Journal of Experimental Botany, Vol. 59, No. 4, pp. 729–737, 2008
doi:10.1093/jxb/erm359
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Altered stomatal dynamics in ascorbate oxidase over-expressing tobacco plants suggest a role for dehydroascorbate signalling

Vasileios Fotopoulos[1], Mario C. De Tullio[2], Jeremy Barnes[3] and Angelos K. Kanellis[1,*]

[1] *Group of Biotechnology of Pharmaceutical Plants, Laboratory of Pharmacognocy, Department of Pharmaceutical Sciences, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece*

[2] *Dipartimento di Biologia e Patologia Vegetale, Universita' di Bari, Via E. Orabona 4, I-70125 Bari, Italia*

[3] *Institute for Research on the Environment and Sustainability, Devonshire Bldg., Newcastle University, Newcastle upon Tyne NE1 7RU, UK*

Received 26 September 2007; Revised 11 December 2007; Accepted 12 December 2007

Abstract

Control of stomatal aperture is of paramount importance for plant adaptation to the surrounding environment. Here, we report on several parameters related to stomatal dynamics and performance in transgenic tobacco plants (*Nicotiana tabacum* L., cv. Xanthi) over-expressing cucumber ascorbate oxidase (AO), a cell wall-localized enzyme of uncertain biological function that oxidizes ascorbic acid (AA) to monodehydroascorbic acid which dismutates yielding AA and dehydroascorbic acid (DHA). In comparison to WT plants, leaves of AO over-expressing plants exhibited reduced stomatal conductance (due to partial stomatal closure), higher water content, and reduced rates of water loss on detachment. Transgenic plants also exhibited elevated levels of hydrogen peroxide and a decline in hydrogen peroxide-scavenging enzyme activity. Leaf ABA content was also higher in AO over-expressing plants. Treatment of epidermal strips with either 1 mM DHA or 100 μM hydrogen peroxide resulted in rapid stomatal closure in WT plants, but not in AO-over-expressing plants. This suggests that signal perception and/or transduction associated with stomatal closure is altered by AO over-expression. These data support a specific role for cell wall-localized AA in the perception of environmental cues, and suggest that DHA acts as a regulator of stomatal dynamics.

Key words: ABA, apoplast, ascorbic acid, ascorbate oxidase, dehydroascorbic acid (DHA), hydrogen peroxide, *Nicotiana tabacum* L., cv. Xanthi, stomata, transgenic plants, water stress.

Introduction

Like other living organisms, plants face a continuously changing environment. They must therefore be capable of perceiving and reacting (often rapidly, given their sessile nature) to changes in environmental conditions. This is achieved via a combination of sensors and receptors that have evolved to monitor the physical and chemical signals triggered by external cues, ultimately delivering a suite of responses at the molecular level aimed at maintaining cellular homeostasis and/or changing the developmental programme (Pastori and Foyer, 2002). Stomata must be particularly responsive to external cues, as the control of stomatal aperture is of central importance for plant survival. Consequently, mechanisms underlying stomatal movement and regulation have been much-studied (Hetherington and Woodward, 2003). The identification of the role of abscisic acid (ABA) in governing stomatal closure (Hiron and Wright, 1973) opened new perspectives to research, but the detailed sequence of events acting upstream and downstream of ABA synthesis remain a matter of conjecture. In the past few years, a role for ABA-dependent hydrogen peroxide and nitric oxide production in mediating stomatal closure has been

* To whom correspondence should be addressed. E-mail: kanellis@pharm.auth.gr

identified (Zhang *et al*., 2001; Bright *et al*., 2006). Moreover, tobacco plants with altered expression of dehydroascorbate (DHA) reductase, the enzyme catalysing conversion of DHA to the reduced form ascorbate (AA), have been reported to exhibit abnormal stomatal behaviour; transgenic plants with increased DHA reductase expression showing increased stomatal opening and reduced capacities to control water loss, whereas antisense plants with low DHA reductase expression exhibited partially closed stomata and improved control over transpiration (Chen and Gallie, 2004). Several lines of evidence suggest a crucial role for apoplast redox status in controlling plant response to environmental conditions (Pignocchi and Foyer, 2003; Noctor and Foyer, 2005). Due to the lack of NAD(P)H and glutathione, the redox buffering capacity of the apoplast is much weaker than inside the cell (Pignocchi *et al*., 2003). It has been hypothesized that the apoplast enzyme ascorbate oxidase (AO) could be a key regulator of extracellular redox status (Noctor and Foyer, 2005). Indeed, tobacco plants over-expressing AO are characterized by the complete oxidation of the cell wall-localized AA pool (Sanmartin *et al*., 2003) plus changes in the expression and activities of several AA-related enzymes (Fotopoulos *et al*., 2006). Therefore, AO over-expressing plants were used to probe the role of apoplastic AA in the regulation of stomatal dynamics. These data reveal altered stomatal behaviour in AO over-expressing plants, and suggest a putative role for apoplast DHA accumulation in the regulation of stomatal aperture.

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 a controlled environment chamber supplying a photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} at plant height as a 16 h photoperiod. Relative humidity was maintained at 65–75%. After 10 d, seedlings were transferred to individual pots containing 5 dm^3 of the same compost. Plants destined for stomatal conductance measurements were transferred to controlled environment chambers described in detail elsewhere (Sanmartin *et al*., 2003), in which the temperature ranged from a midday maximum of 28 °C to a night-time minimum of 17 °C, and plants were subject to a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (at the height of the leaves employed for measurements), in a 16 h photoperiod. RH was maintained at 65–70%. Plants were watered as required and supplied with fertilizer at 14 d intervals (Phostrogen 14N:10P:27K; Monsanto, High Wycombe, Bucks., UK). Fully-expanded leaves from 8–10-week-old plants were used for all analyses. Assays were repeated at least three times using pooled samples (each 'replicate' measurement comprising leaves from a minimum of three independent plants, unless otherwise stated). To avoid rhythmic effects on stomatal aperture, experiments were always started at the same time of day (12.00 noon).

Histochemical detection of reactive oxygen species

The detection of H_2O_2 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 U ml^{-1} catalase (bovine liver, Sigma-Aldrich, St Louis, MO, USA), resulting in clear, unstained samples. Deposits of H_2O_2 were quantified by pixel counts on scanned images using the ImageJ 1.33 program (National Institutes of Health, USA); as described by Romero-Puertas *et al*. (2004). Results were expressed on a projected area basis [(spot area/total projected leaf area)×100]. Four leaves from independent plants were used per genotype. Detection of H_2O_2 in guard cells was examined by loading freehand epidermal peels with DAB as described above, with minor modifications. To reduce variation, epidermal strips from control and AO over-expressing leaves were placed on a microscope slide loaded with 500 μl of 2 mg ml^{-1} DAB-HCl (pH 3.8) for 1 h. After rinsing with dH_2O, H_2O_2 deposits were quantified by measuring the pixel intensity within a defined area (encompassing a guard cell). A minimum of 20 guard cell pairs per genotype were measured. Representative background pixel intensity was obtained from the average of five measurements of similarly sized areas adjacent to the guard cells. The average background value was then subtracted from the average guard cell value, and the difference was presented as the mean pixel intensity per guard cell.

Enzyme assays

Tobacco leaves were ground in buffer (50 mM TRIS–HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, and 1 mM $MgCl_2$), then subject to 30 min centrifugation at 15 000 *g*. CAT (10^{-6} mol H_2O_2 reduced min^{-1} mg^{-1} protein), and APX (10^{-8} mol AA oxidized min^{-1} mg^{-1} protein) activity was determined as described by Aebi (1984) and de Pinto *et al*. (2000), respectively. For the APX assay, 2 mM AA was present in the extract buffer. Protein was estimated by the method of Bradford (1976), using BSA as a standard.

Leaf water content and weight loss following detachment

Leaves were collected and immediately weighed (FW), then rehydrated for 24 h at 4 °C in darkness to determine the turgid weight (TW). Oven-dry weight (DW) was recorded after drying leaf tissue to constant weight at 85 °C. Leaf relative water content (RWC) was determined as:

$$RWC = 100\times(FW - DW)/(TW - DW)$$

Fully-expanded leaves were detached from well-watered plants and immediately weighed (FW). Water loss was then recorded following the incubation of detached leaves under stable environmental conditions at room temperature for 60 min. Measurements were made on four comparable leaves from separate plants. Leaf water loss was plotted as the percentage loss in weight with respect to FW.

Chlorophyll leaching assay

Chlorophyll leaching assays were performed as described by Aharoni *et al*. (2004). In brief, fully-expanded leaves were detached

and rinsed with tap water, weighed, and placed in tubes containing 30 ml of 80% ethanol at room temperature (gently agitating in the dark). One microlitre was removed from each sample after 60 min and 120 min and the absorbance of samples recorded at 664 nm and 647 nm. Chlorophyll concentration was determined using the formulae described by Aharoni *et al*. (2004).

Stomatal measurements

Abaxial stomatal conductance was recorded on comparable fully-expanded leaves of 12 WT and 12 R60 transformants at regular intervals over the course of a diurnal cycle. All measurements were made with the same porometer, calibrated at frequent intervals during the day (Model AP4, Delta-T Devices, Ltd, Cambridge, UK). The experiment was repeated in its entirety with similar outcomes. Only one of the experimental data sets is presented, since baseline conductance varied at the measurement dates selected, due to differences in physiological age, growing conditions, and plant maturity between experiments.

Stomatal aperture was determined by introducing 200 µl of 40 µg ml^{-1} 3,3′-dihexyloxacarbocyanin iodide (DiO_6) to leaf discs mounted (abaxial side up) on glass slides for 5 min in the dark. The stained leaves were rinsed with dH_2O, blotted to remove surface liquid, and imaged using a Zeiss epifluorescent microscope fitted with a 450–490 nm excitation filter and a 526 nm barrier filter. The percentage of open stomata (defined as having a width >1 µm) was determined from records of at least 190 stomata observed on eight independent leaves per genotype. The width and length of 190 pores (i.e. stomatal aperture) were measured and this was used to calculate average stomatal aperture (width/length) (Chen and Gallie, 2004). The width and length was also used to calculate stomatal aperture area

$$\pi\times(\text{width}/2)\times(\text{length}/2)$$

which together with the percentage of stomata that remained open, was used to calculate the total stomatal pore area per unit projected leaf surface (Chen and Gallie, 2004).

$$(\text{average stomatal apenture area})\times$$
$$(\text{percentage of stomata that remained open})\times 100]$$

Induction of stomatal closure by DHA and H_2O_2 was investigated using epidermal strips according to Pei *et al*. (2000). Strips, taken from leaves of similar size and age, were incubated in Petri dishes containing 30 mM KCl, 10 mM MES–KOH pH 6.0. After 2 h equilibration at room temperature and low PPFD (25 µmol m^{-2} s^{-1}), strips were transferred to fresh Petri dishes containing buffer (controls), 1 mM DHA in buffer, or 100 µM H_2O_2 in buffer. 1 mM DHA was chosen after preliminary experiments, showing that it was the most effective concentration without visible signs of damage (5 mM or higher induced some brown spots). After incubating for 2 h, strips were placed onto microscope slides and observed under a light microscope. Opening (width) of at least 200 stomata per treatment were measured using a micrometric grid. To avoid any potential rhythmic effects, all sampling was conducted at midday.

Determination of leaf ABA content

ABA content was determined as described by Lovisolo *et al*. (2002) with minor modifications. In brief, 1 g of freeze-dried homogenized leaf tissue was extracted in 80% v/v methanol. Extracts were centrifuged twice at 4000 *g* for 20 min and the supernatant recovered. Methanol was removed under vaccuum and the aqueous residue partitioned three times against ethyl acetate at pH 3.0. Ethyl acetate of the combined organic fractions was removed under vaccuum. The residue was resuspended in TRIS-buffered saline (150 mM NaCl, 1 mM $MgCl_2$ and 50 mM TRIS, pH 7.8) and ABA detected using an immunological approach (Phytodetek kit supplied by Agdia, Elkhart, USA).

RNA blot analysis

Total RNA was isolated from tobacco leaves as described by Verwoerd *et al*. (1989). Total RNA (10 µg) was fractionated on 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 a ^{32}P-labelled probe for *Cistus creticus NCED* 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). Experiments were performed twice, with similar results. A partial-length cDNA fragment showing high sequence homology to known NCED genes that was originally isolated from a *C. creticus* trichome cDNA library (V Falara and AK Kanellis, unpublished data) was used for the construction of the probe.

Statistical analyses

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

Results

The effect of AO over-expression on stomatal conductance

Measurements made *in situ* on one of the AO over-expressing lines (R60) revealed a consistent and significant (P <0.001) reduction in conductance over the photoperiod in R60 compared with WT plants (Fig. 1).

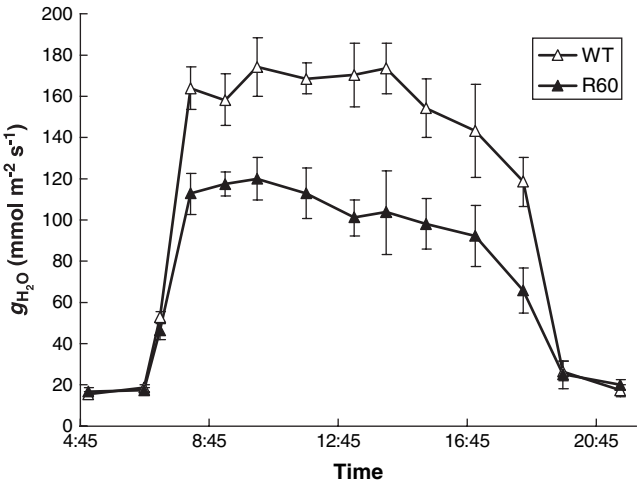

Fig. 1. Diurnal stomatal conductance (g_{H_2O}) profile for fully-expanded leaf of well-watered wild-type and AO over-expressing plants. Bars represent SE (n=12 during photoperiod; n=6 during night-time).

The adoption of a novel guard cell staining method that is rapid and requires no fixation steps, employing 3,3′-dihexyloxacarbocyanin iodide (DiO_6), a lipophilic cationic fluorescent dye previously used to stain mitochondria and the endoplasmic reticulum in living plant and animal cells, as well as fungal structures (Duckett and Read, 1991), revealed effects on stomatal aperture consistent with conductance data (Fig. 2A, B). Measurements revealed average stomatal aperture, average stomatal area μm^{-2} leaf and total open pore area μm^{-2} leaf to be significantly (P <0.05) lower in fully-expanded leaves of AO over-expressing plants compared with WT plants (Fig. 2C–E). AO over-expression resulted in no significant change in stomatal density per unit surface area (Table 1).

Effect of AO over-expression on leaf water management

Leaves of transgenic AO tobacco plants exhibited significantly reduced water loss following detachment, and this was reflected in the maintenance of leaf turgor compared with wild-type plants (Fig. 3A–C). This suggests that the decline in stomatal conductance associated with *in vivo* lowering of apoplast AA redox state by over-expression of AO may yield benefits in terms of improved leaf water management. Chlorophyll leaching assays showed no differences between wild-type and transgenic plants (Fig. 3D), suggesting that AO over-expression yielded no significant changes in cuticular resistance to water loss.

Effect of dehydroascorbate on stomatal closure

Previous studies have shown that, although the total (AA+DHA) content remains unchanged in the apoplast of the AO transformants, the AA/DHA ratio is strongly affected, as only the oxidized form (DHA) can be detected in the apoplast of transgenic plants (Sanmartin *et al.*, 2003). This prompted the investigatation as to whether the partial stomatal closure observed in AO over-expressing plants could be a consequence of increased DHA content. Stomatal aperture was measured in epidermal strips incubated either in buffer or in 1 mM DHA. As an additional control, epidermal strips were also incubated in 100 μM hydrogen peroxide. Data in Fig. 4 show that, at the end of the treatment, 100% of stomata in buffer-incubated strips from WT plants had opened, with a maximum width ranging from 5–10 μm (A). As expected, 100 μM H_2O_2 induced closure in ≈80% of stomata (C). Stomatal closure was also observed in DHA-incubated strips (E). Interestingly, stomata from AO over-expressing plants of the R50 transgenic line appeared insensitive to 100 μM H_2O_2 (D), and only a fraction of the stomata responded to 1 mM DHA (F) as compared to the WT. Higher H_2O_2 concentration (50 mM) induced complete stomatal closure in transgenic plants (data not shown).

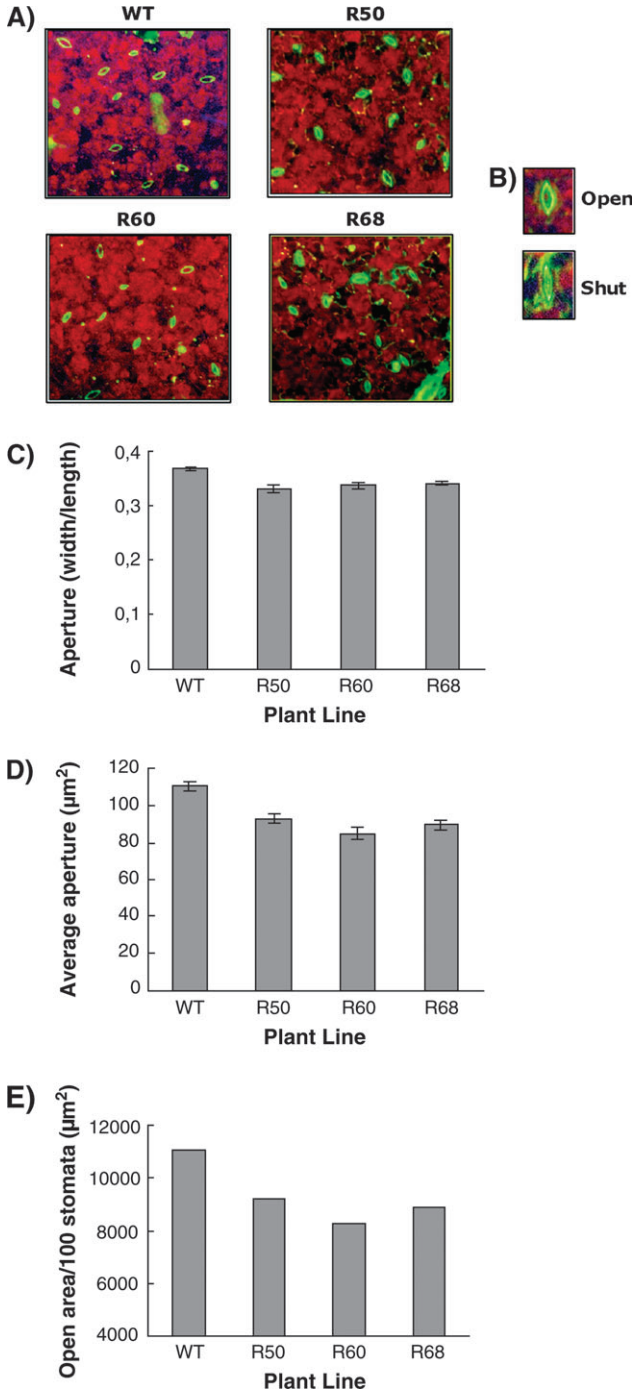

Fig. 2. DiO_6 stain of guard cells, and subsequent stomatal area measurements between WT and AO over-expressing tobacco plants grown under normal conditions. Stomatal bioassay experiments were performed as described in the Materials and methods. (A) Tobacco leaf sections were stained with DiO_6. Red pigment represents mesophyll chlorophyll autofluorescence (Magnification ×250). (B) Individual stomata under higher magnification represent open and shut stomata. (C) The width and length of at least 190 pores were measured and this was used to calculate average stomatal aperture (width/length). The width and length was also used to calculate average stomatal aperture area (D), which together with the percentage of stomata that remained open (defined as having a width >1 μm), was used to calculate the total stomatal open area and reported as the total area (μm^2) per 100 stomata. Bars represent SE (n=190–266).

Table 1. *Stomatal density in WT and transgenic AO tobacco expressed as number of stomata/ mm^{-2} (n=5)*

WT	R50	R60	R68
84±5.5	84±4.9	81±3.1	85±4.2

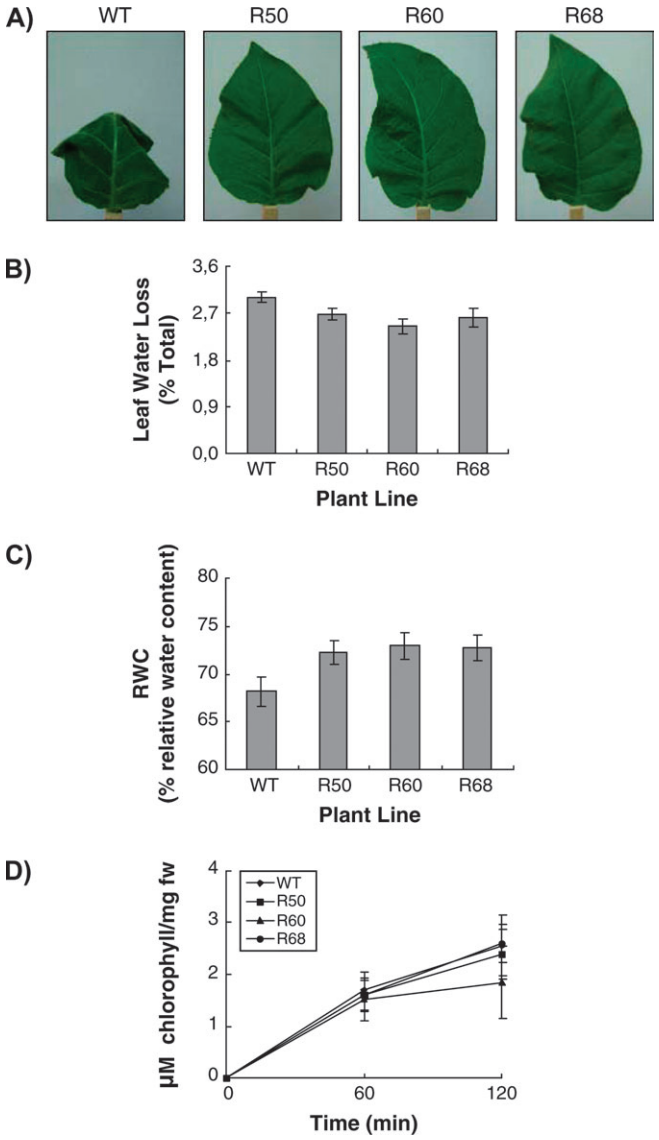

Fig. 3. Detached leaf assays. Fully-expanded leaves were detached from well-watered plants, immediately weighed, and held at room temperature for 1 h. (A) Images of representative leaves 1 h after detachment. (B) Water loss from detached leaves 1 h after detachment. (C) Relative water content of detached leaves 1 h after detachment. Bars and ± represent SE (n=5). (D) Chlorophyll leaching assays with leaves immersed in 80% ethanol for different time intervals. Bars represent SE (n=3). fw, fresh weight.

Histochemical localization of hydrogen peroxide in AO-overexpressing plants

To investigate hydrogen peroxide content and distribution in AO over-expressing plants further, whole leaves (Fig. 5A) and epidermal strips (Fig. 5C) of wild-type and AO over-expressing plants were incubated with DAB, in order to establish the *in vivo* effect of AO over-expression on H_2O_2 content. Quantification of the brown precipitate indicative of H_2O_2 showed significantly (P <0.05) enhanced H_2O_2 accumulation in both whole leaves (Fig. 5B) and guard cells (Fig. 5D). Much of the H_2O_2 present in guard cells was localized in chloroplasts, in agreement with previous reports (Zhang *et al.*, 2001). Leaves of the same transgenic plants have previously been treated with H_2O_2, and this resulted in equal DAB staining levels, suggestive of similar peroxidase activity between R lines and WT plants (Fotopoulos *et al.*, 2006).

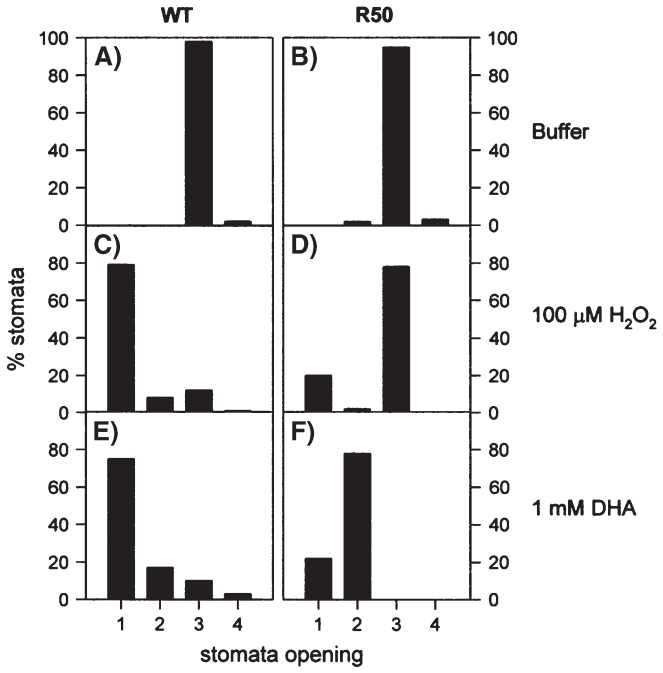

Fig. 4. Stomatal responses to DHA and H_2O_2. Epidermal strips from WT (A, C, E) and AO over-expressing plants (B, D, F) were incubated for 2 h in buffer and then transferred to new dishes containing buffer (A, B), 100 µM H_2O_2 (C, D) or 1 mM DHA (E, F) under low PPFD (25 µmol m^{-2} s^{-1}). Following treatment, strips were observed through a microscope, and stomatal aperture (width) recorded with a micrometer. The percentage of stomata (y axis) is plotted against their aperture (x axis): 1, closed stomata; 2, aperture in the range 1–5 µm; 3, aperture in the range 6–15 µm; 4, aperture >15 µm. At least 150 stomata were measured for each treatment.

Assay of H_2O_2-scavenging enzyme activity

CAT and APX activities were measured in leaves of WT and transgenic plants. A statistically significant (P <0.05) reduction in APX activity was recorded in AO over-expressing lines R60 and R68 compared with wild-type plants (Fig. 6). No differences were found in CAT activity between transgenic and wild-type plants. This suggests that the observed increase in H_2O_2 content could, at least in part, be the result of reduced detoxifying enzymatic capacity in AO over-expressing plants.

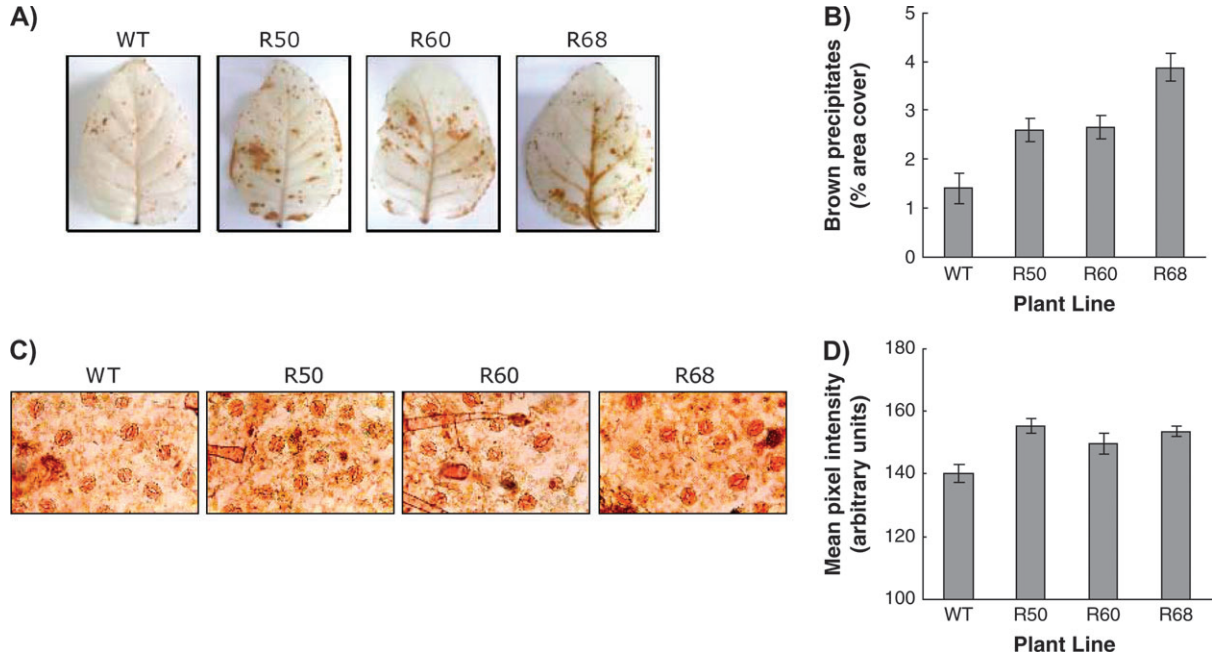

Fig. 5. *In situ* localization of H_2O_2. Whole leaves (A) and epidermal peels (C) were incubated with DAB. H_2O_2 was quantified using ImageJ and expressed as area % covered in whole leaves (B) and mean pixel intensity in guard cells (D). Bars represent SE (*n*b=4, *n*d=20).

Detection of foliar ABA levels and NCED gene expression

Leaves of transgenic plants displayed a 4-fold increase in ABA levels compared with wild-type plants, suggesting a constitutive shift in ABA metabolism as a result of AO over-expression (Fig. 7A). To determine whether the increased foliar ABA content in AO over-expressing plants may be the result of a constitutive increase in the expression of *NCED*, a gene encoding a 9-*cis*-epoxycarotenoid dioxygenase that catalyses the key step in ABA biosynthesis (Tan *et al*., 1997), RNA blot analysis was carried out using a heterologous *NCED* probe from *Cistus creticus* on RNA isolated from leaves of plants grown under normal conditions. However, both wild-type and transgenic AO plants exhibited similar *NCED* mRNA abundance levels (Fig. 7B).

Discussion

Results presented here show that the targeted over-expression of AO (Sanmartin *et al*., 2003) reduces stomatal aperture and, as a consequence, rates of leaf water loss are reduced upon detachment. This is consistent with previous measurements of stomatal conductance reported for additional AO over-expressing transformants as well as the data reported by Chen and Gallie (2004) for plants with reduced expression of DHA reductase (DHAR), which proved more resistant to water stress than WT plants. Both our AO over-expressing plants and Chen and Gallie's (2004) DHAR antisense plants exhibit higher apoplast DHA content than the WT. On the basis of these considerations, epidermal strips of WT tobacco plants were challenged with DHA, in order to understand whether this oxidized form of AA could be responsible for the observed stomatal closure. Notably, stomata of AO over-expressing plants were more closed than the WT in leaf disc assays (Fig. 2) compared with unchallenged epidermal strips (Fig. 4A, B). This apparent contradiction can be explained if one considers that buffer-incubated strips of both WT and over-expressing plants must have similar apoplast DHA content, due to dilution occurring in the free space. Our results clearly show that 2 h incubation in a buffered solution containing 1 mM DHA induces closure of approximately 80% of stomata in WT plants, implying that DHA is the active metabolite causing stomatal closure. This value is comparable to the effect of incubation (under the same conditions) in 100 μM hydrogen peroxide, a well-known inducer of stomatal closure (Bright *et al*., 2006). This observation opens the possibility that H_2O_2 may simply cause stomatal closure by oxidizing apoplastic ascorbate.

Many stresses (including pathogens, soil moisture deficit, salinity, and air pollutants) are known directly or indirectly to cause AA oxidation in the leaf apoplast. The extracellular matrix of epidermal cells and guard cells represents the first line of contact between the plant and its external environment, and there is growing evidence that the redox balance of this compartment plays a key role in regulating plant growth and development, plus perception and response to adverse conditions (Pignocchi and Foyer,

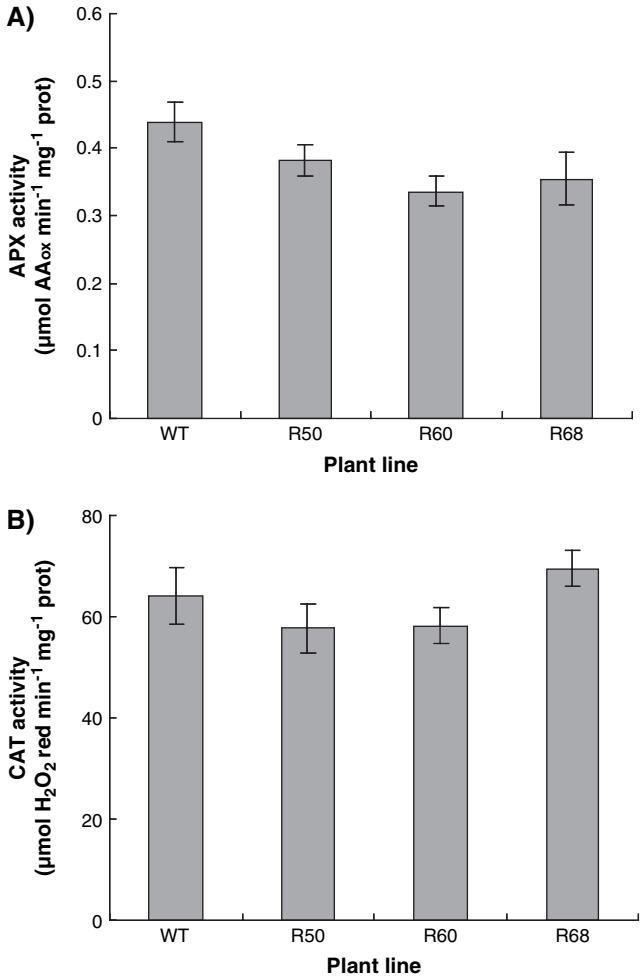

Fig. 6. H_2O_2 scavenging enzyme activity for (A) APX and (B) catalase in WT and AO-over-expressing tobacco plants grown under normal conditions. Bars represent SE (n_a=9, n_b=7).

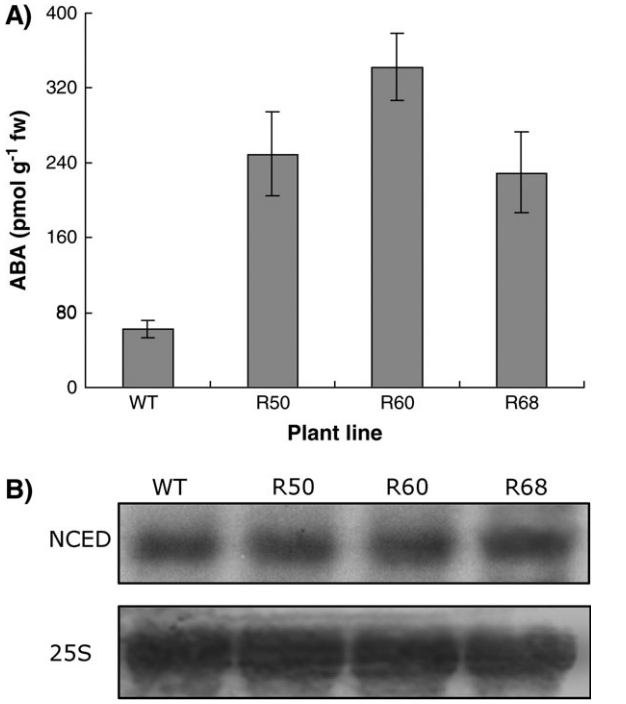

Fig. 7. ABA content and *NCED* mRNA expression. (A) ABA was measured in leaves from AO transgenic and wild-type tobacco plants grown under normal conditions. Bars represent SE (n=5);. fw, fresh weight. (B) Northern analysis of NCED gene expression (ABA biosynthesis). Heterologous ^{32}P probe constructed from a clone initially isolated from a trichome-specific cDNA library of *Cistus creticus* spp. *creticus*.

2003; Pignocchi *et al*., 2003, 2006). Our results lend support to this hypothesis, and also highlight DHA as a potential factor in the signalling pathway. The chemical structure of DHA, with its vicinal carbonyl group, gives the molecule its peculiar reactivity (Deutsch, 2000) with, amongst other compounds, thiols, whereby it induces oxidative protein folding (see for example, Banhegyi *et al*., 2003). Reversible modification of specific proteins by DHA could be important in cell signalling. Although possible DHA-regulated target proteins are still to be identified, it is conceivable that AA oxidation to DHA constitutes a vital signal transduction module governing plant reaction to stressful environmental conditions.

Under challenge by 100 μM H_2O_2, stomata of AO over-expressing plants are not as responsive as those of WT plants. This is quite surprising, since it is well-known that hydrogen peroxide is a powerful inducer of stomatal closure. Presumably this means that the signalling mechanism is altered in AO over-expressing plants. The observation also suggests that both DHA and H_2O_2 probably co-operate by reinforcing one another. *In vivo* localization of H_2O_2 indicated that AO over-expression in the apoplast resulted in H_2O_2 accumulation in both whole leaves and guard cells (Fig. 5). Similarly, Yamamoto *et al*. (2005) demonstrated increased H_2O_2 accumulation in the symplast and apoplast of transgenic tobacco plants over-expressing tobacco AO, while Chen and Gallie reported that *in vivo* lowering of the AA redox state, by manipulation of DHAR expression in the symplast, results in increased H_2O_2 content in guard cells and leaf apoplast (Chen and Gallie, 2004, 2005, respectively). APX activity was observed to be lower in transgenic plant lines R60 and R68 than in WT plants, grown under optimal conditions (Fig. 6A). Such a constitutive shift in ROS detoxifying enzyme capacity is consistent with the findings of Pignocchi *et al*. (2006), who reported that *in vivo* lowering of the AA redox state by AO over-expression resulted in a significant reduction in whole leaf APX activity in tobacco under non-stressed growth conditions. Moreover, these authors also report no differences in CAT activity, which supports our findings. This suggests that the observed increase in H_2O_2 content may, at least in part, be the result of reduced detoxifying enzymatic capacity. APX and CAT rapidly destroy the

majority of H_2O_2 produced by metabolism, but low steady-state levels are allowed to persist to maintain redox signalling pathways (Noctor and Foyer, 1998). Therefore, higher H_2O_2 content in AO over-expressing plants may reflect the lowered APX activity. H_2O_2 can also react directly with AA, yielding DHA. Such self-reinforcement mechanisms are typical of signalling cascades and developmental processes (Meinhardt, 1994). A correlation between AA degradation products and H_2O_2 generation was also recently suggested by Green and Fry (2005). The authors reported that the pathway for AA degradation is located in the apoplast, demonstrating that AA degradation can occur via DHA, yielding oxalate plus L-threonate. This pathway operates extracellularly, potentially generating H_2O_2 at several steps in the pathway.

ABA plays a key role in stomatal regulation, both under non-stressed growth conditions and during soil-water deficit (Raschke, 1987). Leaves of AO over-expressing plants exhibited substantially higher ABA content compared with WT plants (Fig. 7). Although no change could be observed in *NCED* gene expression this is not conclusive. Rossel and colleagues (2006) recently showed no correlation between increased ABA content of *alx8 Arabidopsis* mutants and *NCED3* expression, and there might be other *NCED* genes that are responsible for regulating the ABA content of plant tissues. Interestingly, López-Carbonell *et al*. (2006) recently observed a correlation between DHA content and ABA accumulation in foliage of the AA-deficient *vtc1* mutant of *Arabidopsis*. This mutant, defective in the activity of the AA-biosynthetic enzyme GDP-mannose pyrophosphorylase, is characterized by low AA content (approximately 30% of WT) and low AA/DHA ratio (Conklin *et al*., 1996). The *vtc1* mutant shows abnormal ABA accumulation, which correlates with higher DHA content observed in these plants. These data lend additional support to the hypothesis that DHA acts as an early activator of stomatal closure, and acts supposedly upstream of H_2O_2 production by NADH oxidase and ABA synthesis. ABA leads to stomatal closure via an increase in cytosolic Ca^{2+} concentration through H_2O_2-activated Ca^{2+} channels (Price *et al*., 1994; Grabov and Blatt, 1998; Pei *et al*., 2000; Zhang *et al*., 2001). Notably, AO over-expressing plants show increased expression of a plasma membrane Ca^{2+} channel-associated gene (Pignocchi *et al*., 2006), further supporting the view that imbalanced signalling occurs in the AO over-expressing transformants, leading to constitutive activation of mechanisms inducing stomata closure.

In conclusion, the data presented suggest that *in vivo* shifts in the apoplast AA redox state serve an important role in controlling stomatal aperture. The proposed model in which DHA elicits stomatal closure allows us to accommodate not only our data, but also other observations on stomatal movement (Chen and Gallie, 2004; Bright *et al*., 2006) within the same theoretical framework. A range of stressful conditions cause AA oxidation to DHA and, in turn, this molecule can modulate plant responses to stress in different ways, regulating ABA synthesis and increasing hydrogen peroxide production. The putative DHA-mediated mechanism of signal transduction is still unclear, but is probably related to DHA capability to cause reversible thiol disulphide exchanges in specific proteins, an aspect deserving of further detailed investigation.

Acknowledgements

We thank Dr A Patakas and Mr A Mpeis for their valuable help with ABA detection, Ms G Tanou for her assistance with enzyme activity assays, and Dr D D'Haese and A Kaminska (Newcastle University, UK) for their experimental contribution. This work was supported through grants awarded to AKK (GR-GSRT-Greek British Council Bilateral Agreement) and JB (NERC and UK-DEFRA).

References

Aebi H. 1984. Catalase *in vitro*. *Methods in Enzymology* **105,** 121–126.

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A. 2004. The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties confers drought tolerance when over-expressed in *Arabidopsis*. *The Plant Cell* **16,** 2463–2480.

Banhegyi G, Csala M, Szarka A, Varsanyi M, Benedetti A, Mandl J. 2003. Role of ascorbate in oxidative protein folding. *Biofactors* **17,** 37–46.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. *The Plant Journal* **45,** 113–122.

Chen Z, Gallie DR. 2004. The ascorbic acid redox state controls guard cell signaling and stomatal movement. *The Plant Cell* **16,** 1143–1162.

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, Williams EH, Last RL. 1996. Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. *Proceedings of the National Academy of Sciences, USA* **81,** 9970–9974.

de Pinto MC, Tommasi F, De Gara L. 2000. Enzymes of the ascorbate biosynthesis and ascorbate–glutathione cycle in cultured cells of tobacco Bright Yellow 2. *Plant Physiology and Biochemistry* **38,** 541–550.

Deutsch JC. 2000. Dehydroascorbic acid. *Journal of Chromatography A* **881,** 299–307.

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.

Fotopoulos V, Sanmartin M, Kanellis AK. 2006. Effect of ascorbate oxidase over-expression on ascorbate recycling gene expression in response to agents imposing oxidative stress. *Journal of Experimental Botany* **57,** 3933–3943.

Grabov A, Blatt MR. 1998. Membrane voltage initiates Ca^{2+} waves and potentiates Ca^{2+} increases with abscisic acid in stomatal guard cells. *Proceedings of the National Academy of Sciences, USA* **95,** 4778–4783.

Green MA, Fry SC. 2005. Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-*O*-oxalyl-L-threonate. *Nature* **433,** 83–87.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. *Nature* **424,** 901–908.

Hiron RWP, Wright STC. 1973. The role of endogenous abscisic acid in the response of plants to stress. *Journal of Experimental Botany* **24,** 769–781.

López-Carbonell M, Munné-Bosch S, Alegre L. 2006. The ascorbate-deficient *vtc-1 Arabidopsis* mutant shows altered ABA accumulation in leaves and chloroplasts. *Journal of Plant Growth Regulation* **25,** 137–144.

Lovisolo C, Hartung W, Schubert A. 2002. Whole-plant hydraulic conductance and root-to-shoot flow of abscisic acid are independently affected by water stress in grapevines. *Functional Plant Biology* **29,** 1349–1356.

Meinhardt H. 1994. Biological pattern formation: new observations provide support for theoretical predictions. *Bioessays* **16,** 627–632.

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.

Noctor G, Foyer CH. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell* **17,** 1866–1875.

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

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

Pignocchi C, Fletcher JM, Barnes JD, Foyer CH. 2003. The function of ascorbate oxidase (AO) in tobacco (*Nicotiana tabacum* L.). *Plant Physiology* **132,** 1631–1641.

Pignocchi C, Kiddle G, Hernández I, Asensi A, Taybi T, Barnes JD, Foyer CH. 2006. Apoplast redox state modulates gene transcription leading to modified hormone signalling and defence in tobacco. *Plant Physiology* **141,** 423–435.

Price AH, Taylor A, Ripley SJ, Griffiths A, Trewavas AJ, Knight MR. 1994. Oxidative signals in tobacco increase cytosolic calcium. *The Plant Cell* **6,** 1301–1310.

Raschke K. 1987. Action of abscisic acid on guard cells. In: Zeigler E, Farquhar GD, Cowan IR, eds. *Stomatal function*. Stanford, CA: Stanford University Press, 253–279.

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.

Rossel JB, Walter PB, Hendrickson L, Chow WS, Poole A, Mullineaux PM, Pogson BJ. 2006. A mutation affecting *ASCORBATE PEROXIDASE 2* gene expression reveals a link between responses to high light and drought tolerance. *Plant, Cell and Environment* **29,** 269–281.

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.

Tan BC, Schwartz SH, Zeevaart JA, McCarty DR. 1997. Genetic control of abscisic acid biosynthesis in maize. *Proceedings of the National Academy of Sciences, USA* **94,** 12235–12240.

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.

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

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.

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song CP. 2001. Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiology* **126,** 1438–1448.