Journal of Experimental Botany, Vol. 57, No. 14, pp. 3933–3943, 2006
doi:10.1093/jxb/erl147 Advance Access publication 22 September, 2006

Journal of Experimental Botany

RESEARCH PAPER

Effect of ascorbate oxidase over-expression on ascorbate recycling gene expression in response to agents imposing oxidative stress

Vasileios Fotopoulos, Maite Sanmartin* and Angelos K. Kanellis†

Group of Biotechnology of Pharmaceutical Plants, Laboratory of Pharmacognosy, Department of Pharmaceutical Sciences, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece

Received 14 July 2006; Accepted 15 August 2006

Abstract

Ascorbate oxidase (AO) is a cell wall-localized enzyme that uses oxygen to catalyse the oxidation of ascorbate (AA) to the unstable radical monodehydroascorbate (MDHA) which rapidly disproportionates to yield dehydroascorbate (DHA) and AA, and thus contributes to the regulation of the AA redox state. Here, it is reported that *in vivo* lowering of the apoplast AA redox state, through increased AO expression in transgenic tobacco (*Nicotiana tabacum* L. cv. *Xanthi*), exerts no effects on the expression levels of genes involved in AA recycling under normal growth conditions, but plants display enhanced sensitivity to various oxidative stress-promoting agents. RNA blot analyses suggest that this response correlates with a general suppression of the plant's antioxidative metabolism as demonstrated by lower expression levels of AA recycling genes. Furthermore, studies using *Botrytis cinerea* reveal that transgenic plants exhibit increased sensitivity to fungal infection, although the response is not accompanied by a similar suppression of AA recycling gene expression. Our current findings, combined with previous studies which showed the contribution of AO in the regulation of AA redox state, suggest that the reduction in the AA redox state in the leaf apoplast of these transgenic plants results in shifts in their capacity to withstand oxidative stress imposed by agents imposing oxidative stress.

Key words: Apoplast, ascorbate oxidase, biotic stress, *Botrytis cinerea*, oxidative stress, transgenic plants.

Introduction

Ascorbate (AA) is the most abundant antioxidant in plants and serves as the major contributor to cell redox state (Smirnoff, 2000). Although most of the AA is localized in the cytoplasm, up to 10% of the AA content of the whole leaf is exported and localized in the apoplast, where it is found at millimolar concentrations (Noctor and Foyer, 1998). Apoplastic AA is thought to represent the first line of defence against potentially damaging external oxidants, and may play an important role in mediating response to stresses generating an enhanced oxidative burden (Barnes *et al*., 2002; Pignocchi and Foyer, 2003). In the apoplast, ascorbate oxidase (AO; a glycoprotein belonging to the family of blue copper oxidase enzymes) oxidizes AA to the unstable radical monodehydroascorbate (MDHA), which rapidly disproportionates to yield dehydroascorbate (DHA) and AA (Smirnoff, 2000). The MDHA radical can be recycled back to AA by the activity of NAD(P)-dependent monodehydroascorbate reductase (MDHAR). Despite MDHA regeneration systems, rapid MDHA disproportionation results in DHA production, that can be reduced back to AA through the so-called ascorbate–glutathione (AA–GSH) cycle, involving the co-ordinated action of DHA reductase (DHAR) and NADPH-dependent glutathione reductase (GR) (Smirnoff, 2000).

Ascorbate oxidase expression is modulated by complex transcriptional and translational controls (Esaka *et al*., 1992). The activity and expression of AO are closely correlated with cell expansion (Kato and Esaka, 2000), with transcript levels increased by growth promoters (e.g. auxin, Pignocchi *et al*., 2003; jasmonates, Sanmartin, 2002) and reduced by growth suppressors (e.g. salicylic acid; Sanmartin, 2002; Pignocchi *et al*., 2003). Moreover, AO has been shown to

* Present address: Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología CSIC, E-28049 Madrid, Spain.
† To whom correspondence should be addressed. E-mail: kanellis@pharm.auth.gr

catalyse the oxidative decarboxylation of auxin, which may have impacts on root development (Kerk and Feldman, 2000). These findings imply a role for AO in hormone-mediated cell wall loosening, but a causal relationship remains to be established.

Another interesting function of AO is the regulation of AA redox state. For example, Sanmartin *et al*. (2003) demonstrated that the over-expression of cucumber AO in tobacco resulted in no change in the total ascorbate content recovered in apoplast washing fluid, but reduced the redox state of ascorbate from 30% in wild-type leaves to below the threshold for detection in transgenic plants. Levels of AA and glutathione (GSH) in the symplast were not significantly affected by AO over-expression, but the redox state of ascorbate was reduced. Similar lowering of the apoplast AA redox state was reported by Pignocchi *et al*. (2003) following a parallel experimental programme resulting in the over-expression of cucumber AO in tobacco plants.

The apoplast may play an important role in mediating responses to abiotic stresses because the initial events most likely occur at the apoplasm–plasmalemma interface (Pignocchi and Foyer, 2003). Moreover, plants can sense shifts in the amount and redox state of AA and this may be important in the perception of potentially stressful situations as well as in the modulation of compensatory defence responses (Pastori and Foyer, 2002). In line with these suggestions, transgenic tobacco plants with a lower AA redox state appear to show increased sensitivity to ozone (Sanmartin *et al*., 2003; H Smith, CH Foyer, JD Barnes, unpublished results). Interestingly, alterations in plant responses to oxidative stress have also been mediated by the modification of AA redox state through the genetic manipulation of other enzymes involved in AA metabolism. For example, transgenic tobacco over-expressing human dehydroascorbate reductase (DHAR), show decreased DHA/AA ratio and enhanced salinity tolerance (Kwon *et al*., 2003).

In the present study, transgenic tobacco plants over-expressing cucumber AO were used to probe the effect of altered apoplast AA redox state on the capacity exhibited by plant tissue to detoxify the reactive oxidative burden created by a variety of ROS-inducing agents.

Materials and methods

Plant material and growth conditions

Seeds of wild-type tobacco (*Nicotiana tabacum* cv. *Xanthi*) and three transgenic lines over-expressing AO (R50, R60, and R68, selected on the basis of AO expression and single transgene copy number; Sanmartin *et al*., 2003) were sown in seed trays containing John Innes No. 2 potting compost. After sowing, the seeds were stratified for 2 d at 4 °C and then grown at 23/18 °C day/night in controlled environment chambers supplying a photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} at plant height with a 16 h photoperiod. Relative humidity was maintained at 65–75%. After 10 d, seedlings were transferred to individual pots containing the same compost. Fully expanded leaves from 8–10-week-old plants were used for all molecular and biochemical analyses. Ten-week-old plants were used to monitor susceptibility to *Botrytis cinerea* infection. Experiments were repeated at least three times using pooled samples comprising leaves from a minimum of three independent plants for each replicate, unless otherwise stated.

Carotenoid and chlorophyll content

Leaf pigments were extracted from 9 mm-diameter leaf discs in dimethyl sulphoxide as described by Richardson *et al*. (2002). Carotenoid and chlorophyll concentrations were determined using the equations described by Sims and Gamon (2002).

Abiotic stress assays

Assays probing the tolerance of wild-type and transgenic tobacco plants to oxidative stress essentially followed the methods of Aono *et al*. (1995) with slight modifications. Fully expanded leaves were briefly washed in deionized water, and 9 mm-diameter leaf discs floated (abaxial surface upwards) on 10 ml of a solution containing either 200 μM methylene blue (MB, singlet oxygen inducer; Khan and Kasha, 1979), 10 μM methyl viologen (MV, superoxide inducer; Anthony *et al*., 2005), or 50 mM H_2O_2. Leaf discs were then subjected to vacuum infiltration (−60 kPa for 30 s), and incubated at room temperature for 18 h under low light (25 μmol m^{-2} s^{-1}). 'Control' discs, punched from the same leaves, were floated on distilled water.

Following treatment, electrolyte leakage (a crude indication of membrane damage) from leaf discs treated with MV and H_2O_2 and the corresponding controls was measured using a conductivity meter (Consort, Turnhout, Belgium) following the methods of Vranova *et al*. (2002), and the degree of oxidative damage was expressed as the ratio of conductivity exhibited by treated samples to their corresponding control samples. Damage due to generation of singlet oxygen by MB was estimated using a thiobarbituric acid reacting substances (TBARS) assay. Measurement of TBARS, which are primarily lipid peroxidation products, resulting from the thiobarbituric acid (TBA) reaction was carried out as described by Minotti and Aust (1987), using an extinction coefficient of 155 mM^{-1} cm^{-1}. The degree of oxidative damage was expressed as the ratio of TBARS content of treated samples to the corresponding controls.

Histochemical detection of reactive oxygen species

The detection of H_2O_2 in tissues was performed according to Thordal-Christensen *et al*. (1997) with the following modifications. Detached leaves were incubated in 1 mg ml^{-1} 3,3′-diaminobenzidine (DAB)-HCl, pH 3.8 (Fluka, Buch, Switzerland) in the dark at room temperature for 8–10 h, then chlorophyll was removed by boiling in ethanol (96%, v/v) for 10 min. The assay was based on the instant polymerization of DAB (to form a reddish-brown complex which is stable in most solvents), as soon as it comes into contact with H_2O_2 in the presence of peroxidases. To determine the specificity of DAB staining, leaves were also stained in the presence of 200 units ml^{-1} catalase (bovine liver, Sigma-Aldrich, St Louis, MO, USA) in a control experiment. H_2O_2 deposits were quantified by scanning spots from leaf pictures and the number of pixels was quantified with the ImageJ 1.33 program (National Institutes of Health, USA; http://rsb.info.nih.gov/ij) as described by Romero-Puertas *et al*. (2004). The results were expressed on a projected area basis in terms of percentage spot area [(spot area/total projected leaf area)×100]. Four leaves from independent plants were used per genotype.

In situ localization of superoxide was performed using the nitroblue tetrazolium (NBT) staining method described by Romero-Puertas *et al*. (2004), with minor modifications. When NBT reacts with superoxide a dark-blue, insoluble formazan compound is produced (Flohe and Otting, 1984). Superoxide is believed to be the

major oxidant species responsible for reducing NBT to formazan (Maly *et al*., 1989). In brief, leaf halves were immersed in a 0.1% solution (w/v) of NBT in 50 mM K-phosphate buffer (pH 6.4), containing 10 mM Na-azide, incubated for 1 h in the dark at room temperature and illuminated until the appearance of dark spots, characteristic of blue formazan precipitates. Leaves were then cleared by immersing in boiling ethanol. Superoxide dismutase (10 units ml^{-1}) was added to the staining medium in a control experiment. Four leaves from independent plants were used per genotype.

Cloning of tobacco NtcAPX and NtGR

Isolation and cloning of partial-length cDNA fragments of genes encoding cAPX and GR in tobacco was carried-out in order to use these as templates for the subsequent construction of radiolabelled probes. Total RNA (5 μg) isolated from tobacco leaves was DNase I-treated (Promega GmbH, Mannheim, Germany) and used to synthesize single-stranded cDNA using the M-MuLV (New England Biolabs, Beverly, MA, USA) reverse transcriptase and oligodT primer [5′-ACTAGTCTCGAG$(T)_{19}$-3′] according to the manufacturer's instructions. On the basis of the published sequences, the following gene-specific primers were designed and used for amplification: cAPX (U15933), forward primer, 5′-CAAGGACATGGAGCAAACAAT-3′, reverse primer, 5′-ACTCTTCCTCCTATCGCAAGC-3′; GR (X76293), forward primer, 5′-TTGGGCAGTTGGAGATGTTAC-3′, reverse primer, 5′-GCCTTTATTGGAGCACATCAA-3′. PCR amplification was performed for 40 cycles (94 °C for 30 s, 62 °C for 90 s, and 72 °C for 2 min) followed by a final extension step of 10 min at 72 °C. PCR products (579 bp for cAPX, 549 bp for GR) were gel purified and cloned into pCR 2.1 (Invitrogen, Life Technologies, Madison, USA) plasmid vector. A Li-Cor Long Readir 4200 automated sequencer and a 'Sequitherm EXCELII' kit (Epicentre, Madison, WI, USA) were used to determine the nucleotide sequence of the resulted clones.

RNA blot analyses

Total RNA was isolated from tobacco leaves as described by Verwoerd *et al*. (1989). Total RNA (10 μg) was fractionated in formaldehyde denaturing agarose gels, transferred to nylon membranes (Nytran® 0.45, Schleicher and Schuell, GmbH, Dassel, Germany), stained with 0.04% methylene blue (to observe equality of loading) and hybridized with radiolabelled probes for tobacco cAPX, DHAR, GR, and PR1a cDNAs, and heterologous tomato MDHAR cDNA. Probe labelling was carried out with RadPrime DNA Labelling System (Invitrogen, Life Technologies, Madison, WI, USA) according to the manufacturer's instructions. Blot hybridization and membrane washing were performed as described by Church and Gilbert (1984). All experiments were performed at least twice, with similar results. For the construction of the probes, full-length tobacco cDNAs for DHAR and PR1a (accession nos. AY074787 and X12737, respectively) were kindly provided by Dr D Gallie (University of California, Riverside) and Professor N Panopoulos (University of Crete), respectively, while a tomato MDHAR EST (SGN-C130712) was kindly provided by Dr J Giovannoni (Boyce Thompson Institute for Plant Research, Cornell Campus, Ithaca). Tobacco cAPX and GR probes were constructed using the partial-length cDNA fragments obtained as described above.

Fungal inoculum, inoculation procedures, and measurement of infection

*Botrytis cinerea (*kindly provided by Professor K Tjavella-Klonari, Aristotle University of Thessaloniki), originally isolated from naturally-infected aubergine plants, was cultured on potato dextrose agar (PDA; Difco, Detroit, MI, USA) in the dark at 24 °C. A conidial suspension was obtained by washing potato dextrose agar slant-cultures with sterile water. Conidia were harvested from 10 d-old sporulating plates by washing with 10 ml of sterile water. The conidia were filtered through fine nylon mesh, washed twice by centrifugation (5 min at 200 *g*) and resuspended in sterile water supplemented with 5 mM glucose. Spore numbers were counted using a haemocytometer, with suspensions adjusted to the desired concentration.

For the detached leaf assay, fully expanded leaves were placed abaxial side up in 15 cm-diameter Petri dishes containing filter paper moistened with 5 ml of water. Half the leaf was inoculated with four 5 μl drops of *B. cinerea* conidial suspension (2×10^5 ml^{-1}), with four 5 μl drops of 5 mM glucose inoculated similarly on the opposing half. The dishes were covered with a transparent lid in order to maintain high humidity, and incubated at room temperature under ambient light conditions. Disease spread was evaluated 3 d post-inoculation (dpi) by measuring lesion development (i.e. lesion area) in a total of 12 leaves excised randomly from six individual plants per genotype.

A whole plant assay was performed by spraying plants with a conidial suspension (1×10^6 ml^{-1}). The inoculation solution was sprayed uniformly on all leaves. Mock inoculations were performed with 5 mM glucose. Following inoculation, plants were covered with transparent plastic bags to maintain high humidity. Plants were incubated at ambient light and temperature. The progress of fungal infection was followed for 10 d by viewing the development of necrosis in the infected leaves. Susceptibility to *Botrytis* was evaluated 3 d after inoculation by measuring the percentage of leaves showing necrotic lesions (six plants per genotype; plants used in the experiments had produced approximately 15 leaves). Further evaluation of disease susceptibility was performed by measurement of chlorophyll loss in infected leaves at 3 dpi as described above. Pathogen-associated declines in chlorophyll content were expressed relative to the chlorophyll content of healthy, control leaves. The whole plant infection assay was carried out twice with similar results. Tissue samples were obtained at 0, 1, 2, and 3 dpi, and stored at −80 °C prior to RNA blot analyses.

Detection of fungal structures by microscopy

Fluorescence staining of fungal structures was performed with 3,3′-dihexyloxacarbocyanin iodide (DiO_6) as described by Duckett and Read (1991). Inoculated leaves were detached and immersed for 2–3 min in freshly prepared stain containing 50 mg ml^{-1} DiO_6 in 100% ethanol. The stained leaves were blotted to remove surface liquid, placed on a microscope slide (adaxial surface uppermost), and viewed by epifluorescence (450–490 nm excitation filter and a 526 nm barrier filter) under a Zeiss epifluorescent microscope through a 10× objective lens. Fungal structures including conidia, hyphae, and conidiophores stained bright green/yellow, and the presence or absence of red fluorescence from chloroplasts in underlying healthy palisade mesophyll cells, provided an indication of the extent of necrosis.

Statistical analyses

Statistical analyses were performed using SPSS v.11 (SPSS Inc., Chicago, USA). Biochemical and physiological damage measurements were subject to ANOVA, and then significant differences between individual means determined using Tukey's pairwise comparison test at the 5% confidence level.

Results

Expression of genes involved in AA recycling under optimal growth conditions

The expression of genes involved in AA recycling was examined by RNA blot analysis using leaves of plants grown under optimal conditions (Fig. 1B). No differences

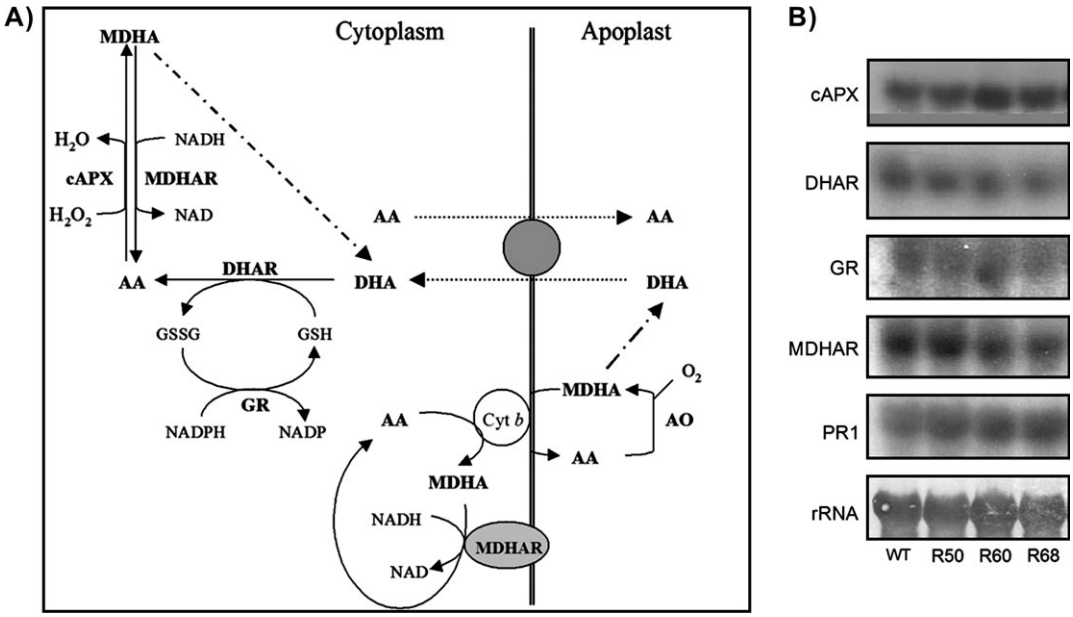

Fig. 1. Expression of genes involved in AA recycling under optimal growth conditions. (A) Schematic representation of the AA-GSH cycle responsible for AA recycling (modified from Smirnoff, 2000). (B) RNA blot expression analysis of AA recycling genes cAPX, DHAR, MDHAR, and GR performed with RNA from leaves of wild-type and AO over-expressing tobacco plants grown under optimal conditions. Expression of the defence-related marker gene PR1a is also included. Equal loading was checked by methylene blue staining (25s rRNA band is shown).

were found in mRNA levels of cAPX, MDHAR, GR, and DHAR between wild-type and transgenic plants, suggesting that cell wall-localized AO over-expression plays no constitutive role in mediating the expression of AA recycling genes. Expression of pathogenesis-related protein (PR) 1a expression (a commonly used marker gene in biotic stress studies that was found to be induced in the AA-deficient *Arabidopsis* mutant *vtc1*; Pastori *et al*., 2003) was also examined. Again, no marked differences in transcript abundance (Fig. 1B) were observed in transgenic plants.

Carotenoid and chlorophyll content in AO over-expressing plants

Carotenoids are low molecular weight compounds that act as non-enzymatic antioxidants primarily quenching singlet oxygen (Horemans *et al*., 2000) with the participation of AA as a substrate during their biosynthesis (Eskling *et al*., 1997), thus ultimately contributing to the plant's general antioxidant capacity. Figure 2 demonstrates that carotenoid and total chlorophyll (*a*+*b*) content is not altered in tobacco plants as a result of AO over-expression (at P=0.05), suggesting that AO over-expression in tobacco has no direct effect on the plant's antioxidant leaf pigment content under optimal growth conditions.

Response of wild-type and AO-overexpressing tobacco plants to agents imposing enhanced oxidative stress

Exogenous application of agents resulting in the enhanced production of ROS resulted in increased oxidative damage

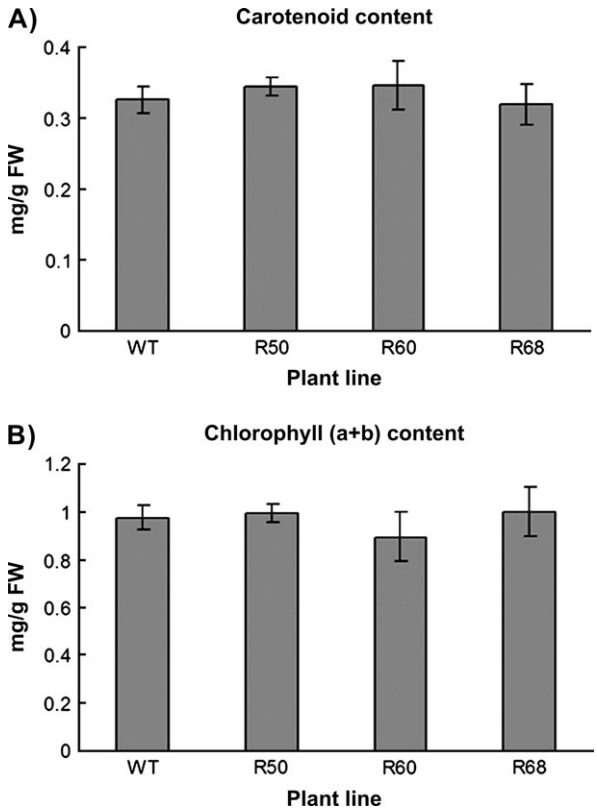

Fig. 2. Carotenoid and chlorophyll content under optimal growth conditions. Total carotenoid (A) and chlorophyll (B) content was determined from leaves of wild-type and transgenic plants grown under optimal growth conditions.

to the leaves of transgenic plants following treatment with MB and MV, while no differences in damage levels were observed following H_2O_2 treatment. More specifically, treatment with 200 μM MB and 10 μM MV for 18 h resulted in a significant (P <0.05) increase in oxidative damage in transgenic lines as indicated by lipid peroxidation (Fig. 3) and electrolyte leakage (Fig. 4A). Increased sensitivity to MV was supported by *in situ* studies determining localization production of superoxide, in which MV-treated leaf halves from transgenic plants stained with NBT for 1 h showed increased amounts of blue formazan precipitates compared with treated wild-type samples (Fig. 4B). Control, untreated samples and samples treated with superoxide dismutase showed no staining (data not shown).

Treatment with 50 mM H_2O_2 resulted in no statistical differences (at P=0.05) in oxidative damage between wild-type and transgenic plants as indicated by electrolyte leakage assays (Fig. 5A). The concentration (50 mM) used in these investigations was based on preliminary findings

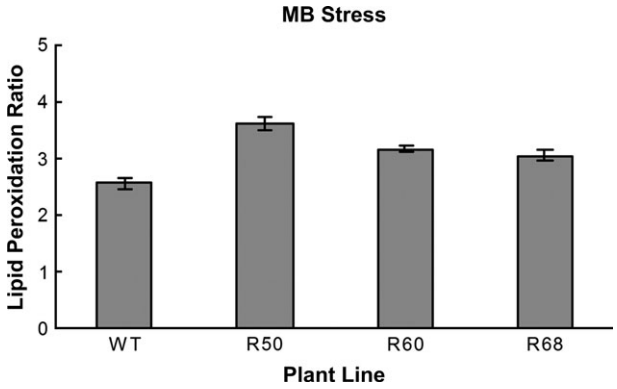

Fig. 3. Oxidative stress induced by singlet oxygen. Response to singlet oxygen was examined using leaf discs from wild-type and transgenic plants incubated with 200 μM MB for 18 h under low light, and measured by estimation of lipid peroxidation. The degree of oxidative damage was expressed as the ratio of TBARS contents of treated samples to their corresponding control samples.

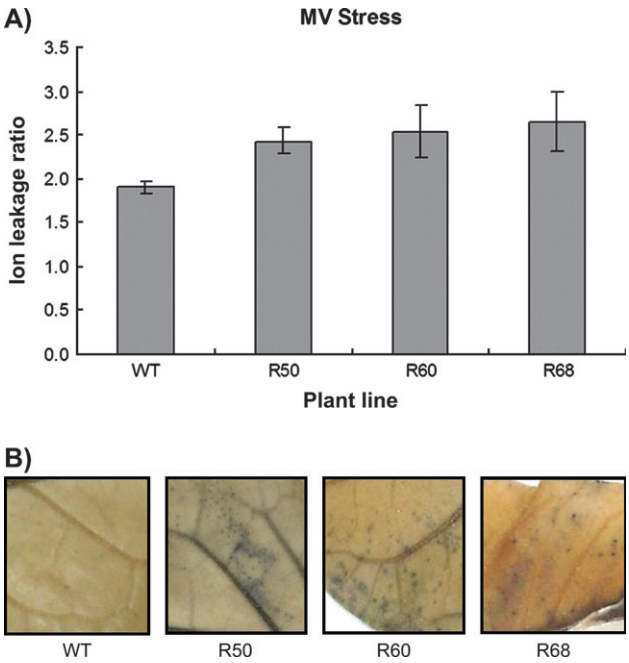

Fig. 4. Oxidative stress induced by superoxide. (A) Response to superoxide was examined using leaf discs from wild-type and transgenic plants incubated with 10 μM MV for 18 h under low light, and measured by estimation of electrolyte leakage. The degree of oxidative damage was expressed as the ratio of conductivity of treated samples to their corresponding control samples. (B) *In situ* localization of superoxide with NBT following MV treatment (superoxide is indicated by the blue spots).

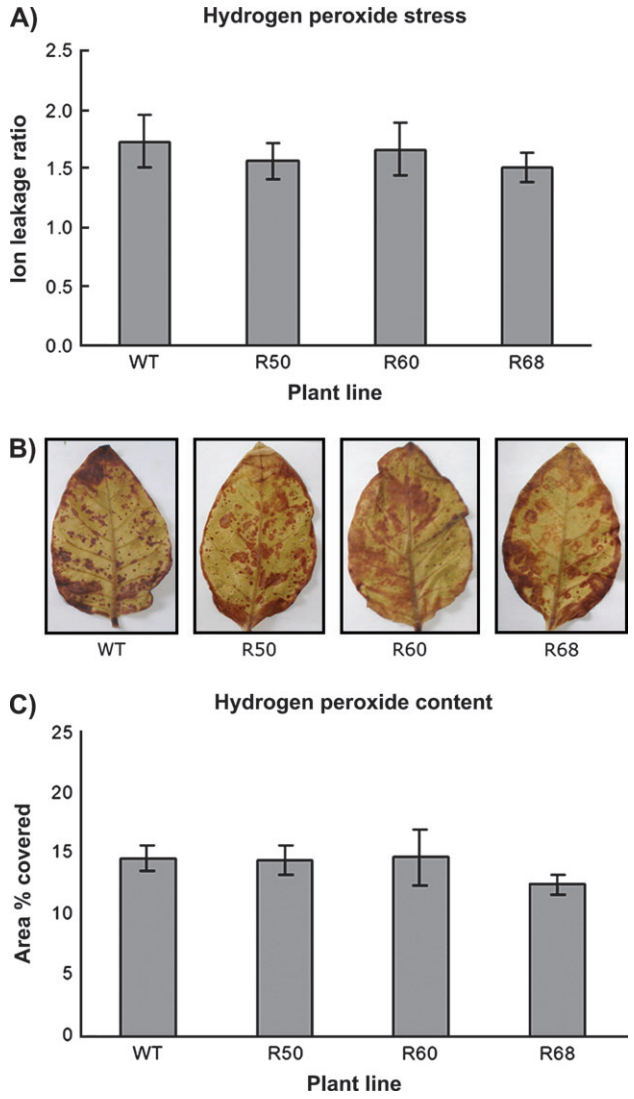

Fig. 5. Oxidative stress induced by hydrogen peroxide. (A) Response to superoxide was examined using leaf discs from wild-type and transgenic plants incubated with 50 mM H_2O_2 for 18 h under low light, and measured by estimation of electrolyte leakage. The degree of oxidative damage was expressed as the ratio of conductivity of treated samples to their corresponding control samples. (B) *In situ* localization of H_2O_2 with DAB following H_2O_2 treatment (reddish-brown coloration indicates the polymerization of DAB at the site of H_2O_2 production). (C) H_2O_2 deposits were quantified with ImageJ and results were expressed as percentage of spots area versus total leaf area.

using a H_2O_2 dilution series (0, 25, 50, 100 mM); 25 mM H_2O_2 did not enhance electrolyte leakage levels compared with control samples (non-damaging), while 100 mM resulted in even higher ion leakage than observed at 50 mM H_2O_2 (data not shown). Subsequent *in situ* localization of H_2O_2, in which H_2O_2-treated leaves were stained with DAB for 8–10 h, indicated comparable amounts of brown precipitation formation in wild-type and transgenic plants (Fig. 5B, C). Interestingly, control, untreated samples of transgenic plants showed enhanced staining compared with WT plants, suggesting that AO over-expression leads to increased leaf H_2O_2 content (V Fotopoulos, AK Kanellis, unpublished data).

Expression of genes involved in AA recycling following exposure to agents imposing enhanced oxidative stress

The increased sensitivity of AO over-expressing plants to singlet oxygen (MB) and superoxide (MV) was associated with the suppression of expression levels of genes involved in AA recycling (cAPX, DHAR, GR) compared with WT plants (Fig. 6). Control samples (incubated in H_2O for 18 h) and leaf discs exposed to H_2O_2 displayed no marked differences in expression levels. The heterologous MDHAR probe failed to produce a specific signal (data not shown).

Physiological responses of wild-type and transgenic tobacco plants to infection with Botrytis cinerea

Transgenic tobacco plants over-expressing AO showed increased susceptibility to infection with the necrotrophic fungus *B. cinerea*. Macroscopic observations of disease symptoms at 3 dpi show increased number and area of necrotic lesions in leaves of transgenic plants (Fig. 7A). Fluorescence microscopy on necrotic lesions indicated more pronounced hyphal growth in infected leaves of transgenic AO over-expressing plants (Fig. 7B), while *in situ* localization of H_2O_2 indicative of the oxidative burst following infection also appeared to be more pronounced in transgenic plants (Fig. 7C). Chlorophyll loss was also more pronounced in infected transgenic plants compared with wild-type plants (Fig. 8A), and the percentage of leaves showing disease symptoms (necrotic lesions) was significantly higher than wild-type plants at 3 dpi (P <0.05; Fig. 8B). Detached leaf assays in which leaves were inoculated with *Botrytis* conidial suspension droplets showed increased lesion development (i.e. size) in transgenic lines compared with wild-type plants, but differences were not statistically significant at P=0.05 (Fig. 8C).

Expression of genes involved in AA recycling following Botrytis cinerea *infection*

Increased susceptibility to *Botrytis* was accompanied by similar trends in cAPX and DHAR gene expression patterns between infected wild-type and transgenic plants (Fig. 9; representative transgenic line, R50, is shown), suggesting that alterations in AA redox state may not have a direct regulatory role in mediating plant response to biotic stress. All infected plants showed peaks in expression of cAPX and DHAR at 2 dpi (relative to their respective control, uninfected samples), which fell back to control levels at 3 dpi. PR1a expression appeared to be increased in wild-type samples at 3 dpi compared with that observed in the transgenic lines (Fig. 9).

Discussion

Plants can sense shifts in the amount and redox state of AA and this may be important in the perception of potentially stressful situations as well as in the modulation of compensatory defence responses (Pastori and Foyer, 2002).

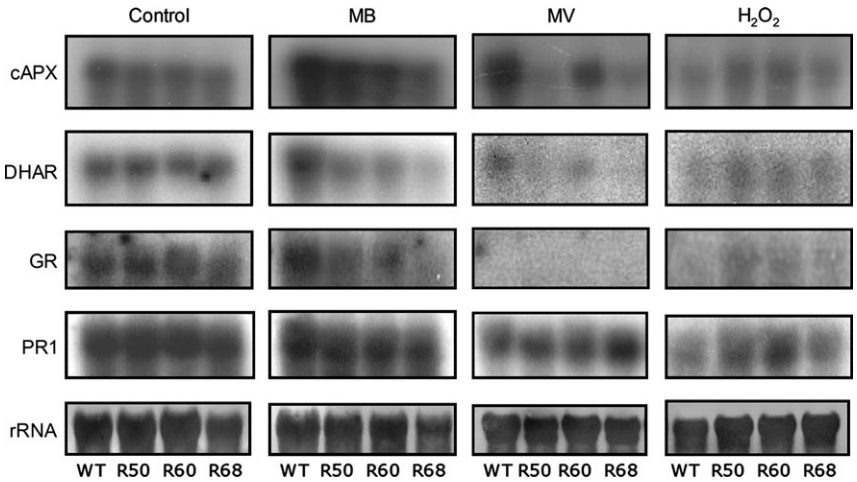

Fig. 6. Expression of genes involved in AA recycling following treatment with ROS-producing abiotic stress agents. RNA blot expression analysis of AA recycling genes cAPX, DHAR, and GR was carried out with RNA from leaves of wild-type and AO over-expressing tobacco plants treated with 200 μM MB (singlet oxygen), 10 μM MV (superoxide) or 50 mM H_2O_2 for 18 h under low light. Expression of the defence-related marker gene PR1a is also included. Equal loading was checked by methylene blue staining (25s rRNA bands are shown).

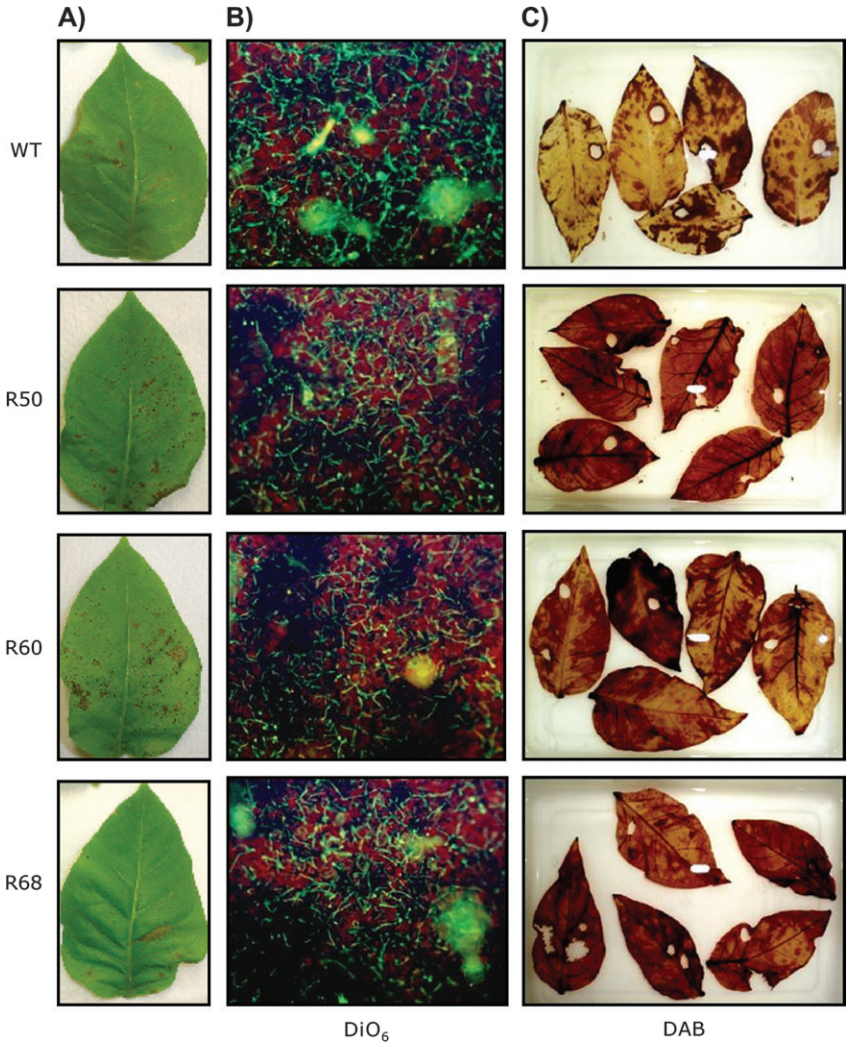

Fig. 7. Response of wild-type and transgenic AO over-expressing plants to infection with *Botrytis cinerea*. (A) Symptom development in infected leaves from wild-type and transgenic plants at 3 dpi. (B) Fluorescence microscopy of fungal structures in infected leaves from wild-type and transgenic plants at 3 dpi as detected by DiO_6 staining. Fungal structures including conidia and hyphae stain bright green/yellow (Magnification ×100). (C) *In situ* localization of H_2O_2 indicative of the oxidative burst at 3 dpi.

The results presented here show that over-expression of AO which results in a lower AA redox state in the cell walls of tobacco results in increased sensitivity to agents imposing an enhanced oxidative burden and this effect correlates with the suppressed expression of genes involved in AA recycling. Transgenic plants also show increased susceptibility to fungal infection, but this response is not accompanied by similar suppression of gene expression associated with AA recycling. These findings imply that reduction in extracellular AA redox state has an indirect effect on the plant's capacity to withstand the oxidative burden imposed by a variety of abiotic and biotic stresses.

No marked difference in the expression of ascorbate recycling genes was observed between wild-type and transgenic plants grown under optimal conditions, suggesting that AO over-expression results in no constitutive shift in AA-associated antioxidative metabolism. These results are consistent with the findings of Chen and Gallie (2005), where *in vivo* lowering of AA redox state by DHAR suppression in tobacco resulted in no differences in APX, catalase, and MDHAR activity under normal growth conditions, while Yamamoto *et al*. (2005) reported similar levels in APX activity between wild-type and AO over-expressing tobacco plants grown under normal conditions. Nevertheless, the potential involvement of other isozymes of ascorbate recycling enzymes should not be excluded, as several genes exist encoding for isozymes of different cellular localization that may contribute towards the regulation of redox metabolism (e.g. cytoplasmic, chloroplastic, and mitochondrial MDHAR; Conklin and Barth, 2004; Mittler *et al*., 2004).

Treatment with singlet oxygen and superoxide-inducing agents resulted in increased damage levels in AO-transgenic plants, being in accordance with the report of

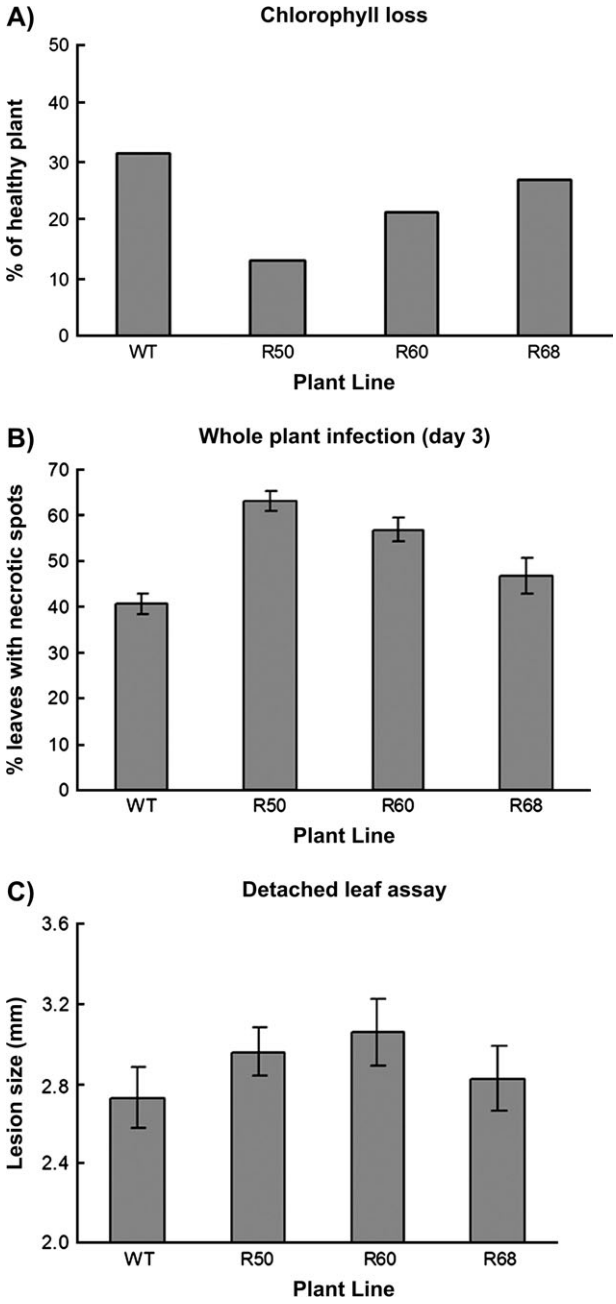

Fig. 8. Evaluation of disease progression in wild-type and transgenic AO over-expressing plants following infection with *Botrytis cinerea*. Susceptibility to *Botrytis* was evaluated at 3 dpi by measuring (A) chlorophyll loss expressed as percentage of chlorophyll content in infected leaves in comparison with healthy, control leaves, (B) the percentage of leaves showing necrotic lesions, and (C) lesion size in a detached leaf assay.

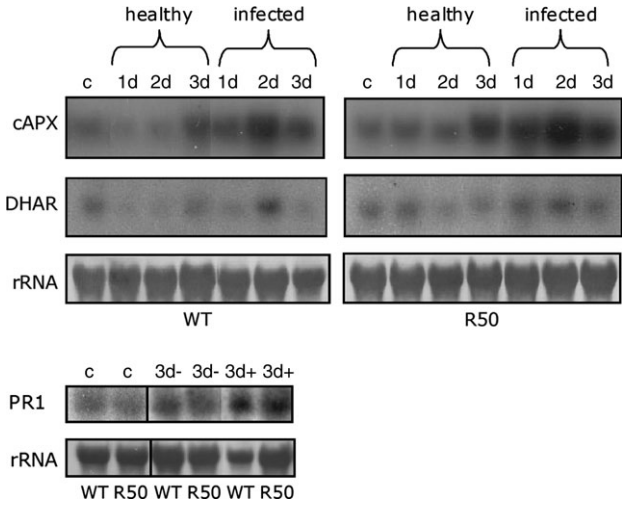

Fig. 9. Expression of genes involved in AA recycling following infection with *Botrytis cinerea*. RNA blot expression analysis of AA recycling genes cAPX and DHAR was carried out with RNA from infected leaves of wild-type and AO over-expressing tobacco plants during a time course of 3 dpi. 'C' indicates the control sample obtained at the start of the experiment (0 dpi). Expression of the defence-related marker gene PR1a at 0 dpi ('C') and 3 dpi is also included. Equal loading was checked by methylene blue staining (25s rRNA bands are shown).

Yamamoto *et al*. (2005) that transgenic tobacco plants over-expressing AO show increased sensitivity to MV, although these findings were based solely on the visual estimation of chlorophyll loss. Conversely, Kwon *et al*. (2003) report that MV treatment leads to reduced membrane damage (as indicated by electrolyte leakage analyses) in transgenic tobacco plants which have increased AA redox state as a result of DHAR over-expression. Interestingly, AO-transgenic tobacco plants showed similar damage levels to wild-type plants when treated with 50 mM H_2O_2. Studies carried out by Kwon *et al*. (2003) showed that transgenic tobacco plants with an increased AA redox state due to DHAR over-expression exhibit reduced levels of membrane damage relative to wild-type plants following treatment with much higher H_2O_2 concentrations. It is possible, therefore, that responses are concentration-specific. In the present study, treatment with such high concentrations was avoided so as to avoid confounding effects on the stability of transcripts in subsequent RNA blot analyses.

Sensitivity of transgenic plants to abiotic stress imposed by MB and MV application correlated with the suppressed expression of genes involved in AA recycling compared with wild-type plants (cAPX, DHAR, GR; Fig. 6), while treatment with H_2O_2 resulted in similar expression levels between wild-type and transgenic plants (Fig. 6). As far as is known, there have been no previous reports of the effects of *in vivo* AA redox state alteration on AA recycling-associated gene expression and responses to oxidative stress. In general, the response of wild-type plants was in agreement with previous studies; for example, treatment of spinach leaves with MV resulted in an increase in cAPX expression (Yoshimura *et al*., 2000), while GR transcript expression was lowered following H_2O_2 and MV treatment in *Arabidopsis* (Xiang and Oliver, 1998). The general trend observed suggests that AO-overexpressing transgenic plants exhibit a reduced capacity to up-regulate

defences against ROS (e.g. AA recycling), thus suggesting a potentially crucial role for apoplastic AA in mediating responses to combat oxidative stress.

The effect of biotic stress was also examined by infecting plants with the necrotrophic fungus *Botrytis cinerea.* As in the case of agents imposing oxidative stress, AO-transgenic plants demonstrated increased susceptibility to fungal infection compared with wild-type plants. Moreover, the oxidative burst typically associated with pathogen attack (Bolwell *et al*., 2002) appeared to be more pronounced in transgenic plants. This finding is in accordance with previous reports by Tiedemann (1997) and Govrin and Levine (2000), who suggested that *Botrytis* utilizes ROS as a virulence factor, triggering cell death and thus facilitating its colonization in plants. RNA blot analyses demonstrated similar trends in AA recycling gene expression between infected wild-type and transgenic plants, with cAPX and DHAR transcripts reaching maximum levels at 2 dpi and then falling to control levels. Kuzniak and Sklodowska (2005) demonstrated a similar increase in APX enzyme activity in tomato leaves infected with *B. cinerea* at 2 dpi, which dropped to control levels at 5 dpi. Presumably this reflects the capacity of the pathogen to overcome the protective antioxidant barrier eventually (Kuzniak and Sklodowska, 2005). PR1a expression (a common marker gene in biotic stress studies associated with the salicylic acid (SA)-dependent resistance pathway; Uknes *et al*., 1992) appeared to be increased in wild-type samples at 3 dpi compared with AO-transgenic plants (Fig. 9), suggesting that wild-type plants induce disease resistance pathways more efficiently and would be expected to control disease progression more efficiently.

Plants with a reduced capacity for the scavenging of ROS in the leaf apoplast (because of the oxidation of the apoplast AA pool in this compartment) exhibit enhanced sensitivity to ozone (Sanmartin *et al*., 2003). This may be due to the important forward-defensive role played by AA in scavenging ozone and/or its primary derivatives en route to the mesophyll plasmalemma (Plőchl *et al*., 2000; Moldau and Bichele, 2002) and/or the role extracellular AA is believed to play in defence gene regulation (CH Foyer, JD Barnes, personal communication). Consistent with the view that co-ordination of the ROS defence network in plants is complex (Karpinski *et al*., 1999; Rizhsky *et al*., 2003), the application of light stress to *Arabidopsis* results in the induction of cytosolic and not chloroplastic defence enzymes (Karpinski *et al*., 1999; Pnuelli *et al*., 2003), even though the ROS produced during the stress is believed to be generated in the chloroplasts and peroxisomes (Asada, 1999). A sensor for ROS would need to be located near the site of its generation to provide optimal sensitivity and specificity (Ledford and Niyogi, 2005), with the exception of H_2O_2 which is a relatively stable molecule that can travel freely across membranes, potentially moving systemically to signal stress and activate defences (Ledford and Niyogi, 2005). Indeed, this study's results are consistent with recent findings by Foyer and Barnes (personal communication) that transgenic tobacco plants over-expressing AO (Pignocchi *et al*., 2003) exhibit reduced *Nt*TPC1B (a Ca^{2+} channel) gene expression. Activation of voltage-dependent Ca^{2+} channels has been suggested to be involved in response to various signals, including abscisic acid (Ward *et al*., 1995), elicitors from pathogens (Kuchitsu *et al*., 1993) as well as H_2O_2 (Pei *et al*., 2000), while Kadota *et al*. (2005) showed that *Nt*NPC1A/B is required for ROS-induced regulation of expression of APX and glutathione peroxidase. These findings suggest that *Nt*TPC1B expression may contribute to the shifting patterns of gene expression observed in the present study in a different suite of AO-overexpressing transformants.

In conclusion, the data presented suggest that AA redox state in the leaf apoplast is regulated by AO and this is an important factor modulating the ROS tolerance of plant tissue, though the mechanisms require further investigation. The transgenic lines used in the present study, as well as recently-produced transgenic melon plants exhibiting suppressed AO levels (ongoing work in this laboratory), should continue to provide a useful tool with which to explore the role of AA in redox signalling.

Acknowledgements

We are grateful to JD Barnes (University of Newcastle) for editing and for his constructive comments on the manuscript. The work was supported in part by a grant awarded to AKK (GSRT-GR–UK joint program in Agricultural Biotechnology).

References

Anthony JR, Warczak KL, Donohue TJ. 2005. A transcriptional response to singlet oxygen, a toxic byproduct of photosynthesis. *Proceedings of the National Academy of Sciences, USA* **102,** 6502–6507.

Aono M, Saji H, Sakamoto A, Tanaka K, Kondo N. 1995. Paraquat tolerance of transgenic *Nicotiana tabacum* with enhanced activities of glutathione reductase and superoxide dismutase. *Plant Cell Physiology* **36,** 1687–1691.

Asada K. 1999. The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 601–639.

Barnes J, Zheng Y, Lyons T. 2002. Plant resistance to ozone: the role of ascorbate. In: Omasa K, Saji H, Youssefian S, Kondo N, eds. *Air pollution and plant biochemistry: prospects for phytomonitoring and phytoremediation.* Berlin, Heidelberg, New York: Springer, 235–252.

Bolwell GP, Bindschedler LV, Blee KA, Butt VS, Davies DR, Gardner SL, Gerrish C, Minibayeva F. 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany* **53,** 1367–1376.

Chen Z, Gallie DR. 2005. Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiology* **138,** 1673–1689.

Church GM, Gilbert W. 1984. Genomic sequencing. *Proceedings of the National Academy of Sciences, USA* **81,** 1991–1995.

Conklin PL, Barth C. 2004. Ascorbic acid, a familiar small molecular intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant, Cell and Environment* **27,** 959–970.

Dangl JL, Dietrich RA, Richberg MH. 1996. Death don't have no mercy: cell death programs in plant–microbe interactions. *The Plant Cell* **8,** 1793–1807.

Duckett JG, Read DJ. 1991. The use of the fluorescent dye, 3,3′-dihexyloxacarbocyanin iodide, for the selective staining of ascomycete fungi associated with liverwort rhizoids and ericoid mycorrhizal roots. *New Phytologist* **118,** 259–272.

Esaka M, Fujisawa K, Goto M, Kisu Y. 1992. Regulation of ascorbate oxidase expression in pumpkin by auxin and copper. *Plant Physiology* **100,** 231–237.

Eskling M, Arvidsson PO, Akerlund HE. 1997. The xanthophyll cycle, its regulation and components. *Physiologia Plantarum* **100,** 806–816.

Flohe L, Otting F. 1984. Superoxide dismutase assays. *Methods in Enzymology* **105,** 93–104.

Govrin EM, Levine A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea. Current Biology* **10,** 751–757.

Horemans N, Foyer CH, Potters G, Asard H. 2000. Ascorbate function and associated transport systems in plants. *Plant Physiology Biochemistry* **38,** 531−540.

Kadota Y, Furuichi T, Sano T, Kaya H, Gunji W, Murakami Y, Muto S, Hasezawa S, Kuchitsu K. 2005. Cell-cycle-dependent regulation of oxidative stress responses and Ca^{2+} permeable channels NtTPC1A/B in tobacco BY-2 cells. *Biochemical and Biophysical Research Communications* **336,** 1259–1267.

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G, Mullineaux P. 1999. Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis. Science* **284,** 654–657.

Kato N, Esaka M. 2000. Expansion of transgenic tobacco protoplasts expressing pumpkin ascorbate oxidase is more rapid than that of wild-type protoplasts. *Planta* **210,** 1018–1022.

Kerk NK, Feldman LJ. 2000. Auxin metabolism in the root apical meristem. *Plant Physiology* **122,** 925–932.

Khan AU, Kasha M. 1979. Direct spectroscopic observation of singlet oxygen emission at 1268 nm excited by sensitizing dyes of biological interest in liquid solution. *Proceedings of the National Academy of Sciences, USA* **76,** 6047–6049.

Kuchitsu K, Kikuyama M, Shibuya N. 1993. *N*-Acetylchitooligosaccharides, biotic elicitor for phytoalexin production, induce transient memebrane depolarization in suspension-cultured rice cells. *Protoplasma* **174,** 79–81.

Kuzniak E, Skłodowska M. 2005. Compartment-specific role of the ascorbate–glutathione cycle in the response of tomato leaf cells to *Botrytis cinerea* infection. *Journal of Experimental Botany* **56,** 921–933.

Kwon SY, Choi SM, Ahn YO, Lee HS, Lee HB, Park YM, Kwak SS. 2003. Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. *Journal of Plant Physiology* **160,** 347–353.

Ledford HK, Niyogi KK. 2005. Singlet oxygen and photo-oxidative stress management in plants and algae. *Plant, Cell and Environment* **28,** 1037–1045.

Maly FE, Nakamura M, Gauchat JF, Urwyler A, Walker G, Dahinden CA, Cross AR, Jones ATG, Weck AL. 1989. Superoxide-dependent nitroblue tetrazolium reduction and expression of cytochrome b_{245} components by human tonsillar lymphocytes and B cell lines. *Journal of Immunology* **142,** 1260–1267.

Minotti G, Aust D. 1987. The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and hydrogen peroxide. *Journal of Biological Chemistry* **262,** 1098–1104.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. *Trends in Plant Science* **9,** 490–498.

Moldau H, Bichele I. 2002. Plasmalemma protection by the apoplast as assessed from above-zero ozone concentrations in leaf intercellular air spaces. *Planta* **214,** 484–487.

Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* **49,** 249–279.

Pastori GM, Foyer CH. 2002. Common components, networks and pathways of cross tolerance to stress: the central role of 'redox' and ABA-mediated controls. *Plant Physiology* **129,** 460–468.

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH. 2003. Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. *The Plant Cell* **15,** 939–951.

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406,** 731–734.

Pignocchi C, Fletcher JM, Wilkinson JE, Barnes JD, Foyer CH. 2003. The function of ascorbate oxidase in tobacco. *Plant Physiology* **132,** 1631–1641.

Pignocchi C, Foyer CH. 2003. Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Current Opinion in Plant Biology* **6,** 379–389.

Plőchl M, Lyons T, Ollerenshaw J, Barnes J. 2000. Simulating ozone detoxification in the leaf apoplast through the direct reaction with ascorbate. *Planta* **210,** 454–467.

Pnuelli L, Liang H, Rozenberg M, Mittler R. 2003. Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (APX1)-deficient *Arabidopsis* plants. *The Plant Journal* **34,** 187–203.

Richardson AD, Duigan SP, Berlyn GP. 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytologist* **153,** 185–194.

Rizhsky L, Liang H, Miller R. 2003. The water–water cycle is essential for chloroplast protection in the absence of stress. *Journal of Biological Chemistry* **278,** 38921–38925.

Romero-Puertas MC, Rodriguez-Serrano M, Corpas FJ, Gómez M, Del Rio LA, Sandalio LM. 2004. Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant, Cell and Environment* **27,** 1122–1134.

Sanmartin M. 2002. Regulation of melon ascorbate oxidase gene expression and effect of its modification in transgenic tobacco and melon plants. PhD thesis, University of Valencia, Spain.

Sanmartin M, Drogoudi PA, Lyons T, Pateraki I, Barnes J, Kanellis AK. 2003. Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. *Planta* **216,** 918–928.

Sims DA, Gamon JA. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment* **81,** 337–354.

Smirnoff N. 2000. Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Current Opinion in Plant Biology* **3,** 229–235.

Thordal-Christensen H, Zhang Z, Wei YD, Collinge DB. 1997. Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in

papillae and hypersensitive response during the barley-powdery mildew interaction. *The Plant Journal* **11,** 1187–1194.

Tiedemann AV. 1997. Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea. Physiological and Molecular Plant Pathology* **50,** 151–166.

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J. 1992. Acquired resistance in *Arabidopsis. The Plant Cell* **4,** 645–656.

Verwoerd TC, Dekker BMM, Hoekema A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Research* **17,** 2362.

Vranova E, Atichartpongkul S, Villarroel R, Van Montagu M, Inzé D, Van Camp W. 2002. Comprehensive analysis of gene expression in *Nicotiana tabacum* leaves acclimated to oxidative stress. *Proceedings of the National Academy of Sciences, USA* **99,** 10870–10875.

Ward JM, Pei ZM, Schroeder JI. 1995. Roles of ion channels in initiation of signal transduction in higher plants. *The Plant Cell* **7,** 833–844.

Xiang C, Oliver DJ. 1998. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis. The Plant Cell* **10,** 1539–1550.

Yamamoto A, Bhuiyan MNH, Waditee R, Tanaka Y, Esaka M, Oba K, Jagendorf AT, Takabe T. 2005. Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and *Arabidopsis* plants. *Journal of Experimental Botany* **56,** 1785–1796.

Yoshimura K, Yabuta Y, Ishikawa T, Shigeoka S. 2000. Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. *Plant Physiology* **123,** 223–233.