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Energy dissipation and radical scavenging by the plant phenylpropanoid pathway

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Environmental stresses such as high light, low temperatures, pathogen infection and nutrient deficiency can lead to increased production of free radicals and other oxidative species in plants. A growing body of evidence suggests that plants respond to these biotic and abiotic stress factors by increasing their capacity to scavenge reactive oxygen species. Efforts to understand this acclimatory process have focused on the components of the 'classical' antioxidant system, i.e. superoxide dismutase, ascorbate peroxidase, catalase, monodehydroascorbate reductase, glutathione reductase and the low molecular weight antioxidants ascorbate and glutathione. However, relatively few studies have explored the role of secondary metabolic pathways in plant response to oxidative stress. A case in point is the phenylpropanoid pathway, which is responsible for the synthesis of a diverse array of phenolic metabolites such as flavonoids, tannins, hydroxycinnamate esters and the structural polymer lignin. These compounds are often induced by stress and serve specific roles in plant protection, i.e. pathogen defence, ultraviolet screening, antiherbivory, or structural components of the cell wall. This review will highlight a novel antioxidant function for the taxonomically widespread phenylpropanoid metabolite chlorogenic acid (CGA; 5-*O*-caffeoylquinic acid) and assess its possible role in abiotic stress tolerance. The relationship between CGA biosynthesis and photosynthetic carbon metabolism will also be discussed. Based on the properties of this model phenolic metabolite, we propose that under stress conditions phenylpropanoid biosynthesis may represent an alternative pathway for photochemical energy dissipation that has the added benefit of enhancing the antioxidant capacity of the cell.

Keywords: antioxidant; chlorogenic acid; phenylpropanoid pathway; high-light acclimation; low temperature acclimation; reactive oxygen species

1. INTRODUCTION

Exposure to environmental stress often results in increased production of oxidative species such as superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) in plants (Alscher *et al.* 1997; Delledonne *et al.* 1998). The ability to survive these cellular toxins depends on the metabolic responsiveness of detoxification mechanisms. Reactive oxygen species (ROS) and reactive nitrogen species have both direct and indirect effects on the cellular redox state and the expression of various stress-related genes, including those involved in antioxidant defence and phenolic secondary metabolism (Levine *et al.* 1994; Karpinski *et al.* 1997; Foyer *et al.* 1997; Durner *et al.* 1998).

Plants possess an array of antioxidants that act in a coordinated fashion to mitigate cellular damage under oxidative conditions. Previous studies of the role of ROS scavenging systems in plant stress responses have focused primarily on the expression of the 'classical' antioxidant enzymes: superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), and glutathione reductase (GR), and the antioxidant metabolites ascorbate and glutathione. Catalase, an enzyme found mainly in peroxisomes, also plays an important role in defence against oxidative injury (Willekens *et al.* 1997). Ascorbate is the primary antioxidant in plant cells, whereas glutathione is both an antioxidant and a nucleophile involved in intracellular transport processes (Noctor & Foyer 1998).

An increase in the synthesis of phenolic compounds is another common response to environmental stress in plants (Dixon & Paiva 1995). Phenolics are a diverse group of secondary metabolites that includes flavonoids, tannins, hydroxycinnamate esters and the structural polymer lignin. These compounds constitute the most abundant class of plant secondary metabolites and share a common origin in the phenylpropanoid biosynthetic pathway. Among their biological activities, phenolics are involved in stress responses by acting as chemical deterrents to herbivores and pathogens, screening agents against harmful ultraviolet (UV) radiation, and potential scavengers of free radicals and other oxidative species.

This review will highlight several themes related to phenolic function in plant tolerance to light stress, with particular emphasis on the properties of chlorogenic acid (CGA; 5-*O*-caffeoylquinic acid), a taxonomically widespread phenylpropanoid metabolite. Despite over four

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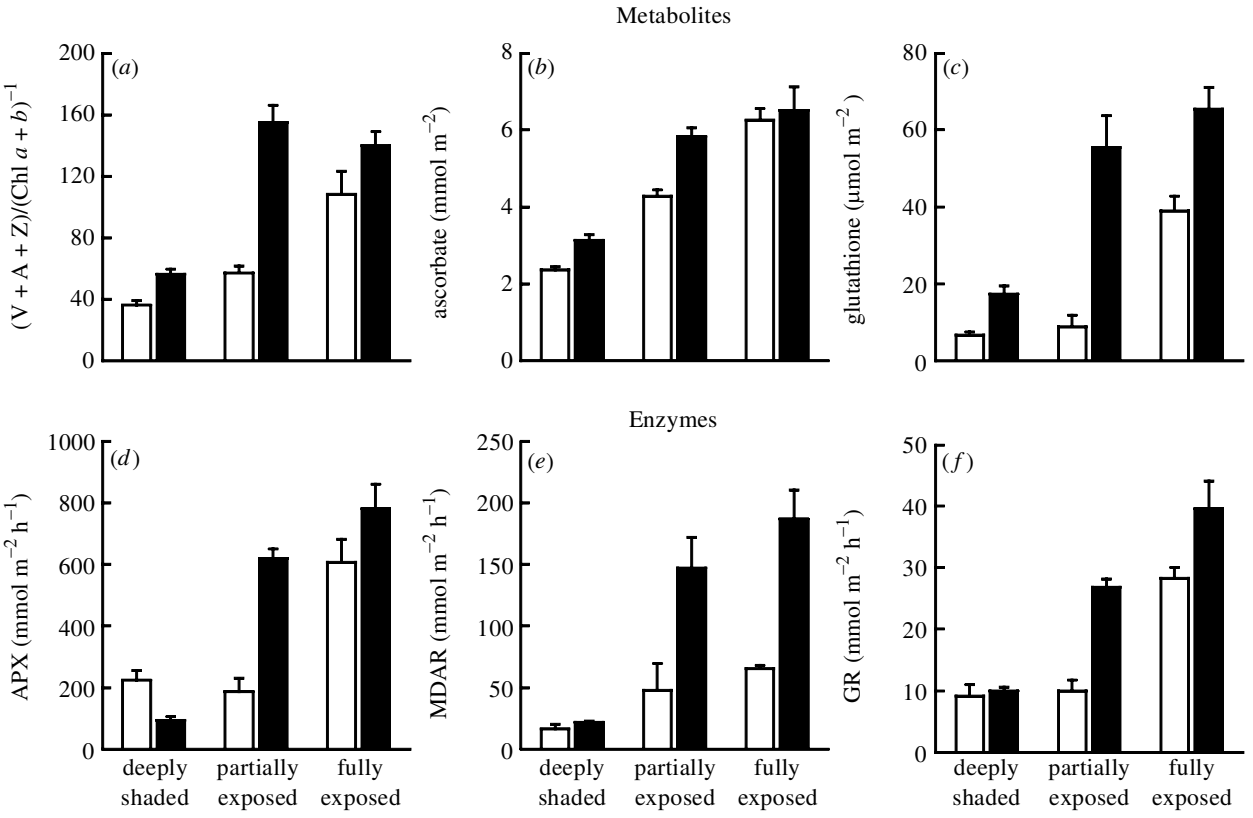

Figure 1. Seasonal changes in photoprotective systems and antioxidants in leaves of *Mahonia repens* in relation to light environment. Leaves were collected from three populations of *M. repens* in summer (open bars) and winter (closed bars). (*a*) Pool sizes of xanthophyll cycle pigments (expressed per total chlorophyll), (*b*) total ascorbate and (*c*) total glutathione. Activities for the antioxidant enzymes (*d*) APX, (*e*) MDAR and (*f*) GR. Data redrawn from Logan *et al.* (1998*a*).

decades of research, no consensus on the function of this compound has emerged. Based on recent studies, we will present evidence that CGA is a novel hydrogen-donating antioxidant that may serve to mitigate the effects of oxidative stress by acting as a direct radical scavenger and, perhaps more importantly, as a reducing substrate for guaiacol peroxidase. We will also present a heuristic case that phenylpropanoid metabolism provides an additional level of photoprotection due its ability to consume photochemical reducing power and to act as an alternative carbon sink under excess light conditions.

2. ECOPHYSIOLOGY OF ANTIOXIDANT RESPONSE

Components of the classical foliar antioxidant system acclimate strongly to levels of excess light absorption in a range of species with widely varying growth habits (Gillham & Dodge 1987; Mishra *et al.* 1995; Grace & Logan 1996; Logan *et al.* 1998*a*,*b*). This suggests that O_2 photoreduction in the chloroplast increases with increasing levels of excess absorbed light, and that plants acclimate to this potential stress by increasing the content and activity of oxidant scavenging systems.

Recently, we focused our attention on acclimation of antioxidants to seasonal temperature changes in relation to ambient light levels in *Mahonia repens*, a broad-leaf evergreen perennial native to the Rocky Mountains. To determine the effect of seasonally colder temperatures, *M. repens* leaf samples were collected in summer (average temperatures 25 °C) and winter (average temperatures 4 °C) from deeply shaded, partially exposed and fully exposed populations. A full description of the environmental conditions can be found in Logan *et al.* (1998*a*). In agreement with our previous growth-chamber studies (Grace & Logan 1996), we observed a general trend towards increasing capacity of photoprotective and antioxidant systems with increasing intensity of the light environment. This was reflected in the pool size of xanthophyll cycle pigments (violaxanthin + antheraxanthin + zeaxanthin per unit of chlorophyll) and levels of ascorbate and glutathione, as well as the activities of APX, MDAR and GR (figure 1). Interestingly, the patterns of seasonal change in photoprotective and antioxidant systems differed among populations. In deeply shaded populations there were slightly larger pool sizes of xanthophyll cycle pigments, ascorbate and glutathione in winter versus summer, whereas the activities of antioxidant enzymes did not change, or in the case of APX, actually declined. In fully exposed populations there were higher levels of xanthophyll cycle pigments, glutathione and antioxidant enzymes in winter versus summer populations, with the most dramatic winter increase observed in MDAR activity. The partially exposed population provides a noteworthy example of the interaction of light and low temperature stress. In summer this population experienced low light intensities under a closed canopy, consistent with the observed low levels of xanthophyll cycle

pigments and antioxidants. However, in winter this population was subjected to much higher light intensities due to a more open canopy and lower solar angles (Logan *et al.* 1998*a*). As a result, it experienced the dual stresses of high light in conjunction with low seasonal temperatures. This population exhibited the most dramatic wintertime increase in the pool sizes of xanthophyll cycle pigments and glutathione, as well as in activities of APX, MDAR and GR. Taken together, these results provide clear evidence that seasonal acclimation of antioxidant systems to low temperature stress depends strongly on the nature of the light environment.

3. A NOVEL ANTIOXIDANT FUNCTION FOR PHENOLIC METABOLITES

Leaves of *M. repens* undergo a pronounced reddening under excess light conditions due to the accumulation of anthocyanins in the epidermis and upper palisade. This response can be observed in juvenile leaves grown under high photon flux densities (PFDs) and normal temperatures and in mature leaves exposed to moderate PFDs and low temperatures. In natural populations leaf reddening is usually observed in conjunction with the onset of low temperatures in the autumn in plants growing under moderate to high levels of irradiance but not in shaded populations. Leaves typically re-green in the spring with rising temperatures, indicating that leaf reddening is part of the process of seasonal acclimation rather than a senescence response. A similar 'depurpling' phenomenon has been observed in needles of *Pinus banksiana*, indicating that seasonal changes in phenolic metabolism occur across a range of woody evergreen species (Nozzolillo *et al.* 1989). Our interest in these seasonal changes was sparked by reports in the literature that anthocyanins and other phenolic products have antioxidant properties (Yamasaki *et al.* 1996).

Spectroscopic and HPLC analysis of *M. repens* leaf extracts unexpectedly revealed that CGA was the major phenolic metabolite in this species, representing over 90% of total soluble phenolic pools (Grace *et al.* 1998*a*). Unlike anthocyanins, which were only present in exposed leaves in winter, CGA was detected in shade- as well as sun-acclimated leaves, although in different amounts. Fully and partially exposed leaves had approximately twofold higher levels of CGA than deeply shaded leaves in summer (figure 2*a*). The differences in CGA content were more pronounced in winter, with levels remaining largely constant in shaded populations but increasing approximately twofold in exposed populations. Thus, seasonal changes in CGA content paralleled changes in the activity and content of photoprotective pigments and antioxidants.

In many plant species CGA biosynthesis is induced by environmental stresses such as high light (Kühnl *et al.* 1987), chilling (Koeppe *et al.* 1970), UV irradiation (Del Moral 1972), wounding (Rhodes & Wooltorton 1978), fungal elicitors (Yao *et al.* 1995), nitrogen deficiency (Del Moral 1972) and phosphate deficiency (Koeppe *et al.* 1976). CGA is one of the initial products formed during the transcriptional activation of the phenylpropanoid pathway by pathogen infection and abiotic stress events (Hahlbrock & Scheel 1989; Dixon & Paiva 1995). It has been suggested that CGA may act as a carbon reservoir

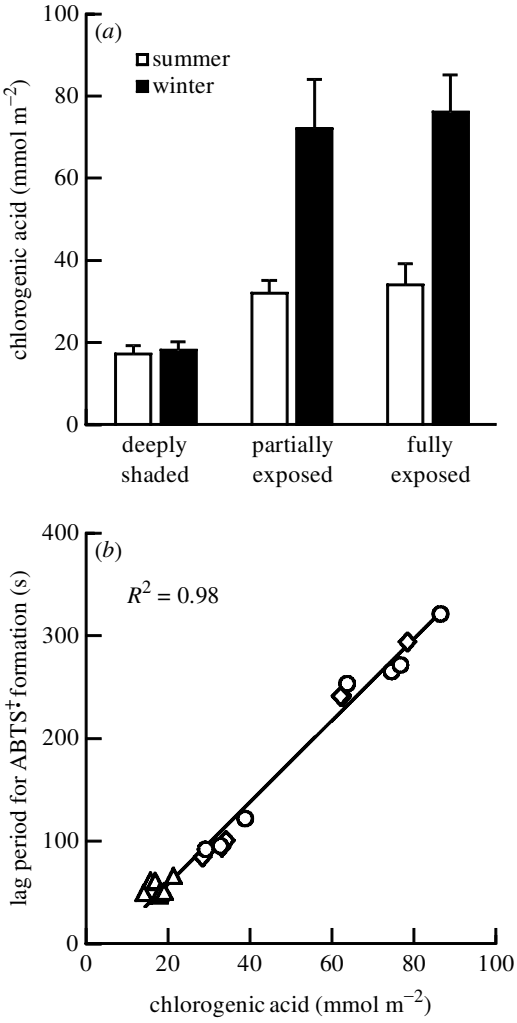

Figure 2. Seasonal changes in (*a*) foliar CGA content and (*b*) free radical scavenging activity in populations of *M. repens*. Symbols refer to deeply shaded populations (triangles), partially exposed populations (diamonds) and fully exposed populations (circles). Data redrawn from Grace *et al.* (1998*a*).

that can be rapidly mobilized to form downstream phenylpropanoid products such as lignin, antimicrobial phytoalexins, and cell wall cross-linking agents (Yao *et al.* 1995; Díaz *et al.* 1997). Mutants defective in phenylpropanoid metabolism provide further evidence that CGA plays an important role in disease resistance in plants. For instance, transgenic tobacco with reduced levels of phenylalanine–ammonia lyase and diminished pools of preformed CGA shows greater leaf damage after infection with the pathogen *Cercospora nicotianae* than wild-type plants (Maher *et al.* 1994). The suppression of CGA synthesis by genetic manipulation has also been shown to cause abnormal palisade development and premature senescence in tobacco leaves (Tamagnone *et al.* 1998). Although these studies suggest that CGA is involved in a wide variety of stress responses, the basis of its protective action *in vivo* remains unclear.

Previous studies of the physiological function of phenolics have emphasized their role as herbivory deterrents, antifungal compounds, and UV screening agents (Hahlbrock & Scheel 1989; Dixon & Paiva 1995; Landry *et al.* 1995). The antioxidant properties of phenolics in relation to plant stress responses have remained largely

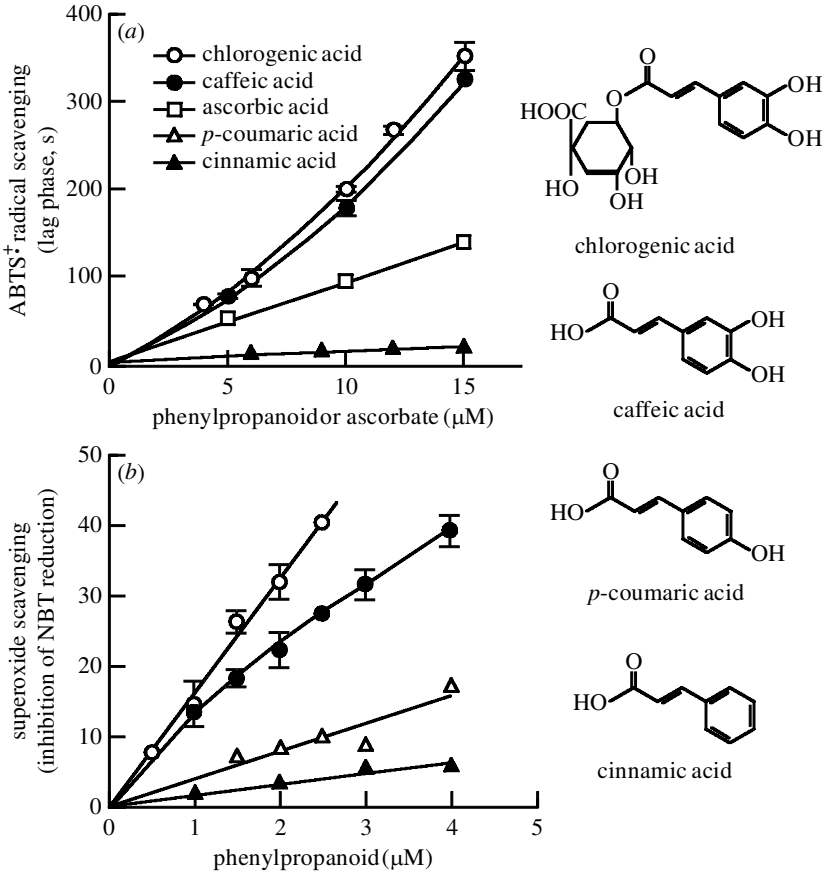

Figure 3. Scavenging activity of phenyl-propanoids against (*a*) the $ABTS^{+\cdot}$ radical cation and (*b*) the superoxide anion ($O_2^{-\cdot}$). The $ABTS^{+\cdot}$ radical cation was generated in the myoglobin–H_2O_2 system, and the lag period for $ABTS^{+\cdot}$ radical formation was measured at 734 nm. Superoxide scavenging was measured as the inhibition of nitroblue tetrazolium reduction in the presence of NADH and phenazine methosulphate as a $O_2^{-\cdot}$ generating system. Data redrawn from Grace *et al.* (1998*a*).

unexplored. Based on reports of the free-radical scavenging properties of CGA (Ohnishi *et al.* 1994; Castelluccio *et al.* 1995; Kono *et al.* 1997) and similar trends in the seasonal response of CGA to other leaf photoprotective systems, we postulated that CGA may play a role in antioxidant defence. To assess the hydrogen-donating (antioxidant) potential of the phenolic constituents in leaves of *M. repens*, we measured the radical scavenging properties of leaf extracts in two separate assays. The substrates used in these assays were the stable green radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and the superoxide anion. We observed a strong correlation between the $ABTS^{+\cdot}$ radical scavenging activity of leaf extracts and foliar CGA content (figure 2*b*). Similar results were obtained in the $O_2^{-\cdot}$ scavenging assay (Grace *et al.* 1998*a*). These data suggest that CGA may play an important role in mitigating the effects of oxidative stress under adverse environmental conditions.

Structure–activity relationships have shown that the catechol group is the principal determinant of the hydrogen-donating (antioxidant) activity of phenolics (Rice-Evans *et al.* 1996). A comparison of the free radical scavenging activity of several phenylpropanoids against the $ABTS^{+\cdot}$ radical cation and $O_2^{-\cdot}$ illustrates the importance of the catechol group for antioxidant activity (figure 3). CGA and caffeic acid are more effective antioxidants in the $ABTS^{+\cdot}$ radical scavenging test than the 'classical' antioxidant ascorbate due to the fact that these catechols and their respective semiquinones both have reducing properties and therefore react stoichiometrically with two radicals rather than one as in the case of ascorbate. CGA and caffeic acid are also effective scavengers of $O_2^{-\cdot}$, exceeding the activity of *p*-coumaric acid, which contains only a single phenolic hydroxyl group. Cinnamic acid, which lacks a phenolic hydroxyl group, does not show appreciable antioxidant properties in either test.

It is noteworthy that, unlike CGA, the $O_2^{-\cdot}$ scavenging activity of caffeic acid shows a nonlinear concentration dependence (figure 3*b*). One possible explanation for this is that caffeic acid can act as both a *scavenger* and a *generator* of $O_2^{-\cdot}$. This latter property is due to the ability of semiquinones to reduce oxygen. To assess this possibility further, we compared the ability of CGA, caffeic acid and dihydrocaffeic acid to reduce oxygen and generate hydroxyl radicals ($^{\cdot}OH$) in the presence of copper ions. Copper(II) catalyses the oxidation of phenolic compounds in a redox cycling process that can cause oxidative damage to DNA and other biological macromolecules (Rahman *et al.* 1989; Li & Trush 1994; Yamanaka *et al.* 1997). Addition of Cu(II) to a solution containing caffeic acid stimulates oxygen uptake (figure 4*a*). In contrast, Cu(II) fails to stimulate O_2 uptake by CGA. Dihydrocaffeic acid, which lacks a side-chain double bond, shows even higher rates of O_2 uptake in the presence of Cu(II) than caffeic acid.

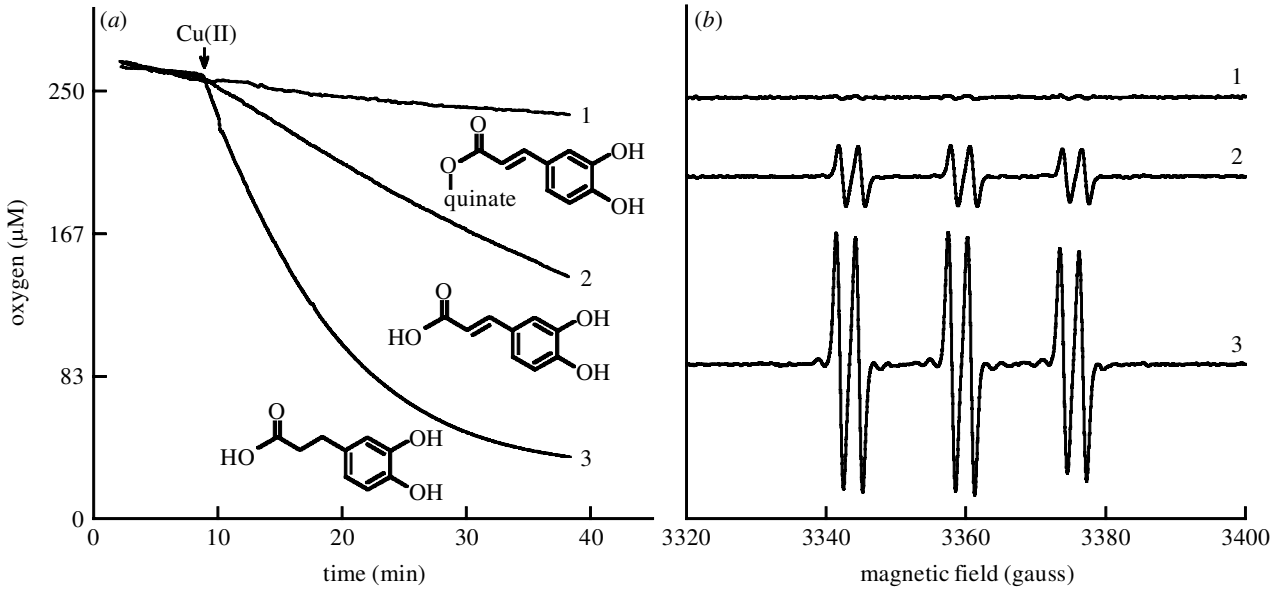

Figure 4. Pro-oxidant activity of phenolics in the presence of Cu(II). (*a*) Oxygen uptake was measured with a Clark-type oxygen electrode in solutions containing chelexed 0.1 M sodium phosphate, pH 7.4, and 1 mM CGA (curve 1), caffeic acid (curve 2) or dihydrocaffeic acid (curve 3). The reaction was started by the addition of 0.33 mM $CuCl_2$. Temperatures were maintained at 37 °C. (*b*) Hydroxyl radical formation was measured by a spin trapping method using DMSO as the primary target compound and POBN as the spin trap. Reaction mixtures contained 25 mM POBN, 2% DMSO (v/v), 0.4 mM $CuSO_4$ and 0.8 mM CGA (curve 1), caffeic acid (curve 2) or dihydrocaffeic acid (curve 3) in chelexed 0.1 M sodium phosphate, pH 7.4. After 1 h solutions were transferred to an aqueous quartz flat cell and ESR spectra were recorded at room temperature using a Varian 109 ESR spectrometer (Varian, Palo Alto, CA, USA) operating at X band (9.5 GHz) and employing 100 kHz field modulation. The methyl radical adduct of POBN generates a six-line ESR spectrum with hyperfine splitting constants of $a^N = 15.9$ G, $a^H = 2.8$ G (Gunther *et al*. 1995).

In parallel experiments we measured $^{\cdot}OH$ radical formation using the electron spin resonance (ESR) spin trap α-(pyridyl-4-*N*-oxide)-*N*-*tert*-butylnitrone (POBN). Since the $^{\cdot}OH$ radical adduct is unstable, detection is greatly facilitated by use of dimethyl sulphoxide (DMSO) as a primary target molecule (Gunther *et al.* 1995). Oxidation of DMSO by the $^{\cdot}OH$ radical generates the methyl radical (CH_3), which reacts rapidly with POBN to form a highly stable adduct with a characteristic six-line ESR spectrum (Gunther *et al.* 1995). Consistent with the O_2 uptake measurements, the highest level of $^{\cdot}OH$ radical formation was observed in the presence of dihydrocaffeic acid and Cu(II). Caffeic acid produced approximately fourfold less $^{\cdot}OH$ radical under these conditions and CGA fails to produce detectable levels of the spin adduct (figure 4*b*). Both O_2 uptake and $^{\cdot}OH$ radical formation were suppressed by the Cu(I) chelator bathocuproine and catalase (data not shown), confirming the involvement of Cu(I) and H_2O_2 in a metal-catalysed Fenton reaction.

These results can be rationalized by differences in the O_2 reducing activity of oxidized phenolic intermediates. The initial oxidation of catechols by Cu(II) generates a semiquinone that can react with O_2 to form $O_2^{-\cdot}$. This reaction has an autocatalytic character since $O_2^{-\cdot}$ will oxidize the parent compound to regenerate the semiquinone and H_2O_2. In the presence of Cu(I) H_2O_2 is rapidly reduced to the $^{\cdot}OH$ radical in a Fenton-type reaction (Gunther *et al.* 1995). Thus, the reactivity of the semiquinone towards O_2 determines the overall pro-oxidant activity of the parent compound. Two factors that apparently limit the reactivity of the semiquinone are greater resonance stabilization of the phenoxyl radical due to the side-chain double bond (e.g. caffeic acid) and esterification of the terminal carboxyl group (e.g. CGA). Thus, dihydrocaffeic acid exhibits strong pro-oxidant behaviour whereas CGA exhibits strong antioxidant behaviour despite the fact that the reducing properties of both catechols are similar (Boyer *et al.* 1988). These chemical features may explain why phenylpropanoids are rarely found in the free state in plants, but are normally bound as esters of sugars or organic acids. Esterification may thus represent a mechanism to limit the pro-oxidant behaviour of phenylpropanoids, enabling these compounds to serve as effective antioxidants *in vivo*.

4. BIOLOGICAL TARGETS OF PHENYLPROPANOID ANTIOXIDANTS

If CGA serves as a low molecular weight antioxidant in plants, then it is important to assess possible targets of biological relevance. This requires knowledge of both the physiological sources of oxidant formation and the intracellular location of phenolic metabolites. During light stress the chloroplast is the major source of free radicals and other oxidative species in plants (Asada 1999). Under normal conditions these oxidants are scavenged within the chloroplast by the concerted action of SOD, APX, MDAR and GR. However, under conditions of severe stress the scavenging capacity of the chloroplast may be exceeded, particularly if plastid ascorbate pools become oxidized (Yamasaki *et al.* 1995). There is growing evidence

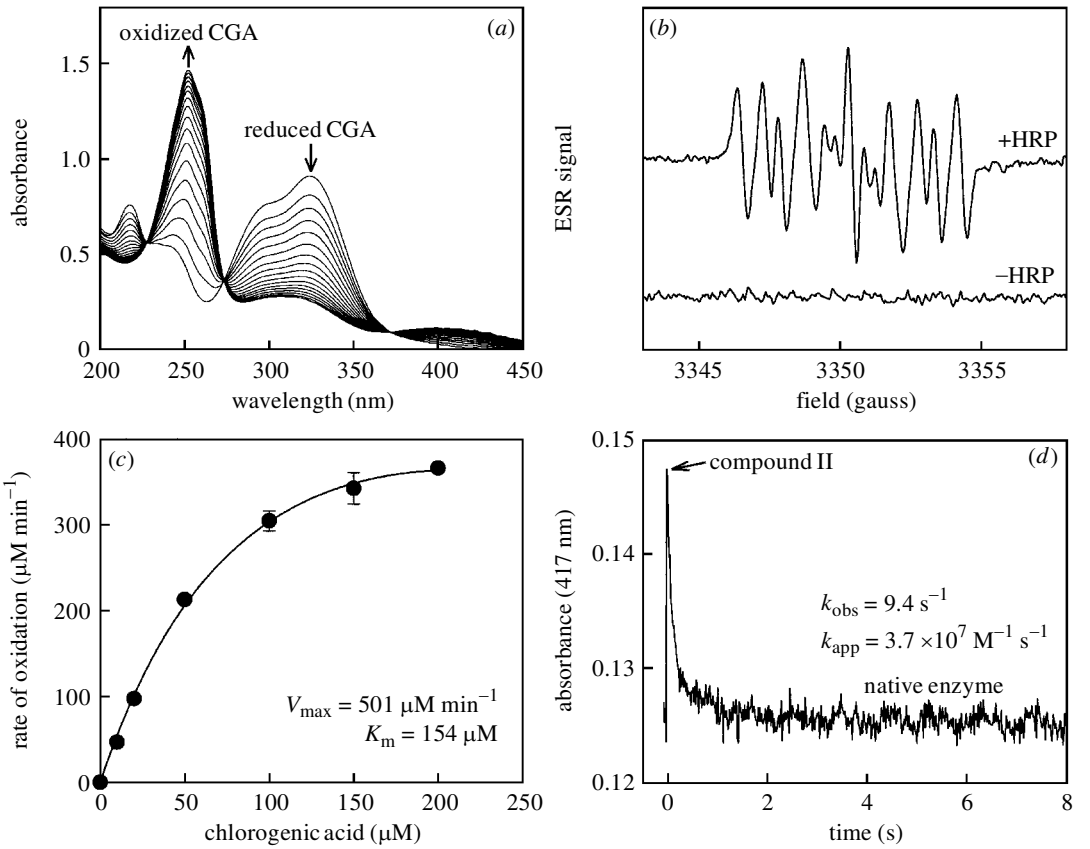

Figure 5. CGA is an electron donor to HRP. (*a*) Spectral changes upon addition of H_2O_2 (0.2 mM) to a solution containing CGA (0.05 mM) and HRP (5 nM) in 50 mM Tris–HCl, pH 7.4. Scans were performed at 1 s intervals. (*b*) ESR spectrum of a semiquinone intermediate formed during the HRP-catalysed oxidation of CGA. The 'spin stabilization' method was used to detect the CGA semiquinone under static ESR conditions (Grace *et al.* 1999). The reaction contained 10 mM CGA, 14 mM H_2O_2 and 0.1 nM HRP in 50 mM 2-(*N*-morpholino)-ethanesulphonic acid–NaOH buffer, pH 5.6 in the presence of 200 mM $ZnSO_4$. Zinc ions stabilize the semiquinone anion by forming a complex. (*c*) Substrate dependence of CGA oxidation in the HRP reaction. Initial rates of oxidation were determined from the absorbance loss at 325 nm, and kinetic parameters were determined from Lineweaver–Burke plots. (*d*) Stopped-flow kinetic analysis of conversion of HRP compound II to the native enzyme by CGA. Compound II was generated by premixing 2 μM HRP with 200 μM peroxynitrite in 100 mM phosphate buffer, pH 7.2 (for details of this reaction, see Grace *et al.* (1998*b*)). The pseudo-first-order rate constant was obtained by an exponential fit of the absorbance loss at 417 nm.

that H_2O_2 can diffuse out of the chloroplast during light stress. For example, sudden exposure of low light-grown *Arabidopsis* or spinach plants to high light or paraquat treatment causes the rapid expression of the cytosolic isoform of APX (Karpinski *et al.* 1997; Yoshimura *et al.* 2000). Recent studies suggest that steady-state H_2O_2 levels can serve as a metabolic indicator of the cellular redox state and act as a signalling agent in the transcriptional activation of a number of stress-related genes (Levine *et al.* 1994; Foyer *et al.* 1997; Wu *et al.* 1997; Hirt *et al.* 2000).

In broad-leaf species the central vacuole is thought to be the major intracellular storage site for phenolic compounds such as anthocyanins, flavonoids and hydroxycinnamate esters (Hutzler *et al.* 1998). Since oxygen radicals cannot readily diffuse into vacuoles from chloroplasts (Takahashi & Asada 1983), it is unlikely that phenolics act as the primary scavengers of photosynthetically generated $O_2^{\cdot-}$. However, $O_2^{\cdot-}$ is rapidly converted to H_2O_2 inside the chloroplast by both enzymatic and non-enzymatic mechanisms. Since H_2O_2 is a diffusable oxidant, it can move freely into the vacuole, which occupies most of the volume of mesophyll cells. Although phenolics do not react directly with H_2O_2, it has been proposed that they can act as secondary scavengers of H_2O_2 in conjunction with guaiacol peroxidase (Takahama 1989; Yamasaki *et al.* 1997).

Plants contain two major types of peroxidase that are distinguished by their substrate specificity and intracellular localization. In leaves APX activity is localized mainly in chloroplasts and the cytosol where the enzyme plays an important role in removing photosynthetically generated H_2O_2 (Asada 1999). In contrast, guaiacol peroxidases are found mainly in the apoplast and cell wall where they are thought to be involved in cell wall deposition and lignification (Nose *et al.* 1995). Oxidation of phenolics by extracellular peroxidases may also be critical in forming a physical barrier in wounding and pathogen responses (Wu *et al.* 1997). For these reasons guaiacol peroxidases have traditionally been considered to serve a 'metabolic' role in providing oxidized substrates for lignin formation and other physiological processes. However, plants also possess an intracellular form of guaiacol peroxidase that is localized exclusively in the vacuole (Bernal *et al.* 1993).

The function of this peroxidase has not been investigated, although it is unlikely to play a role in cell wall deposition since phenoxyl radicals must be generated *in situ* for the oxidative polymerization of lignin (Lewis & Yamamoto 1990). It has recently been proposed that the vacuolar guaiacol peroxidase may function as a scavenger of H_2O_2 under extreme conditions to complement the ascorbate–APX system (Yamasaki *et al.* 1997; Yamasaki & Grace 1998).

Figure 5 summarizes various aspects of the oxidation of CGA by H_2O_2 in the presence of horseradish peroxidase (HRP), the prototype guaiacol peroxidase. CGA is rapidly oxidized in this system to form a product with absorbance maxima at 251 nm and 410 nm, suggesting the formation of the CGA *o*-quinone (Takahama & Oniki 1997) (figure 5*a*). In the presence of 'spin-stabilizing' metal ions such as Zn^{2+}, a transient free-radical intermediate can be detected by ESR (figure 5*b*). This signal arises from the CGA *o*-semiquinone (Yamasaki & Grace 1998), consistent with the known one-electron oxidation mechanism of HRP (Dunford 1991). Figure 5*c* shows the saturation profile of the CGA–HRP reaction, which obeys normal Michaelis–Menten kinetics. Analysis of these data by double-reciprocal plots gave V_{max}- and K_m-values of 501 μM min^{-1} and 154 μM, respectively.

The initial step in the catalytic cycle of HRP is the oxidation of the ferric haem by H_2O_2 to form Compound I, a two-electron oxidation product:

$$HRP^3 + H_2O_2 \rightarrow \text{compound I} + H_2O. \quad (1)$$

This reaction has a second-order rate constant of $1.6 \times 10^7\ M^{-1}s^{-1}$ (Dunford 1991). In the presence of an oxidizable phenolic substrate, the native enzyme is regenerated in a series of one-electron steps with concomitant production of phenoxyl radicals:

$$\text{compound I} + ArOH \rightarrow \text{compound II} + ArO^{\cdot} + H^+, \quad (2)$$

$$\text{compound II} + ArOH \rightarrow HRP^{3+} + ArO^{\cdot} + H^+. \quad (3)$$

Reaction (2) proceeds rapidly owing to the highly oxidizing nature of compound I (Dunford 1991). Therefore, reaction (3) generally determines the overall kinetics of the peroxidase reaction. Figure 5*d* shows the conversion of compound II to native HRP by CGA using a stopped-flow system. This reaction, which can be followed at 417 nm, obeys pseudo-first-order kinetics (Grace *et al.* 1998*b*). Thus, the apparent second-order rate constant (k_{app}) is a linear function of substrate concentration according to the equation $k_{obs} = k_{app}[CGA]$. Based on the preliminary data presented in figure 5, k_{app} was determined to be $3.7 \times 10^7\ M^{-1}s^{-1}$ for the oxidation of CGA by HRP compound II. This is remarkably fast in comparison with other phenols. For example, ferulic acid, the most active phenolic substrate studied to date, is oxidized by compound II with a second-order rate constant of $1.3 \times 10^7\ M^{-1}s^{-1}$ (Henriksen *et al.* 1999), whereas the rate constant for the oxidation of tyrosine is only $1 \times 10^3\ M^{-1}\ s^{-1}$ (Michon *et al.* 1997). These data show that that CGA is an excellent substrate for guaiacol peroxidase and lend further support to the idea that the CGA–peroxidase system may have a physiological role in scavenging H_2O_2 under stress conditions.

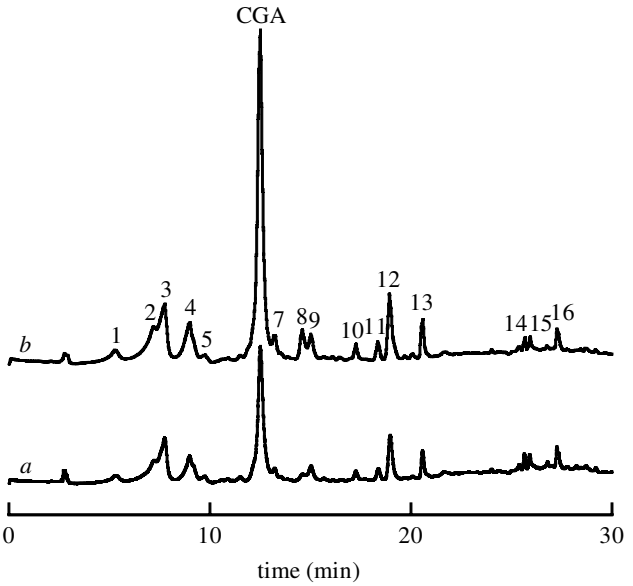

Figure 6. Sucrose stimulation of CGA synthesis in tomato leaves. Leaves were excised at the petiole and infiltrated with 200 mM sucrose through the transpiration stream under natural solar irradiance. Leaf discs (1.5 cm^2) were extracted in 80% methanol, clarified by centrifugation, filtered and subjected to HPLC analysis on a reverse-phase C_{18} column (Spherisorb[1] , Mulford, MA, USA; 25 mm, 4.6 mm). The mobile phase consisted of methanol (solvent A) and 2 mM phosphoric acid–5% methanol (solvent B) using the following conditions: 0–12 min, linear gradient of 10–55% A; 12–15 min, linear gradient of 55–100% A; 15–25 min isocratic at 100% A. The elution profile was monitored at 320 nm. Representative chromatograms are shown for samples collected (trace *a*) before and (trace *b*) 4 h after the treatment.

5. MAINTENANCE OF REDUCED PHENOLIC POOLS

Phenolics must be maintained in the reduced state to function effectively as antioxidants. Given that free radicals and oxidative enzymes continually expose phenolics to oxidation, it is likely that mechanisms exist to maintain reduced pools of phenolic scavengers. These may be enzymatic in nature, but to date no such mechanisms have been identified. However, ascorbate, the major hydrophilic antioxidant in plants, can also act as a chemical reductant of both quinones and semiquinones. In a recent study of the interaction of ascorbate with CGA in the HRP reaction, it was shown that ascorbate retards the oxidation of CGA but does not act as a substrate for HRP itself (Yamasaki & Grace 1998). This 'sparing' behaviour is due to the rapid reduction of the CGA semiquinone by ascorbate. Since ascorbate is present in the vacuole, it may act as a secondary reductant in the peroxidase reaction by recycling the active form of CGA and other phenolic substrates from their respective semiquinones (Takahama & Oniki 1997). The ascorbate radical which is formed in this reaction can decay to non-radical products by disproportionation or can be reduced to ascorbate enzymatically by MDAR or dehydroascorbate reductase or chemically by glutathione (Noctor & Foyer 1998).

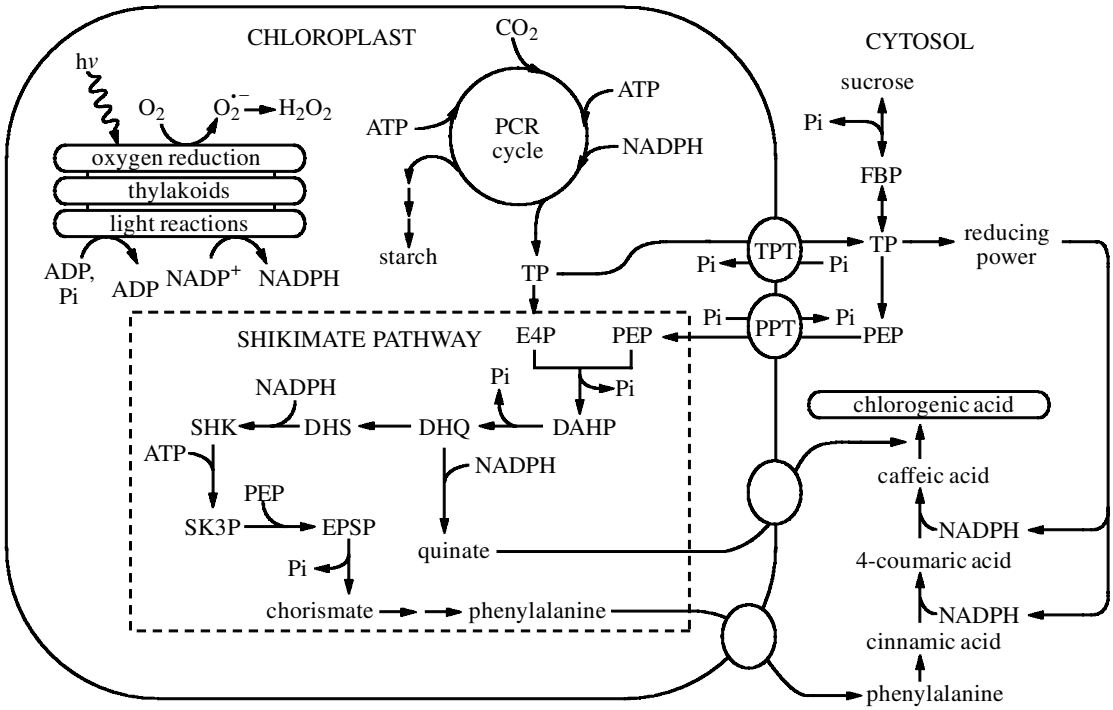

Figure 7. Model describing the relationship between CGA biosynthesis, carbohydrate metabolism, and photosynthetic energy use. Shikimate pathway intermediates are E4P, phosphoenol pyruvate (PEP), 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP), 3-dehydroquinate (DHQ), 3-dehydroshikimate (DHS), shikimate (SHK), shikimate 3-phosphate (SK3P), 5-enolpyruvylshikimate 3-phosphate (EPSP). Intermediates of photosynthetic carbon metabolism include triose phosphate (TP) and fructose 1,6-bisphosphate (FBP). Known metabolite translocators include the TP–phosphate translocator (TPT) and the PEP–phosphate translocator (PPT). See §6 for details.

6. BIOENERGETICS OF PHENOLIC METABOLISM IN STRESS RESPONSES

The initial steps of phenolic biosynthesis involve carbon flow through the phenylpropanoid pathway. The products of this pathway can accumulate as stable sugar and/or organic acid esters, as in the case of CGA, or act as precursors to more complex structures such as flavonoids, tannins and lignin. Among the environmental factors that influence phenolic metabolism, light intensity has a particularly strong effect. For instance, anthocyanin biosynthesis exhibits a fluence rate dependence, typical of the 'high irradiance response' of plant photomorphogenesis (Mancinelli 1985; Beggs & Wellman 1994). This response is not limited to anthocyanins but seems to be a general feature of plant phenolic metabolism (Mole *et al.* 1988).

A useful theory to explain these observations in physiological terms is the carbon–nutrient (C/N) balance hypothesis, which holds that when the C/N ratio is high, photosynthate is used primarily for the production of carbon-based metabolites such as phenolics (Coley *et al.* 1985; Waterman & Mole 1994). Evidence in support of this theory comes from the observation that when nitrogen supply is abundant, leaf phenolic levels tend to decline (Del Moral 1972; Waterman & Mole 1994). Further evidence comes from the observation that production of anthocyanins and phenylpropanoids increases when leaves are supplied with an external source of sucrose (Mancinelli & Rabino 1984; Murray & Hackett 1991). These studies suggest that high levels of phenolics are associated with the accumulation of excess carbohydrate in leaves. According to this view, phenylpropanoid biosynthesis serves as an 'energy overflow' mechanism by diverting photosynthate and cellular reducing power into stable product pools.

Activation of the phenylpropanoid pathway by fungal pathogens and other biotic stresses is well established (Hahlbrock & Scheel 1989; Dixon & Paiva 1995). However, the ability of a wide range of abiotic stress factors to stimulate phenylpropanoid biosynthesis is less widely appreciated. These abiotic factors include high light (Mole *et al.* 1988; Beggs & Wellman 1994), low temperatures (Christie *et al.* 1994; Solecka *et al.* 1999), sugars (Tsukaya *et al.* 1991; Sadka *et al.* 1994) and phosphate deficiency (Koeppe *et al.* 1976; Trull *et al.* 1997; Yamamoto *et al.* 1998). Although the signal transduction pathway that triggers this common biosynthetic response to diverse environmental factors is not fully understood, cellular bioenergetics are thought to play a crucial role in regulating key elements of the pathway (Ehness *et al.* 1997).

Since external feeding of sucrose and other sugars has been shown to stimulate the biosynthesis of phenolic compounds in a wide array of plants (Mancinelli & Rabino 1984; Murray & Hackett 1991; Tsukaya *et al.* 1991; Nose *et al.* 1995), we asked whether sucrose could specifically stimulate the biosynthesis of CGA in tomato, a species that, like other members of the Solanaceae, accumulates CGA as the major phenolic product. Excised leaves were allowed to take up sucrose through the

Table 1. *Reductant use and phosphate recycling in CGA biosynthesis*

(Reactions (2) and (6)–(10) are on the main trunk of the plastid localized shikimate pathway. Reaction (1) is responsible for the formation of quinate from the shikimate pathway intermediate 3-dehydroquinate (DHQ). Reactions (4) and (12) are on the main trunk of the general phenylpropanoid pathway. Reaction (5) is the last step involved in CGA synthesis. It is generally accepted that the hydroxylation reaction giving rise to the catechol moiety occurs via 4-coumaroylquinate rather than 4-coumaric acid (Kühnl *et al.* 1987). Reactions (3) and (11) are involved in the reincorporation of NH_4^+ liberated by the phenylalanine ammonia lyase reaction (Van Heerden *et al.* 1996). For further details, see Hermann 1995.)

reaction	enzyme	site
reductant use		
(1) DHQ + NADPH→quinate + $NADP^+$	quinate dehydrogenase	chloroplast
(2) 3-dehydroshikimate + NADPH→shikimate + $NADP^+$	shikimate dehydrogenase	chloroplast
(3) 2-oxoglutarate + L-glutamine + Fd^{2+}→2 L-glutamate + Fd^{3+}	glutamate synthase	chloroplast
(4) E-cinnamate + NADPH + O_2→4-coumarate + $NADP^+$	cinnamate 4-hydroxylase	cytoplasm
(5) 4-coumaroylquinate + NADPH + O_2→CGA + $NADP^+$	4-coumaroylquinate-3-hydroxylase	cytoplasm
phosphate recycling		
(6) E4P + PEP→DAHP[a] + Pi	DAHP synthase	chloroplast
(7) DAHP→DHQ + Pi	DHQ synthase	chloroplast
(8) shikimate + ATP→shikimate-3-P + ADP	shikimate kinase	chloroplast
(9) shikimate-3-P + PEP→EPSP[b] + Pi	EPSP synthase	chloroplast
(10) EPSP→chorismate + Pi	chorismate synthase	chloroplast
(11) L-glutamate + NH_4^+ + ATP→L-glutamine + ADP + Pi	glutamine synthetase	chloroplast
(12) 4-coumarate + CoA + ATP→4-coumaroyl CoA + AMP + PPi	4-coumarate:CoA ligase	cytoplasm

[a] DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate.
[b] EPSP, 5-enolpyruvylshikimate 3-phosphate.

transpiration stream under natural solar irradiance, and phenolic levels were analysed by HPLC immediately before and 4 h after the treatment. Results are shown in figure 6. Sucrose caused nearly a threefold increase in CGA levels after the 4 h treatment, and smaller increases were also observed in several other metabolite pools (e.g. peak 12). Control leaves infiltrated with water did not show an increase in CGA content (not shown). These results confirm that high soluble sugar levels stimulate carbon flow through the phenylpropanoid pathway and suggest that the cellular redox state plays an important role in regulating the synthesis of CGA.

High soluble carbohydrate levels are associated with an increase in cellular hexose phosphates and a reduction in cytosolic phosphate pools, conditions that inhibit photosynthesis by limiting phosphate cycling from the cytosol to the chloroplast (Walker & Sivak 1986; Krapp *et al.* 1993). It is noteworthy that high levels of soluble carbohydrates and low internal phosphate concentrations have similar effects on the transcription of some stress-related genes, including those involved in phenylpropanoid metabolism (Sadka *et al.* 1994; Ehness *et al.* 1997). By sequestering internal phosphate pools, excess carbohydrate levels can lead to a surplus of reducing power that cannot be used in photosynthesis. The activation of phenylpropanoid metabolism, and in particular CGA synthesis, by light, chilling, low phosphate and sugars can be rationalized by the effects of these environmental stimuli on photosynthesis and cellular energy metabolism. Salient aspects of this complex relationship include the role of phosphate cycling between chloroplast and cytosol and the use of photosynthetic reducing power. Any factor that limits the return of phosphate to the chloroplast has the potential to inhibit the assimilatory reactions of photosynthesis and diminish the availability of acceptors for photosynthetic electron transport. Under conditions where carbohydrate levels become excessive (e.g. high light, chilling), phosphate recycling is impaired due to a build-up in hexose phosphates in the cytosol. In the absence of alternative electron sinks, free radical production in the chloroplast may increase due to increased rates of O_2 photoreduction even under conditions of enhanced thermal energy dissipation (Osmond & Grace 1995; Grace & Logan 1996).

The activation of phenylpropanoid metabolism can alleviate this energetic imbalance in several important ways. Consider the relationship between photosynthetic metabolism and CGA biosynthesis (figure 7). First, the shikimate pathway, which provides aromatic precursors for the phenylpropanoid pathway, is localized largely, if not exclusively, in the plastid (Hermann 1995). Carbon enters this pathway from erythrose-4-phosphate (E4P) and phosphoenol pyruvate (PEP), which is imported into the chloroplast by a PEP-specific translocator (Streatfield *et al.* 1999). Thus, the shikimate pathway can provide an alternative route for the consumption of both carbon intermediates and metabolic reducing power and therefore may sustain turnover of the photosynthetic apparatus under stress conditions. A second important feature of this pathway is that as phosphorylated intermediates are used in the synthesis of aromatic products, phosphate is released inside the chloroplast before the products are exported to the cytosol. This represents an important distinction between the normal path of photosynthetic carbon intermediates en route to sucrose synthesis and the synthesis of aromatic products such as phenolics. Studies with transgenic plants support a link between photosynthesis and phenolic metabolism. An *Arabidopsis* mutant with impaired ability to import PEP into the chloroplast was shown to be compromised in the ability to synthesize anthocyanins when transferred to high light and showed lower light-saturated electron transport rates

than wild-type plants (Streatfield *et al*. 1999). A third bioenergetic aspect of phenolic metabolism is the use of cellular reducing power in both the chloroplast and cytosol. The energy-consuming and phosphate recycling reactions involved in CGA synthesis are summarized in table 1. Finally, the phenolic products can themselves act as hydrogen-donating antioxidants, thereby mitigating the effects of oxidative stress.

The shikimate pathway not only provides phenylalanine for the entry step into the phenylpropanoid pathway but is also the only known source of quinate, an essential precursor for CGA biosynthesis. Surprisingly little is known about the regulation of quinate synthesis in plants, although it can represent a major carbon storage pool in gymnosperms (Bonner & Jensen 1998). In angiosperms quinate is stored primarily as the conjugate depside CGA (Mølgaard & Ravn 1988). It is evident that in order for CGA to accumulate, two conditions must be met. First, there must be coordinated regulation between upstream and downstream segments of the coupled shikimate–phenylpropanoid pathway to provide adequate levels of quinate and hydroxycinnamoyl precursors for CGA biosynthesis. Second, there must be sufficient transferase activity to catalyse the esterification reaction between quinate and hydroxycinnamoyl-CoA intermediates. The enzyme that catalyses this reaction, hydroxycinnamoyl-CoA:quinate transferase (HQT), is just one of several enzymes that competes for 4-coumaroyl-CoA in the multibranched phenylpropanoid pathway. The rapid turnover of CGA in plants (Luckner 1990) is consistent with the reversibility of the transferase reaction *in vitro* (Rhodes & Wooltorton 1976) and underscores the possible storage role of this metabolite. This high degree of metabolic plasticity may be one factor that allows the rapid synthesis of phenolic metabolites such as lignin in response to specific environmental stimuli (Yao *et al*. 1995).

Definitive evidence that CGA acts as an antioxidant *in vivo* will ultimately require genetic manipulation of the biosynthetic pathway. At present the regulatory steps that control CGA synthesis are unknown. More importantly the mechanisms by which CGA synthesis is controlled in relation to other branch pathways of the phenylpropanoid biosynthetic matrix are not known. How are phenylpropanoid intermediates channelled into the CGA pathway in relation to other product pools? Two plausible candidates for enzymic flux control are quinate dehydrogenase, which channels carbon from the shikimate pathway into the synthesis of quinate, and HQT. To date there has been very little study of these enzymes in plants.

7. CONCLUDING REMARKS

Recent research has established the importance of photoprotective processes that minimize photo-oxidative damage that could potentially result from the absorption of excess light. Interactions between xanthophyll cycle-dependent thermal energy dissipation and ROS scavenging systems in the chloroplast provide dynamic regulation of photon use to match the needs of photosynthesis. However, these photoprotective systems may not be adequate to prevent oxidative damage when additional stresses are imposed. The phenylpropanoid pathway generates products that confer increased tolerance to a wide array of stresses and may also provide an alternative route for photon use under conditions of carbohydrate accumulation and/or excess light absorption. There are clear indications that CGA, one of the major products of this pathway, serves a general role in stress responses by acting as a potent hydrogen-donating antioxidant and, perhaps more importantly, as a reductive substrate for guaiacol peroxidase. Even the synthesis of CGA recycles orthophosphate and consumes reductant, enabling photosynthetic use of absorbed photons and thereby further protecting against oxidative stress.

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