliwell 1977; Hossain & Asada 1984; Dipierro & Borranccino 1991; Kato *et al.* 1997). The amino-acid sequence of the purified rice enzyme suggests that it is a specific dehydroascorbate reductase (Kato *et al.* 1997) containing domains that match those encoded by several expressed sequence tags from *Arabidopsis* (Foyer & Mullineaux 1998). A gene sequence encoding a specific chloroplastic isoform has recently been identified (Shimaoka *et al.* 2000), but total dehydroascorbate reductase capacity (whether due to a specific activity or not) may vary widely between species and compartments.

Intercompartmental differences in dehydroascorbate reductase activity mean that the tightness of coupling between glutathione and ascorbate might differ considerably (figure 1). Although the ascorbate–glutathione cycle, as a route for peroxide detoxification, was first proposed for the chloroplast (Foyer & Halliwell 1976), it may be that in this organelle the two antioxidants are coupled less tightly than in other compartments such as the cytosol. One reason for this could be the high capacity of the chloroplast to regenerate ascorbate from MDHA photochemically, through ferredoxin (figure 1, left). The principal physiological significance of high glutathione and glutathione reductase activity in chloroplasts may therefore be in processes other than the regeneration of ascorbate. Nevertheless, there is some redox coupling between ascorbate and glutathione in the chloroplast—addition of exogenous H_2O_2 to spinach chloroplasts was able to drive turnover of both the ascorbate and glutathione pools (Anderson *et al.* 1983).

In plants with decreased catalase, where the increased oxidative load is of extra-chloroplastic origin, it is the glutathione pool that is preferentially oxidized (table 2), which can be interpreted as close coupling of this pool to

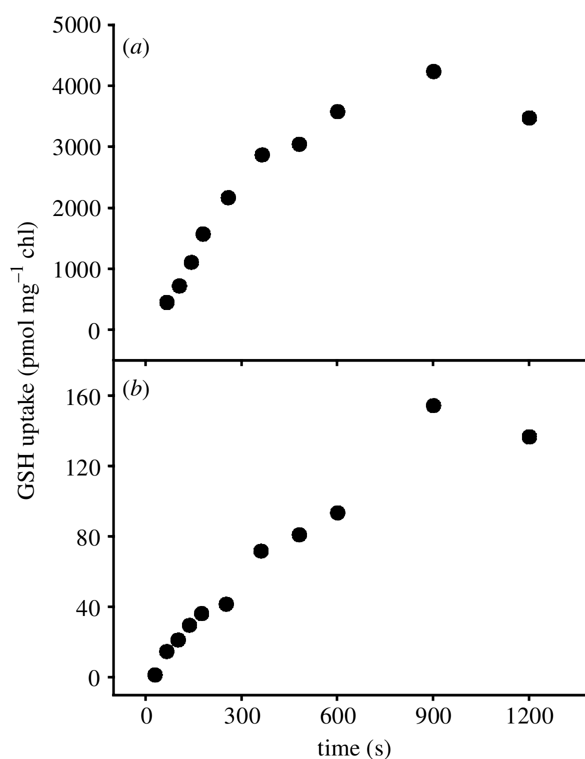

Figure 2. Uptake of glutathione (*a*) 100 μM GSH and (*b*) 1 μM GSH into wheat chloroplasts. Chloroplasts were highly intact as assessed by ferricyanide exclusion and by retention and latency of NADP-glyceraldehyde-3-phosphate dehydrogenase. Uptake was initiated by addition of ^{35}S-GSH. At the times indicated, chloroplasts were separated from the surrounding medium and uptake calculated according to equations given in Heldt (1980).

ascorbate peroxidation (figure 1, right). Other explanations would include direct oxidation of the glutathione pool, for example in GPX activity. This cannot be definitely discounted but fluxes are likely to be relatively low compared to the rate of generation of MDHA and dehydroascorbate. In contrast to effects observed when catalase activity is decreased, 2-cys peroxiredoxin deficiency in the chloroplast was associated with an increased oxidation state of the ascorbate pool (Baier *et al.* 2000). The mechanisms responsible for this effect are not clear but may include, for example, a greater burden on the ascorbate pool for regeneration of α-tocopherol under conditions when the capacity for detoxification of alkyl peroxides is compromised. Maintenance of the glutathione pool in a highly reduced state under these conditions cannot be explained by purely thermodynamic considerations because the lower redox potential of the glutathione couple should make it more oxidized than the ascorbate–dehydroascorbate couple at a given intracellular redox poise. In the 2-cys peroxiredoxin transformants, it therefore seems clear that redox equilibration of the glutathione–ascorbate couples is prevented by kinetic limitations that prevent sufficiently fast reaction between dehydroascorbate and glutathione. While the interpretation must be made with some caution, and considering that the data in the 2-cys peroxiredoxin transformants reflect the redox state of total foliar antioxidant pools, it might be that increased redox coupling inside the

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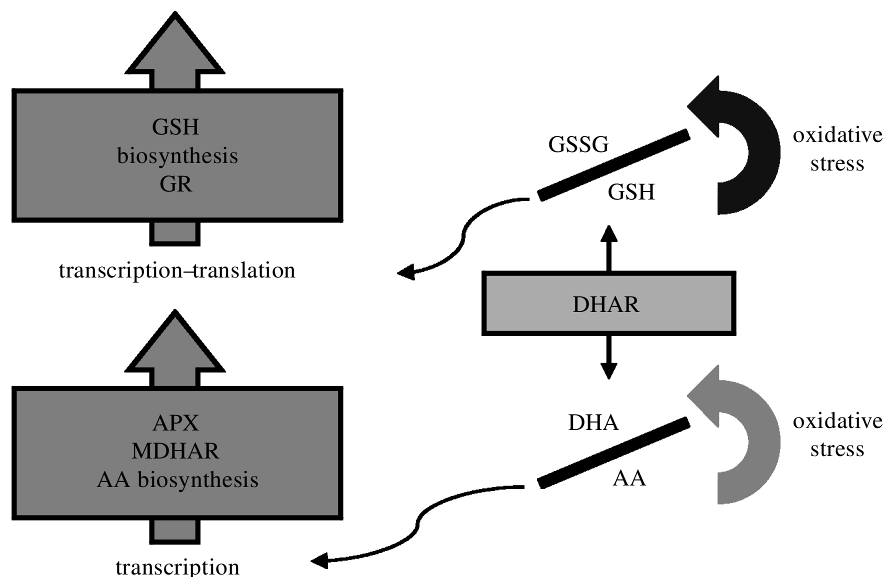

Figure 3. Modulation of components contributing to the homeostasis of the soluble antioxidants ascorbate (AA) and glutathione (GSH). Oxidative stress causes accelerated turnover of the ascorbate–glutathione cycle and severe oxidative stress may cause uncoupling of these redox couples via a limitation at dehydroascorbate reductase (DHAR). A key factor determining whether stress induces changes in the redox state of ascorbate or glutathione will be the relative capacities of DHAR and glutathione reductase (GR). The ratios of oxidized (GSSG) to reduced GSH, and oxidized (DHA) to reduced AA, act as transcriptional–translational controls that tend to restore homeostasis.

chloroplast between ascorbate and glutathione is limited by comparatively low dehydroascorbate reductase activity (figure 2). Higher dehydroascorbate reductase activities outside the chloroplast would allow tighter coupling of the two antioxidants—the preferential oxidation of the glutathione pool under conditions of enhanced oxidant availability could then reflect limiting glutathione reductase activity. Imperfect ascorbate–glutathione redox coupling in the chloroplast could also be explained by the existence of a pool of ascorbate to which glutathione does not have access. An obvious possibility is that an intra-thylakoidal pool of ascorbate becomes oxidized when 2-cys peroxiredoxin activity is insufficient.

Full discussion of the above questions is limited by our incomplete knowledge of the influence of transport on the concentration and redox state of antioxidants. The data in tables 1 and 2 of this paper, and in many other reports, are derived from global foliar antioxidant pools. In the barley catalase mutant, non-aqueous fractionation analysis showed that the marked accumulation of oxidized glutathione occurs in both chloroplastic and extrachloroplastic compartments (Smith *et al.* 1985). Nevertheless, it is still unclear whether this reflects tight redox coupling of ascorbate and glutathione in both compartments, because (i) we do not know the cellular location of those APX isoforms that make the most significant contribution to H_2O_2 detoxification under these conditions, since H_2O_2 readily crosses biological membranes, and (ii) both reduced and oxidized forms of ascorbate cross the chloroplast envelope (Foyer & Lelandais 1996; Horemans *et al.* 1999). In contrast, there is no data in the literature concerning the possibility of glutathione transport across the envelope. Figure 2 presents some preliminary data we have obtained, showing that the reduced form of glutathione is readily taken up into intact wheat chloroplasts. At concentrations of up to 100 μM, the rate is linear for at least 5 min and, at lower concentrations, a linear rate of uptake is observed for up to 20 min (figure 2). When the rates are calculated over the linear portion of the curve and plotted against concentration, a curve is obtained that shows saturation kinetics with half-maximal velocity at around 50 μM glutathione. Chloroplastic uptake of the oxidized form of glutathione is currently being investigated. Like the chloroplast ascorbate transporters, the rate of translocation of reduced glutathione is high enough to play a significant role in determining the total pool size on either side of envelope because the uptake capacity is of the same order as the capacity for synthesis. As these capacities are much lower than that of antioxidant redox cycling, they might be predicted to play a relatively minor role in equilibrating antioxidant redox states between different compartments. However, the high rate of redox cycling means that the slow changes in antioxidant redox state observed, for example, in the catalase mutants, probably result from only slight imbalances in the relative rates of reduction and oxidation. An important 'topping-up' effect of transport activities on redox states cannot, therefore, be excluded, though this would require oxidized and reduced forms to be translocated at significantly different rates.

6. ASCORBATE AND GLUTATHIONE IN SIGNAL TRANSDUCTION

If compartment-specific variation in antioxidant redox states is common, this may have considerable significance for redox signalling (figure 3). First, the activity of the antioxidant system is a key player in determining cellular H_2O_2 concentrations, and therefore in influencing the

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known roles of this oxidant in signal transduction. Recent work suggests that the relatively rapid movement of H_2O_2 through some biological membranes is primarily due to aquaporins, and that the interaction between aquaporins and H_2O_2 decomposition will determine inter-compartmental flux and concentrations (Henzler & Steudle 2000). Intracellular gradients in H_2O_2 concentration are very likely. In some instances, such gradients can be transient—localized accumulation of H_2O_2 in the apoplast is essential in acclimatory responses to pathogens and pollutants. In others, sustained differences must be achieved—concentrations of H_2O_2 sufficient to inactivate CO_2 fixation in isolated chloroplasts are below global leaf concentrations. Second, changes in antioxidant redox state and concentration can be more pronounced than changes in leaf H_2O_2 contents (e.g. in plants with decreased catalase) and effective coordination of gene expression might require that these changes be compartment specific.

Recent work has shown the existence of H_2O_2-sensitive transcription and translation factors (Foyer *et al.* 1997; Xiang *et al.* 2000) and glutathione is also implicated in the control of gene expression (Wingate *et al.* 1988; Dron *et al.* 1988; Hérouart *et al.* 1993; Baier & Dietz 1997; Link *et al.* 1997). The perturbation of the glutathione pool could play a key role in controlling gene expression in catalase-deficient plants (Willekens *et al.* 1997). Genes under glutathione-mediated control not only include components of the antioxidative system, but also genes involved in other processes. While ascorbate is implicated in the regulation of cell growth and division (Noctor & Foyer 1998; Horemans *et al.* 1999), the role of this antioxidant in gene expression has received less attention than that of glutathione. However, the data discussed above suggest that both ascorbate contents and redox state could influence the expression of antioxidative genes in *Arabidopsis*. The decreased ascorbate contents in *vtc1* correlate with increased activity of extra-chloroplastic APX, while the perturbation of the ascorbate redox state in plants with decreased 2-cys peroxiredoxin is linked to enhanced abundance of transcripts for chloroplastic APX and MDHA reductase.

Expression of the cytosolic APX in *Arabidopsis* has been shown to be increased by high light, correlating with changes in the redox state of components of the photosynthetic electron transport chain (Karpinski *et al.* 1997). In our experiments with *vtc1*, enhanced activity of extra-chloroplastic APX was observed in plants grown at low light. We do not know whether the decreased ascorbate content in these leaves is global or is compartment specific. The same caveat applies to the increased oxidation state of ascorbate in plants with decreased 2-cys peroxiredoxin (Baier *et al.* 2000). Nonetheless, in this case, it appears that a stress of chloroplastic origin (compromised 2-cys peroxiredoxin activity) can influence the abundance of transcripts of nuclear genes. The chemical identity of the signal(s) responsible for transmitting information on chloroplast redox state and antioxidant status to the nucleus remains mysterious, but the evidence suggests that such messengers must exist, whether they effect changes in the expression of chloroplastic or cytosolic proteins (Karpinski *et al.* 1997; Baier *et al.* 2000).

Characterization of these signalling systems is likely to be achieved in the near future. Assessment of their significance will require integrated information on the sensitivity of promoters and translation factors to the reduced and oxidized forms of antioxidants, as well as more extensive knowledge of the biochemical factors that determine antioxidant concentrations and redox state in different compartments. Variable redox coupling between ascorbate and glutathione would allow each to evoke different responses in gene expression following changes in the rate of photosynthetic peroxide production. Thermodynamic considerations predict that close coupling should favour oxidation of the glutathione pool. Kinetic effects, due to compartmentalization of the pools, higher capacity of GR than dehydroascorbate reductase (DHAR), or differential transport rates of the reduced and oxidized forms of ascorbate and glutathione, could allow ascorbate pools to become oxidized while glutathione remains reduced. Such redox uncoupling would allow the redox state of ascorbate to mediate specific effects on gene expression.

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Discussion

S. Grace (*Biodynamics Institute, Louisiana State University, USA*). It has been suggested that the majority of leaf glutathione reductase is located in the chloroplast. How does this agree with your model of control of glutathione redox state?

G. Noctor. I agree that most data in the literature suggest that a large fraction of the foliar GR activity is located in the chloroplast. This may reflect the importance of maintaining the chloroplastic glutathione pool in a highly reduced state. As far as intercompartmental difference in redox coupling between ascorbate and glutathione goes, I think this fits pretty well with the model we have presented. If GR capacity is in excess of DHAR capacity, then we would predict that this might allow the chloroplastic glutathione pool to remain highly reduced even in conditions where the ascorbate pool is relatively oxidized. This is the explanation we propose for the observations in *Arabidopsis* with decreases in the chloroplastic component, 2-cys peroxiredoxin.