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Re-evaluating the role of ascorbic acid and phenolic glycosides in ozone scavenging in the leaf apoplast of *Arabidopsis thaliana* L.

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ABSTRACT

Phenolic glycosides are effective reactive oxygen scavengers and peroxidase substrates, suggesting that compounds in addition to ascorbate may have functional importance in defence responses against ozone (O_3), especially in the leaf apoplast. The apoplastic concentrations of ascorbic acid (AA) and phenolic glycosides in *Arabidopsis thaliana* L. Col-0 wild-type plants were determined following exposure to a range of O_3 concentrations (5, 125 or 175 nL L^{-1}) in controlled environment chambers. AA in leaf apoplast extracts was almost entirely oxidized in all treatments, suggesting that O_3 scavenging by direct reactions with reduced AA was very limited. In regard to phenolics, O_3 stimulated transcription of numerous phenylpropanoid pathway genes and increased the apoplastic concentration of sinapoyl malate. However, modelling of O_3 scavenging in the apoplast indicated that sinapoyl malate concentrations were too low to be effective protectants. Furthermore, null mutants for sinapoyl esters (*fah1-7*), kaempferol glycosides (*tt4-1*) and the double mutant (*tt4-1/fah1-7*) were equally sensitive to chronic O_3 as Ler-0 wild-type plants. These results indicate that current understanding of O_3 defence schemes deserves reassessment as mechanisms other than direct scavenging of O_3 by extracellular AA and antioxidant activity of some phenolics may predominate in some plant species.

Key-words: Air pollution; flavonoids; kaempferol; reactive oxygen; sinapoyl malate; vitamin C.

INTRODUCTION

Reactions between plant cell constituents and ozone (O_3) are thought to occur initially in the leaf extracellular space following O_3 uptake through the stomata (Mudd 1996; Moldau 1998). Following dissolution in the liquid phase of the apoplast, O_3 reacts with cellular components to form additional reactive oxygen species (ROS) and reaction products (Mudd 1996; Wohlgemuth *et al*. 2002). Primarily, ascorbic acid (AA) in the apoplast is thought to provide protection against oxidant injury, depending on the concentration, regeneration capacity and plant species involved (Luwe & Heber 1995; Conklin *et al*. 1997; Conklin & Barth 2004). Other compounds in the apoplast such as conjugated polyamines (phenolic amides) and other phenolics might be important as well (Langebartels *et al*. 1991; Takahama 1998).

Phenylpropanoid metabolism and biosynthesis of phenolics are often stimulated by O_3, suggesting that they may be involved in responses to oxidative stress (Grace 2005). Many phenolic compounds are highly effective ROS scavengers because they are easily oxidized and the resulting phenoxyl radicals are less reactive than oxygen radicals (Bors *et al*. 1990; Grace 2005). Phenolics such as flavonoids and hydroxycinnamic acids (HCAs) are primarily located in the vacuole as glycosides or glutathione conjugates or, in the case of HCAs, as covalently bound cell wall components (Wallace & Fry 1994; Grace 2005). The extensive side-chain conjugation of HCAs adds to their antioxidant capacity. However, pro-oxidants formed in reactions between O_3 and HCAs may be toxic and initiate signals as well (Grace 2005). Further, phenolics in the apoplast possibly participate in the decomposition of O_3 by acting as electron donors that result in the production of $OH^{\cdot}$ radicals and O_2 (Moldau 1998). Phenoxyl radicals that form can be reduced by AA (Takahama & Oniki 1997).

Plants often respond to O_3 exposure with a variety of antioxidant-related changes in metabolism and gene expression (Pell, Schlagnhaufer & Arteca 1997; Sharma & Davis 1997; Tosti *et al*. 2006; Heath 2008). One of the early events following an acute O_3 exposure can be an oxidative burst that resembles the hypersensitive response to incompatible plant pathogens (Langebartels *et al*. 2002; Wohlgemuth *et al*. 2002; Baier *et al*. 2005). The ROS generated in this burst are suggested to be both damaging in themselves and potential signalling molecules (Sharma & Davis 1997; Baier *et al*. 2005; Foyer & Noctor 2005). Regulation of the oxidative burst occurs through complex radical scavenging and amplification processes that include antioxidants such as AA and glutathione, superoxide dismutase, NADPH oxidases and peroxidases, redox regulation, and others (Rao & Davis 2001; Baier *et al*. 2005).

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The objective of this study was to determine whether O_3 affected AA and phenolic compounds in the leaf apoplast of *Arabidopsis thaliana* L. Effects of physiological O_3 concentrations on leaf apoplast chemistry and phenylpropanoid gene expression were determined. To investigate the physiological significance of AA and soluble phenolics in plant-O_3 interactions, potential O_3 scavenging by AA, sinapoyl malate and kaempferol glycosides in the apoplast was calculated using a mathematical model. We also used null mutants to test whether the lack of sinapoyl esters and flavonol glycosides affected foliar injury and biomass production in plants exposed to chronic O_3. We found that AA cannot be a primary O_3 scavenger because reduced AA in the apoplast was very low, while genetic reduction of phenolic compounds had minimal consequences in O_3 sensitivity. Other mechanisms controlling plant sensitivity to O_3 must predominate.

METHODS

Genotypes, plant culture, O_3 treatments and plant sampling

Arabidopsis genotypes used in this study were Col-0, Ler-0, the chalcone synthase mutant *tt4-1* (Li *et al*. 1993), the ferulic acid hydroxylase mutant *fah1-7* (Landry, Chapple & Last 1995) and the *tt4-1/fah1-7* double mutant. The tDNA insertion mutants were in a Ler-0 background. Seeds for all genotypes were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA).

Seeds were sown in small pots containing Metro-Mix (Sun Gro Horticulture, Bellevue, WA, USA), stratified for 3 d at 4 °C and then germinated under a photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1} (9 h light/15 h dark cycle) at 23 °C in a growth chamber in the North Carolina State University Phytotron. Seedlings were transplanted into Metro-Mix in $5 \times 5 \times 5$ cm cell-packs and grown for up to 5 weeks in the growth chamber. Plants were fertilized with Phytotron nutrient solution once per week (Downs & Thomas 1983).

On the day preceding an O_3 exposure, plants were transferred to four continuous-stirred tank reactor (CSTR) chambers located in a walk-in growth chamber in the North Carolina State University Phytotron (Booker *et al*. 2004). Temperature, relative humidity and PPFD in the CSTRs were 24 °C, 55% and 360 μmol m^{-2} s^{-1}, respectively. Plants were treated with charcoal-filtered air plus 5, 125 or 175 nL L^{-1} O_3 7 h d^{-1}, as previously described (Booker *et al*. 2012). Following O_3 treatment, fully expanded, mid-whorl leaves were sampled for total RNA, leaf tissue AA, and apoplastic AA and phenolic compounds. Leaf tissues were frozen in liquid N_2 and kept at –80 °C until assayed. Effects of chronic O_3 on vegetative biomass production (rosette diameter and dry mass) were measured in 3-week-old Ler-0, *fah1-7*, *tt4-1* and *tt4-1/fah1-7* plants treated with 10 or 125 nL L^{-1} O_3 for 11–12 d.

Gene expression array

Total RNA in leaf tissue samples (50 mg) obtained after exposure to 5 or 125 nL L^{-1} O_3 for 3 h and 2 d was extracted using Qiagen RNeasy mini kits according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA was quantified by measuring A_{260} on a spectrophotometer and integrity was checked using RNA Nano LabChips and Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Labelled cRNAs were synthesized at the Functional Genomics Core Facility, University of North Carolina – Chapel Hill and hybridized to the GeneChip® Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA), as previously described (Booker *et al*. 2012). Gene expression results were analysed using Genespring GX (Ver. 11.5.1, Agilent Technologies), as previously described (Booker *et al*. 2012). There were three and four independent biological replicate samples (individual plants from separate chambers and experiments) for each treatment combination at the 3 h and 2 d sampling times, respectively.

Intercellular washing fluid extraction

Intercellular washing fluid (IWF) extraction was conducted as described by Booker *et al*. (2012), with modifications. Leaves were infiltrated with 100 mM KCl. For AA assays, IWF was collected in microcentrifuge tubes containing 50 μL of 6% (w/v) metaphosphoric acid and 0.2 mM of diethylenetriaminepentaacetic acid to prevent oxidation of AA (Cheng *et al*. 2007). After extraction, IWF samples were mixed, weighed and frozen at –20 °C for later analysis. Possible contamination of IWF with intracellular components was evaluated by measuring for the presence of glucose-6-phosphate (G6P) in IWF samples (detection limit 2 μM), as previously described (Burkey 1999). Intracellular solute leakage, as indicated by the presence of G6P in IWF extracts, was not evident. Extraction efficiency of infiltrated leaf tissues averaged 99 ± 1% based on fresh weights (FWs) of leaf tissues samples before and after centrifugation. Yield of IWF from infiltrated leaves averaged 204 ± 5 μL g^{-1} FW, and there were no statistically significant differences in yield among treatments ($P \geq 0.05$).

AA assays

Frozen leaf tissue samples (200 mg FW) were homogenized in 6% (w/v) metaphosphoric acid and 0.2 mM of diethylenetriaminepentaacetic acid in a chilled mortar (100 mg FW mL^{-1}) (Cheng *et al*. 2007). The homogenate was centrifuged at 21 000 *g* and the supernatant collected. IWF samples were centrifuged and the supernatant was used in the AA assays.

AA concentration was measured spectrophotometrically using a combination of ascorbate oxidase and dithiothreitol (DTT) to determine reduced AA and dehydroascorbic acid (DHA) concentrations, respectively (Cheng *et al*. 2007). For reduced AA measurements, a 50 μL aliquot of plant extract was mixed with 0.95 mL of 100 mM KPi buffer (pH 7.0) in a

quartz glass cuvette and A_{265} was determined. Ascorbate oxidase (1 μL of a 1 U μL^{-1} solution) was added to the cuvette, mixed, and A_{265} was measured after 1 min. For the DHA measurements, 50 μL of plant extract was mixed with 0.95 mL of KPi buffer and 1 μL of 200 mM DTT. The solution was incubated for 10 min, followed by measurement of A_{265}. AA concentrations were determined by the difference in A_{265} before and after addition of ascorbate oxidase (reduced AA) and before and after addition of DTT (DHA) using $\varepsilon_{265nm} = 14.3\ mM^{-1}\ cm^{-1}$. The sum of reduced AA and DHA equalled total AA. For calculations of apoplastic AA and DHA concentrations, the volume of the aqueous apoplast was estimated to be 11% of leaf FW (Speer & Kaiser 1991; Luwe & Heber 1995). Ascorbate redox status was expressed as AA / (AA + DHA).

IWF phenolic composition assay

IWF samples were filtered (0.45 μm), and 20 μL aliquots were separated by RP-HPLC using Microsorb C_{18} columns (30 mm × 4.6 mm guard column; 100 mm × 4.6 mm analytical column, 3 μm) (Pro-Star/Dynamax System, Agilent Technologies). Gradient conditions: Solvent A: 0.1% formic acid; Solvent B: 0.1% formic acid in methanol; 10% B for 0.25 min; 10 to 30% B over 14 min; 30% B for 2 min; 30 to 45% B over 10 min; 45 to 80% B over 10 min; 80% B for 3 min; and 80 to 10% B over 4 min; flow rate: 1 mL min^{-1}. Column temperature was 30 °C. Absorbance at 330 nm and spectra (190–400 nm) were measured using a photodiode-array detector (ProStar model 335, Agilent Technologies) and analysed using Star Chromatography Workstation software (Ver. 6.41, Agilent Technologies). Phenolic aglycones were identified on chromatograms by retention time, co-elution with standards, and UV spectra following incubation in 2 N HCl at 80 °C for 1 h. Concentrations of sinapoyl and flavonol glycosides in IWF extracts were based on peak areas of sinapic acid and kaempferol standards. Apoplastic concentrations of phenolic glycosides were determined as described above for AA.

The composition of IWF samples extracted with water from Col-0 control plants was also investigated by high resolution, exact mass measurement using electrospray ionization (ESI) in positive and negative ion modes on an LC-TOF mass spectrometer (model 6213, Agilent Technologies) at the North Carolina State University Mass Spectrometry Facility. Samples were analysed via a 5 or 10 μL flow injection at 1 mL min^{-1} using an Agilent Eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm). Gradient conditions (negative ion mode): Solvent A: 0.1% NH_4OH in water; Solvent B: 0.1% NH_4OH in methanol; 10% B for 0.23 min; 10 to 30% B over 13.77 min; 30% B for 1.5 min; 30 to 40% B over 10 min; 40 to 80% B over 9.5 min; 80% B for 3 min; and 80 to 10% B over 2 min. Gradient conditions (positive mode): Solvent A: 0.1% formic acid; Solvent B: 0.1% formic acid in methanol; 10% B for 0.23 min; 10 to 30% B over 13.77 min; 30% B for 1.5 min; 30 to 40% B over 10 min; 40 to 80% B over 9.5 min; 80% B for 2 min; and 80 to 10% B over 2 min. The mass spectrometer was operated with a capillary voltage of 4 kV, nebulizer pressure of 30 psig and a drying gas flow rate of 12 L min^{-1} at 350 °C. Fragmentor voltage was 180, 200 or 350 V (nozzle/skimmer dissociation) and skimmer voltage was 60 V. Reference ions of purine at m/z 121.0509 and HP-0921 at m/z 922.0098 were used as calibrants.

Apoplast ROS scavenging capacity

To investigate potential O_3 scavenging by AA or sinapic acid derivatives in the apoplast, the fraction of the O_3 flux that is decomposed by direct reaction with AA or sinapic acid before reaching the plasmalemma (FR) was calculated according to the method derived by Chameides (1989), as shown in Moldau (1998):

$$FR = 1 - \mathrm{sech}(L\sqrt{k[M]/D}, \quad (1)$$

where L is wall thickness in cm, k is the bimolecular rate constant of the O_3 + AA or O_3 + sinapic acid reaction in $M^{-1}\ s^{-1}$, [M] is the molar apoplast AA or sinapic acid concentration, and D is the aqueous phase molecular diffusion coefficient for O_3 ($2 \times 10^{-5}\ cm^2\ s^{-1}$). The cell wall thickness value used was 0.32 μm (Teng *et al*. 2006). The bimolecular rate constant, k, for the AA reaction was $4.8 \times 10^7\ M^{-1}\ s^{-1}$ (Moldau 1998). The O_3 + sinapic acid reaction rate constant ($1.9 \times 10^6\ M^{-1}\ s^{-1}$) was based on estimates for ferulic acid ($1.6 \times 10^6\ M^{-1}\ s^{-1}$) (Beltran-Heredia *et al*. 2001) and syringic acid ($2.2 \times 10^6\ M^{-1}\ s^{-1}$) (Benitez *et al*. 1997), both determined at 20 °C, pH 5. Ferulic and syringic acids are structurally similar to sinapic acid, whose k was not found in the literature. Equation 1 was also used to calculate $OH^{\cdot}$ and $^1O_2$ quenching by sinapic acid using $k = 2.2 \times 10^{10}$ and $3.0 \times 10^7\ M^{-1}\ s^{-1}$, respectively (Buxton *et al*. 1988; Foley *et al*. 1999). Scavenging of $OH^{\cdot}$, $^1O_2$ and $O_2^{\cdot-}$ by kaempferol was calculated using $k = 4.6 \times 10^9$, 4.8×10^5 and $5.5 \times 10^5\ M^{-1}\ s^{-1}$, respectively (Bors *et al*. 1990; Tournaire *et al*. 1993).

Experimental design and statistics

Results were analysed by analysis of variance using a mixed model (Littell *et al*. 1996) (PC SAS for Windows, ver. 9.2, SAS Institute, Inc., Cary, NC, USA). In the experiment with Ler-0, *fah1-7* and *tt4-1*, the genotypes were treated in the same chamber, and results were analysed as a split-plot with O_3 treatments as the main plot and genotype as the sub-plot. Reported values are the least squares means and associated standard errors. Average results from plants in a chamber were treated as biological replicates and each experiment was conducted at least three times.

RESULTS

AA

Apoplastic AA was largely oxidized and intracellular AA was largely reduced, even without O_3 treatment. Apoplastic concentrations of AA and DHA were 0.002 ± 0.001 and

0.36 ± 0.03 mM (mean ± SE), respectively. Leaf tissue concentrations of AA and DHA were 4.54 ± 0.26 and 0.41 ± 0.04 mM, respectively. Differences in AA concentration among O_3 treatments were not statistically significant ($P \geq 0.05$). The leaf tissue AA / (AA + DHA) ratio was 0.92 ± 0.01.

Phenylpropanoid and flavonoid gene expression

Given the extremely low levels of reduced AA in the leaf apoplast, we sought other potential reducing compounds that could participate in the plant's primary response to O_3 toxicity. A number of genes in the phenylpropanoid and flavonoid pathways were up-regulated after treatment with 125 nL L^{-1} O_3 for 3 h and 2 d (Table 1). Notably, there was increased expression of genes encoding hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase (*HCT*), caffeoyl-CoA O-methyltransferase (*CCoAOMT*), caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (*COMT*), aldehyde dehydrogenase (*ALDH1A*), cinnamoyl-CoA reductase (*CCR*) and cinnamyl alcohol dehydrogenase (*CAD*) in the pathways leading to sinapic acid derivatives and lignin biosynthesis (Fig. 1). Transcription of a UDP-glucosyltransferase gene (*UGT84A2*, *SGT*) for 1-*O*-sinapoyl glucose synthesis was also stimulated after O_3 exposure for 2 d. With regard to flavonoids, expression of an early pathway gene, chalcone isomerase, was unchanged after 3 h and down-regulated by O_3 after 2 d. However, a gene in the flavonol synthase family (At3g19010) was stimulated by O_3 after 3 h but not at 2 d. Several oxidoreductases putatively involved in flavonoid biosynthesis were stimulated by O_3 after 2 d. The most numerous up-regulated genes due to O_3 in the flavonol pathway were glycosyltransferases (*UGT71C*, *UGT73B*, *UGT73C*, *UGT73D1*, *UGT78D2*).

Table 1. List of phenylpropanoid and flavonoid pathway genes up- or down-regulated at 3 h and 2 d after exposure of Col-0 plants to 125 nL L^{-1} O_3 ($P \leq 0.05$)

AGB ID	Gene symbol	Name or description	Fold change 3 h	Fold change 2 d
At3g44720	ADT4	Arogenate dehydratase (involved in phenylalanine biosynthesis)	2.6	2.0
At5g48930	*HCT*	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase	1.8	ns
At4g34050	*CCoAOMT1*	Caffeoyl-CoA 3-*O*-methyltransferase	ns	1.8
At1g67980	*CCoAOMT6*	Caffeoyl-CoA 3-*O*-methyltransferase	ns	2.1
At1g21100	*COMT-like1*	Caffeic acid/5-hydroxyferulic acid *O*-methyltransferase	3.1	ns
At1g21110	*COMT-like2*	Caffeic acid/5-hydroxyferulic acid *O*-methyltransferase	14.1	ns
At1g21120	*COMT-like3*	Caffeic acid/5-hydroxyferulic acid *O*-methyltransferase	13.6	ns
At1g21130	*COMT-like4*	Caffeic acid/5-hydroxyferulic acid *O*-methyltransferase	3.0	–2.0
At1g33030	*COMT-like5*	Caffeic acid/5-hydroxyferulic acid *O*-methyltransferase	3.4	ns
At3g24503	*ALDH1A*	Aldehyde dehydrogenase	2.0	1.6
At3g21560	*UGT84A2*	Sinapic acid:UDP-glucose glucosyltransferase	ns	2.9
At1g80820	*CCR2*	Cinnamoyl-CoA reductase	1.7	ns
At1g72680	*CAD1*	Cinnamyl alcohol dehydrogenase	ns	3.0
Flavonoids				
At3g63170	*CHI*	Chalcone-flavanone isomerase	ns	–1.6
At1g53520	*CHI*	Chalcone-flavanone isomerase-related	ns	–3.6
At3g19010	*FLS*	Flavonol synthase family	2.1	ns
At4g25300/10		Oxidoreductase	ns	1.6
At5g24530		Oxidoreductase	ns	3.6
At2g29750	*UGT71C1*	UDP-glycosyltransferase/ quercetin 3-O-glucosyltransferase/quercetin 7-O-glucosyltransferase	2.5	ns
At1g07240	*UGT71C5*	Quercetin 3-O-glucosyltransferase/ quercetin 7-O-glucosyltransferase	1.7	–1.5
At2g36790/ At2g36800	UGT73C6	UDP-glucose:flavonol-3-O-glycoside-7-O-glucosyltransferase	1.4	ns
At4g34131/ At4g34135	UGT73B3/ UGT73B2	UDP-glucosyl transferase /flavonol 7-O-glucosyltransferase	3.7	3.7
At2g15480	UGT73B5	UDP-glycosyltransferase/ quercetin 3-O-glucosyltransferase/ quercetin 7-O-glucosyltransferase	4.5	ns
At3g53150	UGT73D1	UDP-glycosyltransferase/ quercetin 4′-O-glucosyltransferase/ quercetin 7-O-glucosyltransferase	ns	3.8
At5g17050	*UGT78D2*	UDP-glucosyl transferase/ flavonoid 3-*O*-glucosyltransferase	–1.7	1.5
At5g54060	UF3GT	UDP-glucose:flavonoid 3-O-glucosyltransferase	–2.7	ns
At1g06000		Flavonol-7-O-rhamnosyltransferase	ns	2.6
At1g30530		UDP-rhamnose:flavonol-3-O-rhamnosyltransferase	–2.2	ns

ns, not statistically significant.

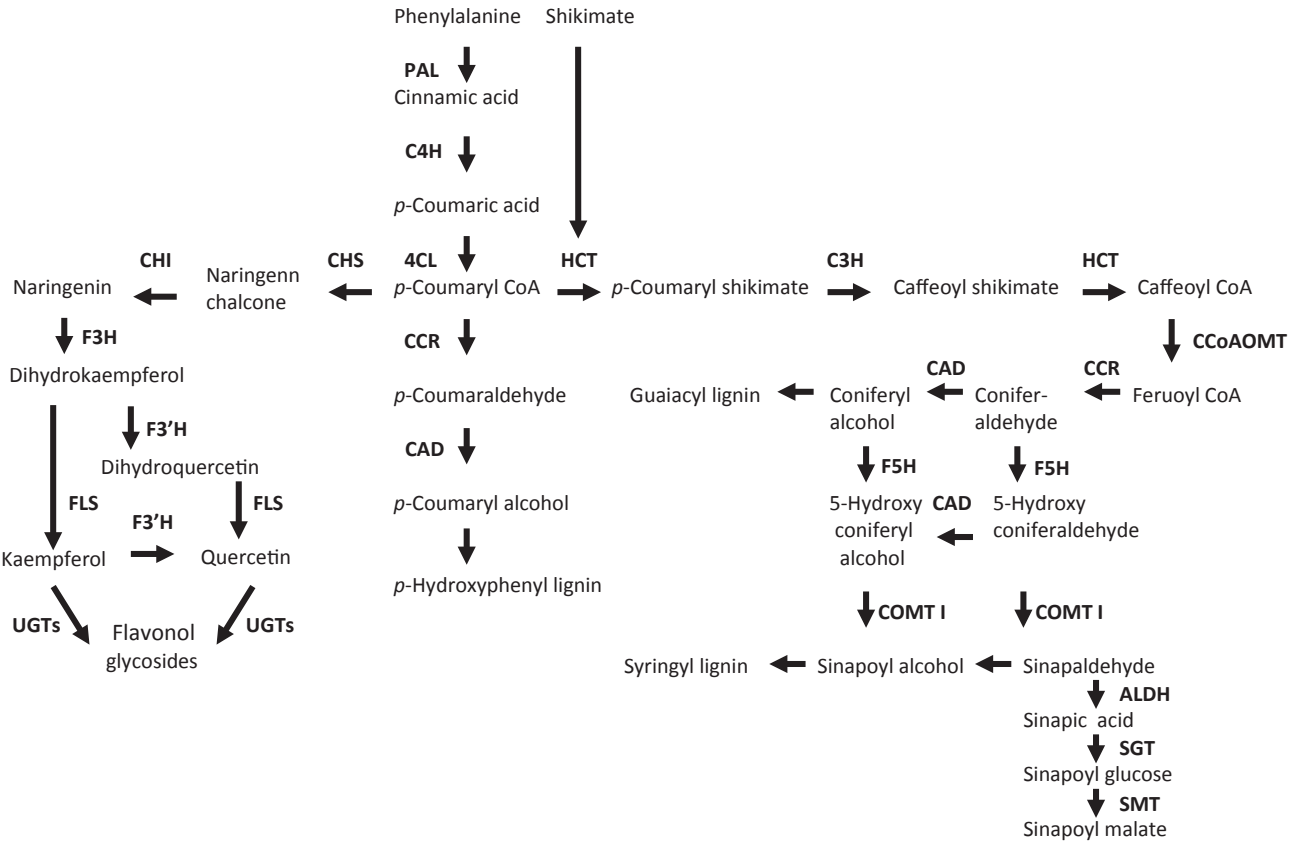

Figure 1. The phenylpropanoid pathway in *Arabidopsis*. ALDH, aldehyde dehydrogenase; C3H, C3-hydroxylase; C4H, C4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumaroyl-CoA ligase; COMT I, caffeic acid O-methyltransferase of class I; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F5H, ferulate 5-hydroxylase; FLS, flavonol synthase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; PAL, phenylalanine ammonia lyase; SGT, sinapate UDP-glucose sinapoyl transferase; SMT, sinapoylglucose malate sinapoyl transferase; UGTs, UDP sugar glycosyltransferases. Redrawn from Besseau *et al.* (2007).

Phenolic glycoside composition of IWF extracts

Separation by RP-HPLC of IWF extracts revealed a number of compounds (Fig. 2a,b), some of which were identified as sinapoyl (peak 2) and flavonol (peaks 1, 3, 4) glycosides, based on their UV spectra. The presence of sinapoyl glycosides was also indicated by acid hydrolysis of IWF extracts which produced a phenolic aglycone matching the retention time and UV spectrum of sinapic acid (Fig. 3, inset). In addition, IWF extract from the sinapoyl malate-deficient *fah1*-7 mutant lacked peak 2, while the flavonoid-deficient *tt4-1* mutant lacked peaks 1, 3 and 4 (Fig. 2c,d), confirming these identities. Four peaks were found in common between the $UV_{330\ nm}$ and total ion chromatograms determined by LC-ESI-TOF mass spectrometry. Peak 2 contained sinapoyl malate, as indicated by: (1) accurate mass of the compound in negative ion mode; (2) accurate mass of the sodiated dimer and monomer in positive mode; and (3) fragmentation patterns (Table 2). Peaks 1, 3 and 4 contained kaempferol-3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside, kaempferol-3-O-glucoside 7-O-rhamnoside, and kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside, respectively (Table 2). Identifications were based on accurate mass determinations of the compounds and fragmentation patterns in positive and negative ion modes. Sinapoyl glucose was not detected in the analysis.

The concentration of sinapoyl malate (peak 2) in the leaf apoplast increased 2.4-fold in the elevated O_3 treatments compared with the control ($P \leq 0.05$) (Fig. 3). The total average concentration of flavonol glycosides in peaks 1, 3 and 4, expressed as kaempferol equivalents, was $72 \pm 10\ \mu M$. There were no significant differences in kaempferol equivalents among O_3 treatments ($P \geq 0.05$).

Apoplastic ROS scavenging capacity

The fraction of O_3 flux that was scavenged before reaching the plasmalemma (FR) by AA ($2\ \mu M$) or sinapoyl malate in the leaf apoplast ($20\ \mu M$) was calculated to be 0.2%. Hydroxyl radicals would be highly reactive with sinapoyl and kaempferol glycosides in the apoplast. Quenching of $^1O_2$ by sinapoyl and kaempferol glycosides in the leaf apoplast was calculated to be less than 3%. The FR of $O_2^{\cdot-}$ by kaempferol derivatives was 14%.

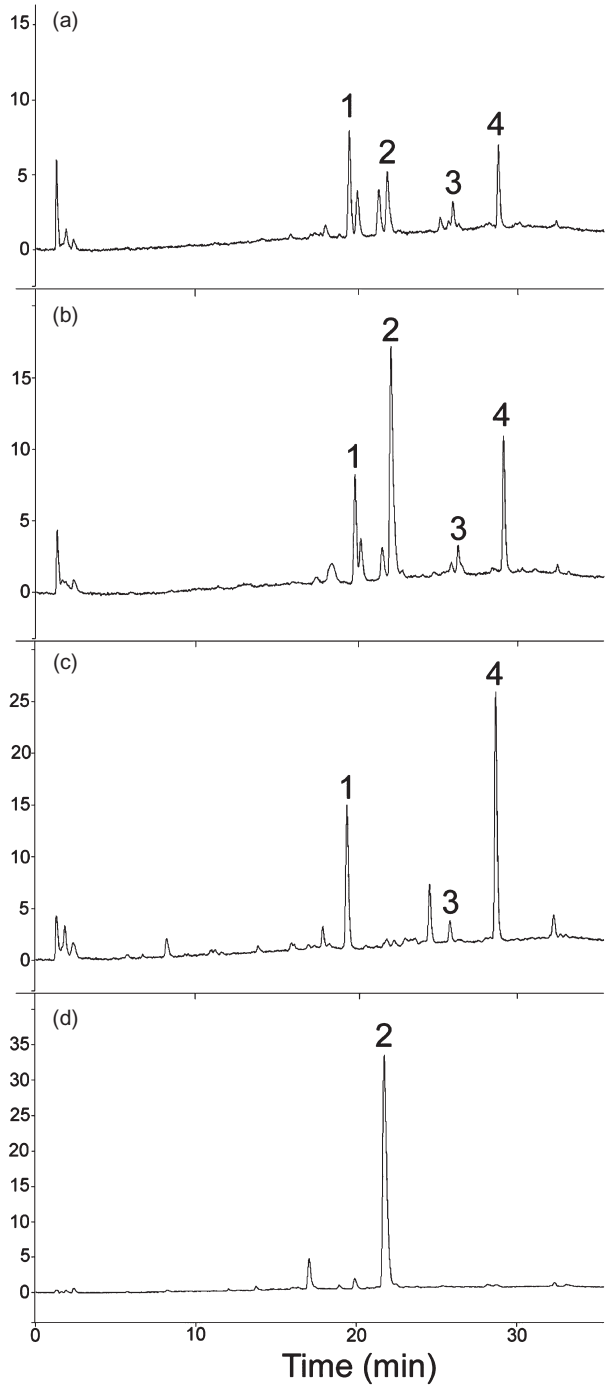

Figure 2. Separation by RP-HPLC of phenolic compounds in IWF extracts from Col-0 plants treated with either 5 nL L^{-1} (control) (a) or 175 nL L^{-1} O_3 (b) for 7 h. Also shown are separations of IWF extracts from *Arabidopsis* mutants *fah1-7* (c) and *tt4-1* (d). Absorbance was measured at 330 nm. Compounds in the numbered peaks were identified as: 1 = kaempferol-3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside; 2 = sinapoyl malate; 3 = kaempferol-3-O-glucoside 7-O-rhamnoside; and 4 = kaempferol 3-O-rhamnoside 7-O-rhamnoside. IWF, intercellular washing fluid.

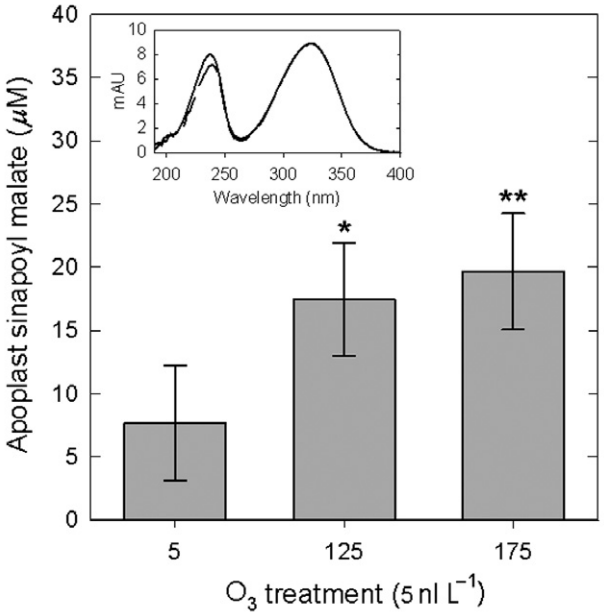

Figure 3. Sinapoyl malate concentrations in the leaf apoplast of Col-0 plants treated with a range of O_3 concentrations 7 h d^{-1} for 2 d. Inset: UV spectra of sinapic acid (solid line) and acid-hydrolyzed IWF sample from control plants (dashed line). Statistically significant pairwise comparisons with the 5 nL L^{-1} O_3 control treatment are indicated as: *, $P \leq 0.05$; **; $P \leq 0.01$. Values are least squares means ± SE. IWF, intercellular washing fluid.

Biomass production of Ler-0 and phenolic null mutants

Treatment of 3-week-old Ler-0, *fah1-7* (lacks sinapate esters), *tt4-1* (lacks kaempferol derivatives) and *tt1-4/fah1-7* plants with 125 nL L^{-1} O_3 for 11–12 d decreased rosette diameter by 24 to 34% and aboveground biomass production by 50 to 61% ($P \leq 0.01$) (Table 3). Ozone injury, apparent as longitudinal leaf curling and senescence of older leaves, occurred to the same extent in all genotypes. There was no significant difference in relative O_3 effects on biomass production among genotypes ($P \geq 0.05$). The proposed protective effect of the soluble phenolics against O_3 injury was not evident.

DISCUSSION

It is often proposed that reduced AA in the apoplast is important for scavenging O_3 as it enters the leaf and as an antioxidant to detoxify ROS (Castillo & Greppin 1988; Chameides 1989; Luwe, Takahama & Heber 1993; Luwe & Heber 1995; Ranieri *et al.* 1996, 2000; Conklin *et al.* 1997; Moldau 1998; Conklin & Barth 2004). In IWF extracts from spinach (*Spinacia oleracea* L.) exposed to 300 nL L^{-1} O_3, for example, AA was slowly oxidized, and the ratio of AA to total ascorbate (AA + DHA) decreased from 0.9 to 0.1 within 6 h and remained at a low level (Luwe *et al.* 1993). At lower O_3 concentrations (56–113 nL L^{-1}), AA and DHA in

Table 2. LC-ESI-TOF mass spectrometry analysis of leaf intercellular washing fluid (IWF) extract from Col-0

Peak	Component	Ion mode	Ion species	Exact mass (m/z)	IWF sample (m/z) (ppm error)	Fragmentor voltage	RT (min)	Reference
1	Kaempferol-3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside (MW 740)	Negative	$[M-H]^-$	739.2091	739.2096 (0.67)	180	6.3	(Stobiecki *et al.* 2006)
		Positive	$[M+Na]^+$	763.2056	763.2063 (0.92)	200	17.2	(Kachlicki *et al.* 2008)
			$[M+H]^+$	741.2237	741.2246 (1.21)	200		(Stobiecki *et al.* 2006)
			$[M+H^+ - \text{rhamnose} - \text{glucose}]$	433.1129	433.1134 (1.15)	200		(Stobiecki *et al.* 2006)
			$[M+H^+ - \text{rhamnose} - \text{glucose} - \text{rhamnose}]$	287.0550	287.0548 (–0.70)	200		(Stobiecki *et al.* 2006)
2	Sinapoyl malate (MW 340)	Negative	$[M-H]^-$	339.0722	339.0735 (3.83)	180	1.3	(Stobiecki *et al.* 2006)
			$[\text{Sinapic acid } M-H]^-$	223.0612	223.0614 (0.90)	180		(Stobiecki *et al.* 2006)
		Positive	$[2M+Na]^+$	703.1481	703.1474 (–1.00)	180	21.6	(von Roepenack-Lahaye *et al.* 2004)
			$[M+Na]^+$	363.0687	363.0685 (–0.55)	180		(von Roepenack-Lahaye *et al.* 2004)
			$[\text{Sinapic acid } M+H-H_2O]^+$	207.0652	207.0652 (0.00)	180		(von Roepenack-Lahaye *et al.* 2004)
3	Kaempferol-3-O-glucoside 7-O-rhamnoside (MW 594)	Negative	$[M-H]^-$	593.1512	593.1520 (1.35)	180	9.1	(Stobiecki *et al.* 2006)
		Positive	$[M+Na]^+$	617.1482	617.1477 (–0.81)	200	24.9	(Kachlicki *et al.* 2008)
			$[M+H]^+$	595.1657	595.1663 (1.01)	200		(Stobiecki *et al.* 2006)
			$[M+H^+ - \text{glucose} - \text{rhamnose}]$	287.0550	287.0556 (2.09)	350	25.2	(Stobiecki *et al.* 2006)
4	Kaempferol 3-O-rhamnoside 7-O-rhamnoside (MW 578)	Negative	$[M-H]^-$	577.1563	577.1569 (1.04)	180	12.0	(Stobiecki *et al.* 2006)
		Positive	$[M+Na]^+$	601.1528	601.1534 (1.00)	200	28.7	(Kachlicki *et al.* 2008)
			$[M+H]^+$	579.1708	579.1716 (1.38)	200		(von Roepenack-Lahaye *et al.* 2004)
			$[M+H^+ - \text{rhamnose}]$	433.1129	433.1134 (1.15)	200		(Kachlicki *et al.* 2008)
			$[M+H^+ - \text{rhamnose} - \text{rhamnose}]$	287.0550	287.0549 (–0.35)	200		(Kachlicki *et al.* 2008)

The IWF sample was extracted with water, freeze dried, re-dissolved in water, and 5 or 10 μL aliquots were separated by HPLC ($A_{330\,nm}$) using a Zorbax eclipse XDB-C18 column followed by accurate ESI-TOF mass spectrometry in negative and positive ion modes (skimmer voltage – 60 V; fragmentor voltage – 180, 200 or 350 V).
RT, retention time.

Table 3. Effect of chronic O_3 on rosette diameter and dry weight of 4-week-old Ler-0, *fah1-7*, *tt4-1* and *tt4-1/fah1-7* genotypes

Genotype	O_3 (nL L^{-1})	Rosette diameter (cm)	Dry weight (g)
Ler-0	10	6.8 ± 0.3 [a]	0.073 ± 0.011 [a]
	125	5.0 ± 0.3 [b]	0.034 ± 0.011 [b]
fah1-7	10	6.8 ± 0.3 [a]	0.075 ± 0.011 [a]
	125	4.8 ± 0.3 [b]	0.032 ± 0.011 [b]
tt4-1	10	7.6 ± 0.4 [a]	0.091 ± 0.011 [a]
	125	5.0 ± 0.4 [b]	0.036 ± 0.011 [b]
tt4-1/fah1-7	10	7.2 ± 0.4 [a]	0.088 ± 0.012 [a]
	125	5.4 ± 0.4 [b]	0.044 ± 0.012 [b]

Plants were treated with 10 or 125 nL L^{-1} O_3 7 h d^{-1} for 12 d. Within genotypes, values followed by a different letter are significantly different, $P \leq 0.01$. Values are means ± SE ($n = 64$).

IWF extracts transiently increased, suggesting that AA transport into the apoplast increased in spinach in response to O_3 (Luwe & Heber 1995). In contrast, reduced AA in the leaf apoplast of broad bean (Vicia *faba* L.) declined from 93 to 81% of total AA following exposure to 56 nL L^{-1} O_3 for 3 d (Luwe & Heber 1995). In pumpkin (*Cucurbita pepo* L.) exposed to 150 nL L^{-1} O_3 for 5 d, IWF extracts showed an increase in total AA concentration, while only small variations in ascorbate concentrations in leaf tissue were found (Ranieri *et al*. 1996). Sunflower (*Helianthus annuus* L.) subjected to 150 nL L^{-1} O_3 for 4 d exhibited an increase in total ascorbate content, accompanied by a marked oxidation of reduced AA, leading to a decrease in redox state in both the intracellular and apoplast compartments (Ranieri *et al*. 2000).

In *Arabidopsis*, however, we show that protection against O_3 injury via direct scavenging by AA appears rather limited because reduced AA concentrations in the leaf apoplast were very low. Veljovic-Jovanovic *et al*. (2001) also found that reduced AA concentrations were low in the leaf apoplast of *Arabidopsis*, especially in the ascorbate-deficient, O_3-sensitive *vtc-1* mutant. Ascorbic acid oxidation in the apoplast can result from ascorbate oxidase and peroxidase activity there (Takahama & Oniki 1997; Pignocchi *et al*. 2003; Booker *et al*. 2012). In addition, AA can be oxidized via reduction of phenoxyl radicals formed in reactions with peroxidases in the apoplast (Takahama & Oniki 1997). For example, Padu *et al*. (2005) calculated that in birch (*Betula pendula* Roth) leaves, re-reduction of the peroxidase phenolic substrate coniferyl alcohol in the apoplast oxidized a significant portion of the reduced AA in control plants. In O_3-treated plants, direct O_3 scavenging by AA was predicted to occur, as was AA oxidation by peroxidases if H_2O_2 concentrations increased (Padu *et al*. 2005). Utilization of AA in enzymatic reactions involving phenolic substrates and H_2O_2 may thus constitute significant sinks for AA in the apoplast, particularly in plants exposed to O_3, contributing to low concentrations of reduced AA in the apoplast in some plants.

Challenges have arisen about the proposed efficacy of AA in the leaf apoplast of some plants exposed to O_3 (Luwe & Heber 1995; Kollist *et al*. 2000; Burkey & Eason 2002). While AA in the apoplast appears to be an important factor for protecting plants from O_3 injury (Moldau, Bichele & Hüve 1998; Conklin & Barth 2004), the generality of this mechanism among plant species is shown here and elsewhere to be limited (Luwe & Heber 1995). For example, differences in O_3 sensitivity among snap bean (*Phaseolus vulgaris* L.) lines were correlated with AA concentrations in IWF extracts of some cultivars, but not others (Burkey & Eason 2002; Burkey, Eason & Fiscus 2003). Two soybean lines exhibiting differential O_3 sensitivity both had low reduced AA concentrations in IWF extracts (Cheng *et al*. 2007). Reduced AA concentrations in IWF extracts from milkweed (*Asclepias exaltata* L.) were negatively correlated with foliar injury from ambient O_3 but not in cutleaf coneflower (*Rudbeckia laciniata* L.) and crown-beard (*Verbesina occidentalis* Walt.) wildflowers, which had very low reduced apoplastic AA concentrations (Burkey *et al*. 2006). We show here that AA in IWF extracts from *Arabidopsis* was almost entirely oxidized. Transport of reduced AA from the cell to the apoplast might be adequate for maintaining AA-coupled peroxidase reactions important for countering O_3 injury, especially as AA export is an early response to H_2O_2 perception in *Arabidopsis* cell cultures (Parsons & Fry 2010), but steady-state concentrations of apoplast AA in *Arabidopsis* were too low to contribute significantly in direct scavenging of O_3 based on FR calculations. Therefore, we conclude that in a number of plants, AA in the apoplast contributes little to the direct scavenging of O_3.

AA in leaf tissue extracts, in contrast with IWF fractions, was essentially all reduced. There was no significant effect of O_3 on AA concentrations in leaf tissue extracts, which has been observed in several other studies (Luwe *et al*. 1993; Burkey *et al*. 2003; Cheng *et al*. 2007). Glutathione concentrations, however, declined in some studies (Luwe *et al*. 1993; Mahalingam *et al*. 2006), suggesting that the AA-glutathione system was maintaining AA in predominately reduced form. At high O_3 concentrations, intracellular redox status became more oxidized, followed by a decline in total ascorbate, suggesting that metabolic processes had become compromised (Luwe & Heber 1995).

The results here contribute to an expanded view of leaf apoplast chemistry and plant response to O_3 stress. We report for the first time to our knowledge that sinapoyl and flavonol glycosides are located in the apoplast of *Arabidopsis* leaves (Figs 2 & 3). Hydroxycinnamic and flavonoid derivatives are located mainly in the vacuole, and in the case of HCAs, bound to cell walls (Wallace & Fry 1994; Grace 2005). Flavonoids are also found in the nucleus, cytoplasm, chloroplast, endoplasmic reticulum (ER), small vesicles and as exudates on plant surfaces (Zhao & Dixon 2010). The major flavonoid compounds in *Arabidopsis* are kaempferol derivatives while HCA conjugates mainly comprise soluble sinapate esters (Veit & Pauli 1999; Milkowski & Strack 2010). *Arabidopsis* and other members of the Brassicaceae produce sinapoyl malate in leaves while seeds

contain sinapoyl choline, with lesser amounts of sinapoyl glucose in leaves and seeds (Li *et al*. 1993; Ruegger *et al*. 1999). Flavonols and sinapate esters have important roles as UV protectants in *Arabidopsis* and other plants (Li *et al*. 1993; Landry *et al*. 1995). Phenolic compounds are important in biotic defence interactions and may function as antioxidants, thereby helping plants counter oxidative stress induced by various environmental factors (Grace 2005). However, most previous studies investigated these compounds in either whole tissue extracts or, in the case of insoluble phenolics, in extractive-free cell wall preparations. Here we showed that sinapoyl and flavonol glycosides were present in IWF extracts and that the concentration of sinapoyl malate increased with O_3.

Soluble, low molecular weight phenolics in the apoplast may be widespread in plants (Wallace & Fry 1994). For example, various HCAs have been found in the media of cell cultures (Wallace & Fry 1994). Chlorogenic acid (3-caffeoylquinic acid) was found in IWF extracts of tobacco (*Nicotiana tabacum* L.) leaves (Takahama 1998). The concentration of the flavone glycoside, apiin, increased twofold in IWF extracts from parsley (*Petroselinum crispum* L.) seedlings treated with 200 nL L^{-1} O_3, accompanied by extensive foliar injury (Eckey-Kaltenbach *et al*. 1993). In the O_3-tolerant Bel-B tobacco cultivar, the concentration of monocaffeoyl-putrescine was found to increase fourfold in IWF extracts following treatment with 150 nL L^{-1} O_3 (Langebartels *et al*. 1991). The radical scavenging capacity of the conjugated polyamine was ascribed primarily to the phenolic moiety of the molecule (Bors *et al*. 1989).

The O_3 treatment used in our study was sufficient to induce a number of changes in gene expression associated with phenolic compound biosynthesis (Table 1), as observed in previous gene expression studies with O_3-treated *Arabidopsis* (Ludwikow, Gallois & Sadowski 2004; D'Haese *et al*. 2006; Li *et al*. 2006; Tosti *et al*. 2006). In the pathway leading to sinapate ester biosynthesis (Fig. 1), for example, expression of *HCT*, *COMT-like* and *ALDH1A* increased after exposure to 125 nL L^{-1} O_3 after 3 h, while *CCoAOMT* genes were up-regulated after 2 d (Table 1). Importantly, expression of *UGT84A2*, which catalyzes the glucosylation of sinapate (Milkowski & Strack 2010) was up-regulated by O_3 after 2 d. However, regulation of sinapate ester biosynthesis is not well understood (Besseau *et al*. 2007), and glucosylation may be complicated by functional redundancy among *UGT84A* enzymes (Milkowski & Strack 2010).

In the flavonoid pathway branch, O_3 effects were less clear (Table 1). Expression of *CHS* was not significantly different with O_3, and *CHI* expression declined after 2 d. However, *FLS* expression was enhanced, as were a number of glucosyltranserases, thus suggesting that O_3 stimulated flavonol glycoside biosynthesis via transcriptionally regulated mechanisms.

In our study, the functional significance of sinapoyl and flavonol glycosides in O_3-plant responses was explored using a model of apoplastic ROS scavenging capacity and several null mutants for these compounds. In agreement with Chameides' (1989) assessment, our estimates of soluble phenolic compound concentrations in the apoplast and their reactivity with O_3 indicated that they would be ineffectual in scavenging significant amounts of O_3 before it reached the plasmalemma. The reaction rate coefficient between phenolic compounds and O_3 is two orders of magnitude lower than what is required to be effective at the concentrations observed. Similarly, $^1O_2$ and $O_2^{\cdot-}$ scavenging by sinapoyl and kaempferol glycosides in the apoplast would be minimal. Concentrations of sinapoyl malate increased in response to O_3, suggesting that defensive mechanisms towards ROS assault were stimulated but a protective role against O_3 injury was not immediately apparent. Biomass production and visible injury of null mutants *fah1-7*, *tt4-1* and *tt4-1/fah1-7* were no more or less sensitive to chronic O_3 than Ler-0 wild-type plants (Table 3). Although this was not a definitive test of apoplastic leaf chemistry changes as both intracellular and extracellular phenolic production was blocked, the lack of effect on O_3 sensitivity strongly suggests that a protective role against O_3 injury and biomass production was not a major factor.

In conclusion, it was found that AA in the leaf apoplast was essentially all oxidized, while AA in leaf cells was mainly reduced. Current models of apoplastic AA as the first line of defence against O_3 toxicity may involve less direct mechanisms than currently thought, at least in *Arabidopsis* and some other plant species. Soluble flavonol and sinapoyl glycosides were found for the first time in the leaf apoplast of *Arabidopsis*, but probably have little effectiveness in scavenging O_3. Null mutants lacking sinapoyl and flavonol glycosides were not more sensitive to chronic O_3 than wild types, suggesting that other pathways leading to O_3 damage predominate in these plants.

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