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Are diverse signalling pathways integrated in the regulation of *Arabidopsis* antioxidant defence gene expression in response to excess excitation energy?

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When low-light-grown *Arabidopsis* rosettes are partially exposed to excess light (EL), the unexposed leaves become acclimated to excess excitation energy (EEE) and consequent photo-oxidative stress. This phenomenon, termed systemic acquired acclimation (SAA), is associated with redox changes in the proximity of photosystem II, changes in foliar H_2O_2 content and induction of antioxidant defences. The induction of extra-plastidial antioxidant systems is important in the protection of the chloroplast under EL conditions. A larger range of transcripts encoding different antioxidant defence enzymes may be induced in the systemically acclimated leaves and these include those encoded by the glutathione peroxidase (*GPX2*) and glutathione-S-transferase (*GST*) genes, which are also highly induced in the hypersensitive response and associated systemic acquired resistance (SAR) in incompatible plant–pathogen interactions. Furthermore, the expression of the SAR-inducible pathogenesis-related protein gene, *PR2*, is enhanced in SAA leaves. Wounded leaf tissue also shows enhanced systemic induction of a cytosolic ascorbate peroxidase gene (*APX2*) under EL conditions. These and other considerations, suggest H_2O_2 and other reactive oxygen species (ROS) could be the common factor in signalling pathways for diverse environmental stresses. These effects may be mediated by changes in the level and redox state of the cellular glutathione pool. Mutants with constitutive expression of a normally EL-inducible *APX2* gene have much reduced levels of foliar glutathione.

The expression of *APX1* and *APX3*, encoding cytosolic and peroxisome-associated isoforms, respectively, are also under phytochrome-A-mediated control. The expression of these genes is tightly linked to the greening of plastids in etiolated seedlings. These data suggest that part of the developmental processes that bring about the acclimation of leaves to high light includes the configuration of antioxidant defences. Therefore, the linkage between immediate responses of leaves to EL, acclimation of chloroplasts to EEE and the subsequent changes to leaf form and function in high light could be mediated by the activity of foliar antioxidant defences and changes in the concentration of ROS.

Keywords: excess excitation energy; oxidative stress; signalling; acclimation; wounding; pathogens

1. INTRODUCTION

Plants encounter a multiplicity of biotic and abiotic stresses to which they must mount both immediate and acclimatory responses. These stresses include limitations in nutritional and water status, fluctuating light intensities and temperatures, physical damage brought about by grazing by insect and animal herbivores, disease agents of varying severity, gaseous pollutants, increased ambient ultraviolet B (UVB) levels and contaminating xenobiotics such as herbicides and heavy metals. All of this suggests that many completely different pathways may exist for sensing and responding to environmental cues. However, the phenomenon of cross-tolerance (Bowler & Fluhr 2000; Noctor & Foyer 1998, and references therein), in which exposure to one form of stress can be shown to bring about a marked increase in tolerance to different modes of stress, suggests that underlying cellular responses might have a common origin. The title of this paper sets the question about commonality of responses to biotic and abiotic stress in the context of our recent work.

In considering plants' tolerance to environmental stress both immediate and acclimatory responses have to be considered. Furthermore, one may have to distinguish between acclimatory mechanisms in existing tissues,

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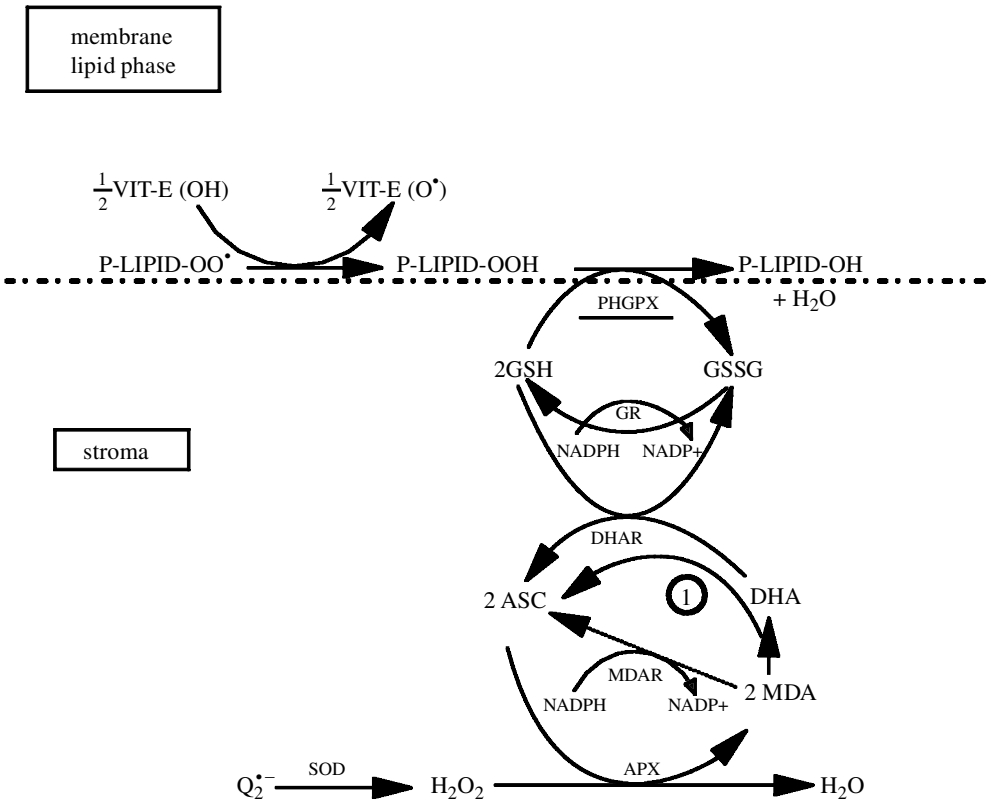

Figure 1. The scavenging of active oxygen species in the chloroplast in both lipid membrane phase and the stroma, linked to redox cycles for ascorbate and glutathione and the oxidation of α-tocopherol (vitamin E). The displayed scheme is combined from the ascorbate–glutathione cycle (Foyer & Halliwell 1976) with modifications described in Scandalios (1997). The reactions involving vitamin E are from Scandalios (1997). The presence of the linking PHGPX-catalysed reaction (underlined) are combined from Mullineaux *et al*. (1998) and Beeor-Tzahar *et al*. (1995). Abbreviations are as follows: P-LIPID-OO, phospholipid peroxy radical; P-LIPID-OOH, phospholipid peroxide; P-LIPID-OH, phospholipid alcohol; VIT-E(OH), α-tocopherol (vitamin E); VIT-E (O·), α-chromanoxyl radical; PHGPX, phospholipid hydroperoxide-dependent glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulphide; GR, glutathione reductase; DHAR, dehydroascorbate reductase; ASC, ascorbic acid; DHA; dehydroascorbate; MDA, mondehydroascorbate free radical; MDAR, monodehydroascorbate free radical reductase; APX, ascorbate reductase; SOD, superoxide dismutase; $O_2^{\cdot -}$, superoxide anion. Reaction 1 is the non-enzyme-catalysed spontaneous dismutation of two MDA molecules to one ASC and one DHA, respectively.

perhaps manifested as adjustments to metabolism, as distinct from changes in developmental processes leading to the growth of new tissues. Therefore, a central point to consider is the regulation of antioxidant gene expression as part of the process whereby plants make the transition from an immediate to an acclimatory response to changing environmental circumstances.

In this paper, we describe our recent work on the regulation of the ascorbate peroxidase gene (*APX*) family of *Arabidopsis thaliana* L. in response to excess excitation energy (EEE) brought about by exposure of low-light (LL)-grown plants to excess light (EL). We aim to set this work in the context of both immediate responses and systemic acclimation to stress and compare this with more advanced studies on responses to pathogens and physical damage. Light also plays a central role in leaf and chloroplast development (Walters *et al*. 1999; Nagy & Schäffer 2000) and the control of expression of some members of this gene family are linked to this process. The impact of developmental processes on the establishment of antioxidant defences and the ability to respond to a fluctuating environment might provide a further connection between immediate responses and acclimation.

2. THE PRODUCTION AND CONSUMPTION OF REACTIVE OXYGEN SPECIES

There is now a wealth of evidence to suggest that at the cellular level, many adverse environmental factors affecting plants act by promoting, at least in part, oxidative stress at the cellular level (Scandalios 1997; Noctor & Foyer 1998; Asada 1999). When the production of reactive oxygen species (ROS) exceeds the capacity of antioxidant metabolism to remove them, oxidative damage to cellular macromolecules and structures occurs, which if unchecked, leads to cell death. It is the stress-induced cellular responses associated with increased levels of ROS that are suggested to be the biochemical basis of cross-tolerance phenomena in plants (Bowler & Fluhr 2000; Noctor & Foyer 1998).

Oxidative stress is often manifested as leaf chlorosis (and subsequent necrosis) and bleaching in a wide variety of both abiotic and biotic stresses. This common feature of stress responses, emphasizes that the chloroplast, with its production of oxygen in the presence of high electron fluxes through photosystem (PS) II is a prime source of cellular ROS in adverse conditions (Asada 1999; Creissen

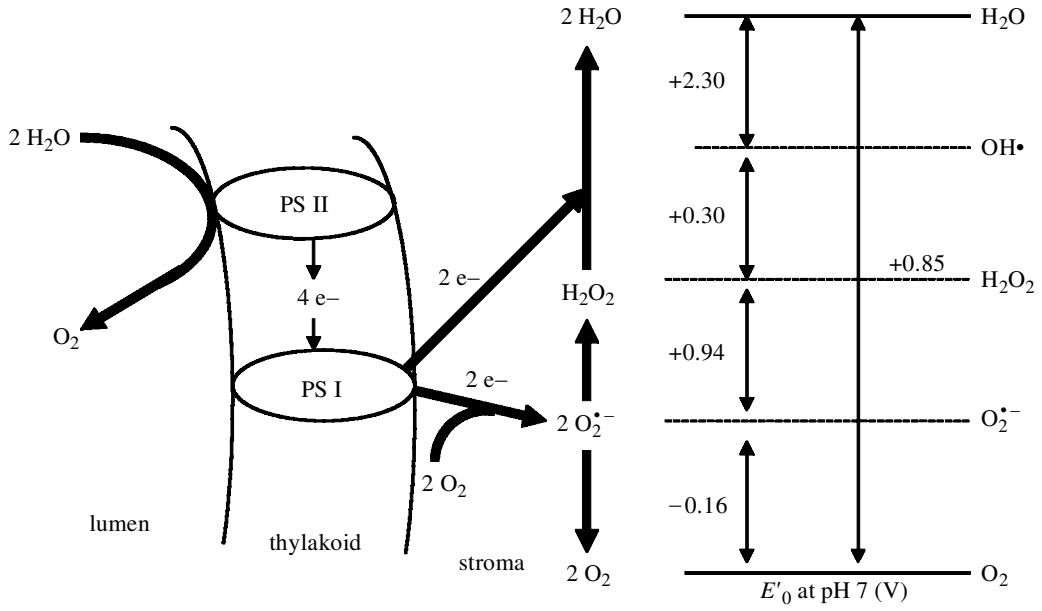

Figure 2. A schematic outline of the Mehler–peroxidase reaction in chloroplasts and the redox potentials of the ROS that are the reduced intermediates of oxygen (reproduced from Asada (1999)). The APX-catalysed reaction is the reduction of H_2O_2 to water using ascorbate as the electron donor. The oxidized ascorbate, mondehydroascorbate free radical or its disproportionation product dehydroascorbate, are reduced back to ascorbate using two electrons ultimately derived from PS II.

et al. 1999; Karpinski *et al.* 1999). However, it is important to realize that even under non-stress conditions, there is continual production of ROS, principally associated with photosynthesis and allied processes such as photorespiration. Therefore, under both stressful and benign conditions, ROS accumulation must be kept in check by a network of low molecular weight antioxidants and antioxidant enzymes present in all subcellular compartments (figures 1 and 2; Noctor & Foyer 1998; Asada 1999).

In addition to oxidative damage brought about by external factors, programmed cell death, as part of developmental processes (e.g. pollen maturation and senescence) and in the hypersensitive response (HR) in incompatible plant–pathogen interactions, is initiated and driven by the production of ROS (Pennell & Lamb 1997).

3. DISSIPATORY PROCESSES AND THE PRODUCTION OF REACTIVE OXYGEN SPECIES

Under all but the lowest of light conditions, photosynthetic cells have to dissipate excitation energy in excess of that required for the production of ATP and reducing equivalents needed by cellular metabolism, most notably carbon fixation. Under saturating light conditions, only about 10% of absorbed light energy is used to fix carbon (Asada 1999). Plants have several diverse mechanisms for dissipating EEE either by quenching it within the light-harvesting apparatus or consuming it in alternative electron sinks (Horton *et al.* 1996; Huner *et al.* 1998; Li *et al.* 2000). A failure to dissipate EEE would rapidly cause over-reduction of components of photosynthetic electron transport, ultimately leading to irreversible photodamage of leaf tissues (Huner *et al.* 1998; Karpinski *et al.* 1999).

In this paper, there will be consideration only of those dissipatory processes that involve ROS metabolism. For a consideration of the central importance of the dissipation of absorbed light as heat by the light-harvesting apparatus (non-photochemical quenching (NPQ)) the reader is referred to Horton *et al.* (1996, this issue) and Li *et al.* (2000).

In the chloroplast, the balance between ROS production and consumption is described in some detail as the 'water–water' cycle and is regarded as an important mechanism for dissipating EEE (figure 2; Asada 1999). In this cycle there is proposed to be a stoichiometric relationship between the electrons abstracted from water and their use in the reduction of ROS back to water. This may account for up to 30% of total electron flux through the photosystems under high light (HL) conditions (Asada 1999), although more recent evidence suggests that this rate may be lower (Badger, this issue). This consumption of electrons, is achieved by the combined action of the reduction of molecular oxygen at PS I to superoxide (the Mehler reaction) and the chloroplast antioxidant system and is known as the Mehler–peroxidase reaction (figure 2).

Another potential source of ROS is photorespiration in C_3 plants (Willekens *et al.* 1997). The oxygenase reaction of Rubisco (ribulose-1,5-bisphosphate carboxylase–oxygenase) consumes energy and reducing equivalents and under HL conditions is an important dissipatory mechanism (Kozaki & Takeba 1996; Willekens *et al.* 1997; Asada 1999). The oxygenase reaction of rubisco in ambient O_2 and CO_2 concentrations is significant with 1 mol O_2 fixed for 3 mol CO_2 fixed. The carbon in the glycollate formed by the oxygenase reaction is recovered in the photorespiratory cycle. However, the photorespiratory cycle involves the production of H_2O_2, catalysed by the peroxisomal glycollate oxidase reaction, which has to be reduced by antioxidant systems, principally in, or associated with the peroxisome. Under saturating light conditions the rate of photorespiratory H_2O_2 production

by a C_3 leaf would be high, at *ca*. 70 μmol min^{-1} g fresh $weight^{-1}$ (calculated from Willekens *et al*. (1997)).

The amount of EEE that must be dissipated will vary according to environmental conditions. In particular, the many environmental factors that can cause a lessening of rates of CO_2 fixation, such as limitations in nutrient availability, drought, salinity and low temperatures, will increase the amount of EEE and accompanying oxidative stress (Asada 1999; Walters *et al*. 1999). Also, the often rapid changes in light intensities that plants can encounter will cause the amount of excitation energy to fluctuate continually between limiting and excessive levels (Asada 1999). In addition, developmental processes will determine the amount of EEE generated and the capacity for its dissipation. These could include examples such as the number of photosynthetic reaction centres per unit area of leaf (Walters *et al*. 1999) and the number of available sinks for the consumption of electrons derived from photosynthesis (Huner *et al*. 1998).

4. THE POTENTIAL FOR CONTROL OF CELLULAR STRESS RESPONSES BY REACTIVE OXYGEN SPECIES

An increase in EEE would have to be matched by increased activity or number of dissipatory processes. In the case of those dissipatory processes that generate ROS directly (the Mehler–peroxidase reaction; figure 2) or indirectly (e.g. via photorespiration) there is never a complete removal of them (Noctor & Foyer 1998; Asada 1999). Therefore potentially, any increase in EEE that is not matched by an increase in the efficiency of the associated antioxidant systems will lead to a rise in ROS levels. While this could lead to oxidative stress, on the positive side, the monitoring of even small changes in ROS levels or turnover in any part of the plant cell potentially provides a very sensitive means of responding to fluctuating environments. ROS sensing in microbes is achieved by redox-sensitive transcription factors such as *oxy*R, *sox*R and *Yap*1 (Jamieson & Storz 1997; Åslund & Beckwith 1999). More complex mechanisms for ROS sensing exist in animals such as those involving the apoptosis regulator, Bcl-2, the antioxidant responsive factor, AP-1, and the ROS responsive transcription factor, NF-κB (Hockenbery *et al*. 1993; Meyer *et al*. 1993; Jamieson & Storz 1997). For plants, the first descriptions of a regulatory framework for oxidative stress regulation have been made with the recent report of a mitogen-activated kinase kinase kinase, ANP1, being strongly activated by H_2O_2 (Kovtun *et al*. 2000). While no redox-sensitive transcription factor has yet been identified, the protein encoded by the *Arabidopsis NPR1* gene, which is a component of the signalling leading to the HR in incompatible plant–pathogen interactions (R/Avr), has been suggested to be an orthologue of I-κB (Cao *et al*. 1997). This protein in animals is an inhibitory subunit of NFκ-B that is released from a heterodimeric complex in response to external stimuli, including ROS (Hockenbery *et al*. 1993; Jamieson & Storz 1997). Additionally, a Rac-like GTP-binding protein, OsRac1, analogous to those found in animal phagocytes, has been suggested to be an initiator of H_2O_2 production associated with developmental cell death processes in rice (Kawasaki *et al*. 1999).

5. THE *APX* GENE FAMILY IN *ARABIDOPSIS*

An important component enzyme of antioxidant defences is APX, which catalyses the reduction of H_2O_2 to water using ascorbate (vitamin C) as the electron donor (figures 1 and 2). In various plant species, APX activity and protein (of the cytochrome *c* peroxidase family) have been located in the chloroplast (one stromal and one anchored to the stromal side of the thylakoid membrane), the cytosol, the outer surface of microbodies (glyoxysomes and peroxisomes) and the mitochondrion (reviewed by Creissen & Mullineaux 2000). In *Arabidopsis*, a total of five genes have been identified coding for isoforms in the chloroplast (*APX4* and *APX5*), cytosol (*APX1* and *APX2*) and microbodies (*APX3*; Kubo *et al*. 1993; Santos *et al*. 1996; Karpinski *et al*. 1997; Jespersen *et al*. 1997). This probably represents the full gene set but other isoforms may arise by some form of post-transcriptional or post-translational processes. It is important to note that the expression of all members of the *APX* gene family, irrespective of which isoform they encode, is intimately associated with the functioning, stress responses, signalling processes and development of the chloroplast.

6. REGULATION OF THE *APX* GENE FAMILY IN *ARABIDOPSIS* SUBJECT TO EXCESS EXCITATION ENERGY

When LL-grown (250 μmol m^{-2} s^{-1}) *Arabidopsis* rosettes (ecotype Columbia) are exposed to greater than tenfold EL, then rapid (< 30 min) photoinhibition of photosynthesis is initiated. If the exposure times are short (typically not greater than 90 min), photoinhibition is reversible (Russell *et al*. 1995; Karpinski *et al*. 1997, 1999). During exposure to EL, the resulting EEE rapidly leads to an increase in foliar ROS (typically measured as H_2O_2) and a sustained rise in the concentration of oxidized glutathione (GSSG). Glutathione (γ-glutamylcysteinylglycine), in the reduced form (GSH), is a thiol antioxidant present at about 0.3 mM in leaves and as such is regarded as a major determinant of cellular redox state (Creissen *et al*. 1999). Under these conditions, *APX1* mRNA levels rise approximately 18-fold and in the post-stress period are still higher than in control plants never exposed to EL (Karpinski *et al*. 1997; also see figure 5). *APX2* transcripts are not detectable in LL-grown leaves (even using PCR-based techniques), but are induced within 7 min upon exposure to EL (Karpinski *et al*. 1997, 1999) and are rapidly lost within a 2 h post-stress period. If EEE persists permanent photodamage to leaves ensues and under such conditions *APX2* expression is inhibited (Karpinski *et al*. 1999). Furthermore, it is striking that the EEE and ensuing oxidative stress that is imposed on the chloroplasts induce genes encoding components of cytosolic antioxidant defences, indicating considerable coordination between antioxidant defences in different subcellular compartments.

The induction of *APX1* and *APX2* expression by EEE can be inhibited by the photosynthetic electron transport inhibitor DCMU (3(3,4-dichlorophenyl)-1,1-dimethylurea), which blocks reduction of plastoquinone (PQ) (figure 3). Conversely, treatment of leaves with the inhibitor DBMIB (2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone), which inhibits PQ oxidation

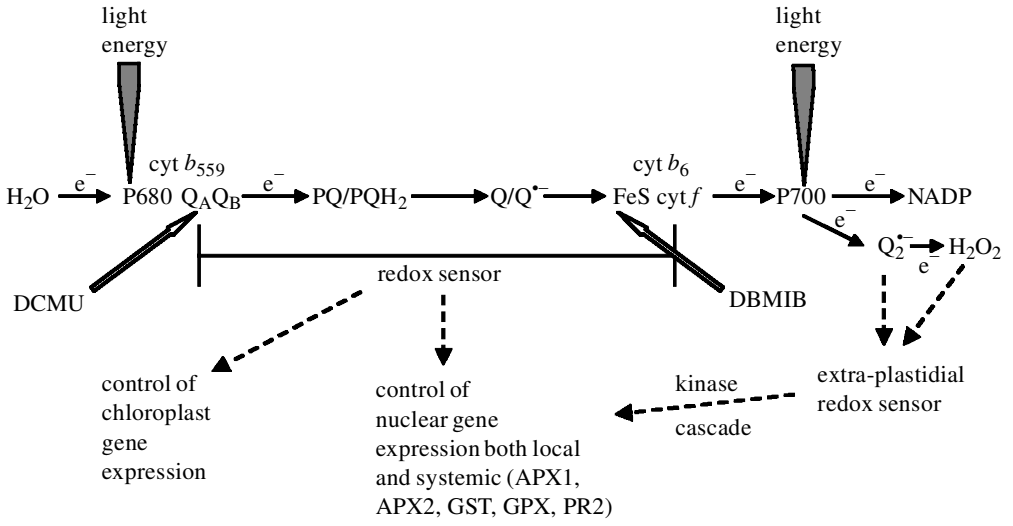

Figure 3. Redox control of chloroplast and nuclear gene expression mediated by a proposed sensor associated with PQ of PS II. The basic scheme is redrawn from Allen & Nilsson (1997). The Mehler–peroxidase reaction, which generates superoxide (O_2) from O_2 at PS I, is one possible source of ROS which, along with the PQ-associated redox sensor, regulates antioxidant defence gene expression. ROS-mediated regulation may involve the activation of a protein kinase cascade (Karpinski *et al.* 1999; Kovtun *et al.* 2000). The sites of action of the photosynthetic electron transport inhibitors DCMU and DBMIB (see §6) are shown.

(figure 3), induces expression of *APX1* and *APX2* in the absence of EL (Karpinski *et al.* 1997, 1999). Taken together, these data suggest the redox changes in PQ (or possibly Q_B; figure 3) are an important part of the regulatory system controlling *APX* gene expression (figure 3). Changes in electron flux through the PQ pool are also important in determining the relative stoichiometry of PS I and PS II. This is achieved by regulating the transcript levels of several key chloroplast-encoded genes (figure 3; Pfannschmidt *et al.* 1999; Allen, this issue). Thus, redox-sensing regulation via PQ coordinates both chloroplast and nuclear gene expression in response to sudden changes in the light environment (figure 3).

In the case of the PS-II-mediated regulation of nuclear gene expression, a signal would have to be transmitted from the chloroplast to the nucleus. There is no firm proof of what this signal might be but the possibilities considered here all implicate ROS and are not mutually exclusive. First, H_2O_2 could diffuse down a concentration gradient out of EL-stressed chloroplasts and activate a cytosol-located redox-sensitive regulator. Second, EL would stimulate photorespiratory rates and would rapidly lead to increased H_2O_2 production in the peroxisome, where a redox-sensing mechanism could be located. The EEE signal would in effect be transmitted by increased flux through the glycollate pool. Finally, the recent description of an electron transport chain ending in oxygen as the terminal electron acceptor in the cytosol (Jäger-Vottero *et al.* 1997) provides an intriguing possibility for transmitting a signal out of the chloroplast.

Treatment of leaves with H_2O_2 can partly induce *APX1* and *APX2* expression and this is associated with increased tolerance of such leaves to EL (Karpinski *et al.* 1999). However, the inhibition of *APX2* expression in EL by DCMU cannot be overridden by treatment with H_2O_2 (Karpinski *et al.* 1999). This indicates that the sequence of events leading from EL sensing to induction of nuclear gene expression is not solely mediated by an increase in ROS levels 'leaking' from the water–water cycle. Therefore, either ROS must be required to have some more direct effect on the functioning of the photosynthetic apparatus or ROS and some component associated with photosynthesis have to work concomitantly. One possibility is direct redox sensing of the PQ pool, independent of ROS generation, and this could be the same redox sensor that is proposed to control chloroplast gene transcription (figure 3; Pfannschmidt *et al.* 1999). An alternative to direct redox monitoring of PQ could be some monitoring of changes in the transthylakoid membrane pH gradient. This is required also for the functioning of NPQ mechanisms for dissipating absorbed light as heat (Horton *et al.* 1996; Li *et al.* 2000). None of the data presented to date (Karpinski *et al.* 1999; Pfannschmidt *et al.* 1999) can distinguish between changes in the redox state of PQ and in transthylakoid pH as the primary initiator of the signal which controls chloroplast and nuclear gene transcription. The recent identification of *Arabidopsis* mutants with reduced capacity for NPQ, that brought about perturbations in transthylakoid ΔpH and alterations in the redox state of Q_B (Tarantino *et al.* 1999; Peterson & Havir 2000; Li *et al.* 2000), may help to clarify the precise requirements for the initiation of induction of *APX* expression by EL.

7. THE ROLE OF GLUTATHIONE IN THE REGULATION OF *APX* GENE EXPRESSION

Treatment of leaves with glutathione (either as GSH or GSSG) prior to EL renders them more sensitive to stress and causes an inhibition of induction of *APX1* and *APX2* (Karpinski *et al.* 1997). This effect can be achieved by other antioxidants, such as ascorbate. Therefore, the inhibitory effect of these antioxidants on APX gene expression is most likely brought about by the 'damping down' of an inductive signal derived from ROS. This idea is supported by the observations that transgenic tobacco lines, with enhanced capacity to synthesize glutathione in their chloroplasts, show an oxidative stress phenotype as a consequence of incorrect configuration of antioxidant

defences (Creissen *et al*. 1999). This effect was suggested to be caused by the accumulation of oxidized γ-glutamylcysteine (the reduced form of which is an intermediate in GSH biosynthesis), interfering with the functioning of a redox-sensing mechanism. Interestingly, this same manipulation in transgenic poplar does not elicit an oxidative stress phenotype (Noctor *et al*. 1999) and emphasizes that the way in which plants are configured to respond to oxidative stress at the cellular level may be quite different.

A more central role for glutathione might also be indicated from our recent isolation of two *Arabidopsis* mutants that express *APX2* in the absence of EL stress (figure 4*a*). These are mutations in a locus encoding a regulator of APX expression (*RAP*). These independently isolated mutants are allelic and have therefore been designated as *rap1-1* and *rap1-2*. Both *rap1* mutants have levels of glutathione at about 40% of wild-type (figure 4*b*). However, the *RAP1* locus is not allelic to *CAD2*, a locus encoding chloroplastic γ-glutamylcysteine synthetase (γ-ECS), the enzyme that catalyses the first step of glutathione biosynthesis (Howden *et al*. 1995; Cobbett *et al*. 1998). *Cad2* mutants, like *rap1* mutants, have reduced GSH levels (figure 4*b*) (Howden *et al*. 1995) but, unlike the *rap1* mutants, do not have any detectable *APX2* expression under non-stress conditions (L. Ball, S. Karpinski and P. Mullineaux, unpublished data). Equally, the *vtc1* mutation, which causes a depletion in foliar ascorbate content (Conklin *et al*. 1997), has no regulatory impact on *APX2* expression (L. Ball, S. Karpinski and P. Mullineaux, unpublished data). These data suggest that foliar redox status, determined by antioxidant content, is not the sole determinant of *APX2* expression but works in conjunction with some other factor(s) not yet identified, but perhaps encoded by, or more indirectly associated with *RAP1*.

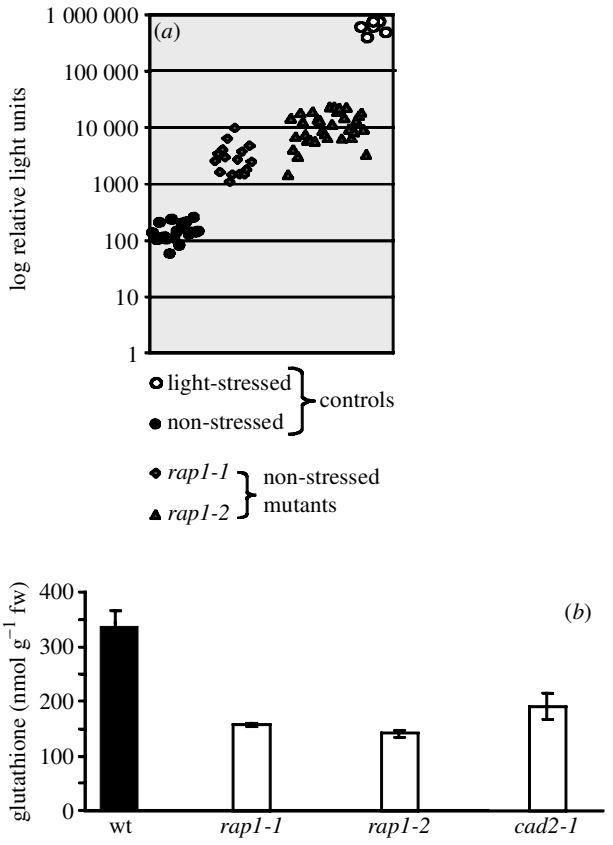

Figure 4. (*a*) Expression of the *APX2–LUC* transgene in *rap1* mutants of *Arabidopsis*. The mutants were selected by screening for luciferase expression of an *APX2*-promoter–*LUC*-gene fusion in a transgenic *Arabidopsis* line (Karpinski *et al*. 1999) in the absence of EL stress. The mutants were then shown to be expressing their native *APX2* gene in an identical manner and thus were designated as lesions in a locus that is a regulator of APX expression (*RAP1*). The luciferase activity of the mutants is compared with the non-induced and EL-induced levels of *APX2–LUC* in the parental wild-type transgenic line. Note the logarithmic scale on the vertical axis. (*b*) Foliar glutathione levels in leaves ($n = 3$) of the two *rap1* mutants compared with the *cad2-1* mutant, which is defective in the first step of glutathione biosynthesis in the chloroplast (Howden *et al*. 1995; Cobbett *et al*. 1998).

8. SYSTEMIC INDUCTION OF ANTIOXIDANT DEFENCES AND ACCLIMATION TO EXCESS EXCITATION ENERGY

Exposure of part of the rosette of an LL-grown *Arabidopsis* plant to EL results in enhanced tolerance to EEE in its unexposed leaves, and has been called systemic acquired acclimation (SAA) (Karpinski *et al*. 1999). SAA is associated with redox changes in PS II in the chloroplasts of unexposed leaves, which renders them more resistant to subsequent episodes of exposure to EL. Thus, chloroplasts suffering EEE emit a systemic signal that promotes changes in PS II electron transport in remote chloroplasts that have never been exposed to the stress, initiating an acclimation response at the level of the chloroplast (Karpinski *et al*. 1999; Walters *et al*. 1999). These changes are coincident with an increase in foliar H_2O_2 and a partial induction of *APX2* expression, being most pronounced in the youngest developing leaves. Furthermore, the systemic induction of *APX2* can be abolished in detached leaves infiltrated with catalase, but not superoxide dismutase, implying that H_2O_2, but not superoxide, is one component of SAA (Karpinski *et al*. 1999). The systemic induction also occurs of *APX1* in a manner similar to *APX2*, although the effect is not so marked in leaves but is most noticeable in the stems of inflorescence (Karpinski *et al*. 2000). This is not too surprising considering there is induction of the expression of this gene in EL-exposed leaves as well (Karpinski *et al*. 1997). However, of more significance is the recent observation that the levels of transcripts encoded by several antioxidant defence-associated genes are increased only in systemic leaves and not in EL-exposed leaves (figure 5). These include genes that encode peroxisomal catalase (*CAT2*), chloroplast phospholipid hydroperoxide-dependent glutathione peroxidase (*GPX2*) and glutathione-S-transferase (*GST*). Thus, the antioxidant defences of systemic leaves in which an acclimation to higher light conditions has been initiated, are constituted differently to those suffering an initial bout of EEE.

9. INTEGRATION OF SYSTEMIC SIGNALLING FOR RESPONSES TO EXCESS EXCITATION ENERGY, PATHOGENS AND WOUNDING

The possibility of diverse stresses operating by a common signalling network is raised by the observed

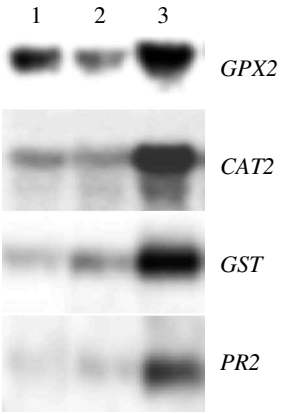

Figure 5. RNA gel blot of total RNA (10 μg) prepared from pooled leaves ($n = 6$) of LL-grown (200 $\mu mol^{-1} m^{-2} s^{-1}$) *Arabidopsis* plants either not exposed to EL (2500 $\mu mol^{-1} m^{-2} s^{-1}$; track 1) or partially exposed to EL for 80 min. EL-exposed leaves from the rosettes (track 2), or the systemic leaves, which did not experience direct EL exposure (track 3), were harvested as described by Karpinski *et al.* (1999). The probes are all derived from expressed sequence tag cDNAs, identified on the basis of published sequences and detect transcripts encoding plastidial phospholipid hydroperoxide glutathione peroxidase (*GPX2*), peroxisomal catalase (*CAT2*), glutathione-S-transferase (*GST*) and pathogenesis-related protein, type 2 (*PR2*).

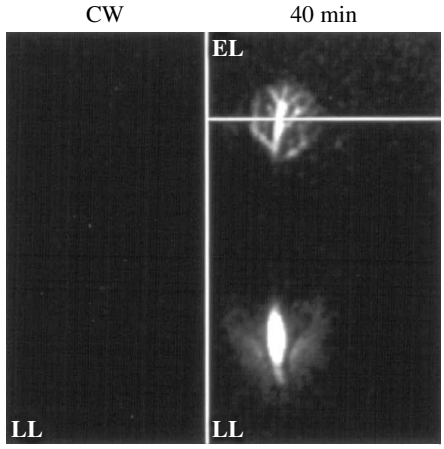

Figure 6. Luciferase expression in a detached wounded leaf from a five-week-old short-day-grown *Arabidopsis* rosette transgenic for an *APX2*-promoter–*LUC* fusion (Karpinski *et al.* 1999). Leaves were detached from the plant at the petiole and were subjected to a series of parallel slashes in the lowest quarter of the leaf. EL was applied for 40 min to the top one-quarter of the leaf, the remainder being shaded with aluminium foil. At the end of the light stress period, the leaves were sprayed with 1 mM D(−)luciferin and 30 min later an image was recorded using a charged couple device camera (Karpinski *et al.* 1999). CW, control wounded leaf.

systemic induction of *GPX2* and *GST* expression (figure 5). The levels of these transcripts are also induced strongly in the HR and associated systemic acquired resistance (SAR) in incompatible (resistant) plant–pathogen interactions (Levine *et al.* 1994; Alvarez *et al.* 1998). Furthermore the SAR-inducible *PR2* gene (Maleck & Dietrich 1999) is also inducible in systemic leaves of EL-exposed plants (figure 5; Karpinski *et al.* 2000). *PR-2* encodes a pathogenesis-related protein, β-1,3-glucanase, and is inducible by salicylic acid (SA) treatment of plants (Van Loon & Van Strien 1999). These data suggest that SAR, induced in incompatible plant–pathogen interactions, and SAA, induced in response to EEE, share common signalling pathways. However, PR-2-like proteins are induced during acclimation to chilling in winter rye (Van Loon & Van Strien 1999), a process that also requires an adjustment to increased EEE (Huner *et al.* 1998) and might indicate that *PR2* genes are subject to more than SA-mediated regulation.

Wounding of *Arabidopsis* leaves potentiates the tissue in the vicinity of the wound sites to respond more strongly to a systemic signal induced by EEE (figure 6), suggesting that signalling for SAA and wound responses might also share common components. It is tempting to speculate that H_2O_2 released by wounded tissue (Orozco-Cardenas & Ryan 1999) is responsible for the enhanced systemic induction of *APX2* by EL, but we have no proof of this to date. Furthermore, the existence of two distinct pathways for wound responses, one local and one systemic involving ethylene and jasmonic acid (JA), respectively (Rojo *et al.* 1999), complicate interpretation of these results.

This potentially complex overlap of signals for SAA, SAR and wounding become clearer with the observation that pre-treatment of *Arabidopsis* leaves with ⩾1 mM SA, prior to exposure to EL, abolishes *APX2* induction (S. Karpinski, unpublished data). SA suppresses the JA and ethylene signalling pathways (at least in tomato; Maleck & Dietrich 1999; Van Loon & Van Strien 1999), perhaps by causing a switch in oxylipin metabolism away from the synthesis of JA into the reductase part of the pathway leading to hydroxy-polyunsaturated fatty acids (Weichert *et al.* 1999). This observation suggests systemic signalling for responses to both wounding and EEE do indeed have considerable overlap and that the *PR-2* induction during SAA might be a reflection of this gene's regulation by non-SA-mediated pathways.

Recent work by Rao & Davis (1999) on ozone-fumigated *Arabidopsis* leaves suggests that SA is linked to the maintenance of the redox state of the glutathione pool (i.e. the ratio of GSH to total glutathione) under oxidative stress conditions. In EL-exposed leaves, the redox state of the glutathione pool declines during exposure and takes several hours to be restored during post-stress recovery (Karpinski *et al.* 1997). It is possible that SA undergoes a similar decline in EL-treated leaves and this could have the effect of activating wound response pathway(s) as part of the onset of SAA. Given that the glutathione pool has an influence on the expression of *APX1* and *APX2* and the degree of tolerance to EEE (Karpinski *et al.* 1997), these preliminary data suggest that glutathione could provide a connection between SA-, wound- and EEE-mediated signalling.

The possible overlap in signalling pathways is supported by the observation that *APX1* and *APX2* can also be induced in *Arabidopsis* leaves challenged with both compatible and incompatible pathovars of *Pseudomonas syringae*, which promote disease and induce SAR, respectively (Escobar 1998). A similar SAR response of tobacco to tobacco mosaic virus is associated with marked changes in antioxidant defences (Fodor *et al.* 1997), and

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suppression of catalase or APX levels in transgenic tobacco renders such plants hyperresponsive to pathogen attack (Mittler *et al*. 1999). Furthermore, SAA is associated with a dramatic increase in the appearance of microscopic zones of cell death, reminiscent of the so-called 'micro-HRs', required for the establishment of SAR (Alvarez *et al*. 1998).

There is also integration of the phosphorylation cascades mediated by the SA and JA pathways for the R–Avr interaction, non-race-specific elicitors, wounding and damage by UVB irradiation (Romeis *et al*. 1999; Mackerness *et al*. 1999).

10. COMMONALITY AND DISCRIMINATION

From the above considerations, we propose that there is a wide network of defence-related genes, whose induction directly or indirectly involves ROS, but nevertheless require additional factors that permit the perception of distinct types of environmental stress. Increases in ROS can be caused either via redox changes in PS II (Karpinski *et al*. 1997, 1999), or via the R–Avr interaction in the HR response (Bi *et al*. 1995; Neuenschwander *et al*. 1995; Pennel & Lamb 1997), or via elicitors in wound responses (Watanabe & Sakai 1998; Orozco-Cardenas & Ryan 1999). In none of these cases are ROS alone sufficient to elicit a response and this provides the discrimination that permits recognition of the duration and intensity of specific environmental challenges. For example, nitric oxide has been suggested to act with ROS to orchestrate the HR and SAR (Delledonne *et al*. 1998). H_2O_2 production in wound responses requires a functional JA-mediated signalling pathway (Watanabe & Sakai 1998; Orozco-Cardenas & Ryan 1999). Photosynthetic electron transport is required to promote a response to EEE, over and above its role in the generation of ROS (see § 6; Karpinski *et al*. 1997, 1999).

11. PHOTORECEPTOR-MEDIATED RESPONSES TO HL AND EL

Plants' responses to light conditions produce many changes in leaf and plant morphology at every stage of their life cycle (Walters *et al*. 1999; Nagy & Schäffer 2000). In the context of this paper, HL is that photosynthetically active photon flux density (PPFD) to which the plant is acclimated. The same PPFD applied to non-acclimated (e.g. LL-grown plants) would elicit a stress response and in this respect would be EL. In the context of the chloroplast and photosynthesis, HL-grown plants display increased leaf thickness and changes in chloroplast number per cell and/or per unit area of leaf (reviewed in Walters *et al*. 1999). These processes are often termed 'leaf-level' acclimation to the light environment as distinct from 'chloroplast-level' acclimation described previously (see § 8). These two distinct pathways of acclimation to light are proposed to have a functional link (Walters *et al*. 1999).

These developmental responses to HL are regulated by at least two distinct sets of photoreceptors: the phytochromes that detect spectral quality of light in the red–far red range and the cryptochromes (blue/ultraviolet A (UVA) receptors) that detect the quantity of light (Nagy & Schäffer 2000). The blue/UVA receptors may be carotenoids such as the xanthophylls (reviewed in Tlałka *et al*. 1999) and therefore there is a potential link to the dissipation of EEE.

The phytochromes are proteins that undergo reversible conformational changes in response to red and far red light and exert their effects on gene expression via the DET–COP–FUS regulatory cluster that regulates the activation state of several transcription factors (e.g. Hy5). These, in turn, bind to the promoters of light-regulated genes, thus increasing their expression (Walters *et al*. 1999; Nagy & Schäffer 2000). Other, as yet only partly characterized, pathways for light-receptor-mediated control of gene expression may involve Ca^{2+} and cyclic GMP as secondary messengers (Nagy & Schäffer 2000).

A model has been proposed recently which links leaf-level and chloroplast-level acclimation to light via either direct or indirect interactions between the DET–COP–FUS regulatory cluster and redox sensing of photosynthetic electron transport (Walters *et al*. 1999). This model is based on experiments on *Arabidopsis* mutants defective in various steps of the light-receptor-mediated response grown under LL and HL conditions.

Photoreceptors are also implicated in more immediate responses of LL-grown plants to EL. When leaves undergo a transition from LL to EL conditions, chloroplast movement within cells is a rapid response. In leaves of duckweed (*Lemna trisulca* L.), chloroplasts move to anticlinal walls within 20–40 min of the onset of HL to escape the effects of this exposure (Tlałka *et al*. 1999). This response is mediated by blue/UVA receptors and is associated with an accumulation of the xanthophyll carotenoid, zeaxanthin. Calcium may act as a secondary messenger in the control of chloroplast movement (Tlałka & Fricker 1999). There is also the known involvement of a protein kinase (NPH1) with a putative redox-sensing domain in blue-light-induced responses (Huala *et al*. 1997). Therefore, there is the potential involvement of a redox-sensitive phosphorylation cascade and at least one mechanism of dissipating EEE. Consequently, it is not unreasonable to suggest that even in immediate responses to EL, the different pathways controlling leaf-level and chloroplast-level acclimation are as tightly coordinated as the processes that bring about longer-term developmental changes associated with light and cellular metabolism (Walters *et al*. 1999).

12. PHYTOCHROME-MEDIATED CONTROL OF *APX* GENE EXPRESSION

The expression of *APX1* and *APX3*, encoding non-plastidial isoforms of APX, is highest in green tissue and their transcript levels increase strongly during the greening of etiolated seedlings. *APX1* and *APX3* expression is induced by a 5 min pulse of red light but is not reversible by subsequent illumination with far-red light. This indicates that the regulation of *APX* gene expression may correspond to a very low fluence response (Escobar 1998; Nagy & Schäffer 2000). Furthermore, in a phytochrome A (PhyA) mutant background, *APX1* and *APX3* expression shows far-red reversibility following their induction by red light, indicating the involvement, at least in part, of PhyA in *APX1* and *APX3* light-mediated

responses. The timing of maximal transcript levels for *APX3* induced by a 15 min red light treatment of etiolated seedlings was up to 16 h ahead of the maximal appearance of the *CAB* transcript that encodes chlorophyll *a*/*b* binding protein. This suggests that the configuration of extra-plastidial antioxidant defences is established ahead of the development of photosynthetically active chloroplasts. These data provide evidence that antioxidant defences are under the same developmental control as other aspects of chloroplast and leaf development. The integration of developmental and stress responses is also underlined in the inappropriate expression of stress-related genes (associated with hypoxia and response to pathogens) in mutants defective in DET–COP–FUS function (Mayer *et al*. 1996). This implies that the configuration of antioxidant defences by developmental processes will have an impact on the turnover of ROS in a plant's immediate response to environmental factors, thus establishing a threshold above which additional defence systems are induced. Finally, the recent report that the *ROOT MERISTEMLESS 1* and *CAD2* are the same gene, encoding γ-ECS, and that glutathione depletion causes a block in cell division in the G_1–S transition in tobacco suspension culture cells (Vernoux *et al*. 2000) brings the considerations in this paper full circle. There is now the exciting prospect of linking changes in the glutathione pool, the way antioxidant systems respond to stress and how acclimatory processes at the cellular level influence the pattern of plant development.

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