The Function of Ascorbate Oxidase in Tobacco[1]

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The function of the apoplastic enzyme ascorbate oxidase (AO) was investigated in tobacco (*Nicotiana tabacum*). The abundance of AO mRNA was up-regulated by light. Cytosolic ascorbate peroxidase (APX1) transcripts were also highest in the light. In contrast, L-galactono-γ-lactone dehydrogenase, stromal APX, and thylakoid APX transcripts remained constant over the day/night cycle. Salicylic acid inhibited growth, increased expression of the pathogenesis-related protein (PR) 1a, and decreased AO transcript abundance. In contrast, the application of auxin enhanced growth and increased AO and PR 1a gene expression. Therefore, AO transcript abundance varied in a manner similar to hormone-mediated changes in plant growth. To study the effects of modified AO expression on growth, transformed tobacco plants expressing AO in the sense and antisense orientations were generated. The resultant large changes in apoplastic AO activity in the transformed tobacco plants had little effect on whole leaf ascorbate (AA) content, but they had dramatic effects on apoplastic AA levels. Enhanced AO activity oxidized the apoplastic AA pool, whereas decreased AO activity increased the amount of AA compared with dehydroascorbate. A relationship was observed between AO activity and plant height and biomass. Native AO transcript levels were no longer subject to light/dark regulation in AO sense and antisense plants. Taken together, these data show that there is an interaction between hormone, redox, and light signals at the level of the apoplast via modulation of ion of AA content.

Ascorbate (AA) plays a key role in defense against oxidative stress and is particularly abundant in photosynthetic tissues (Foyer et al., 1983; Smirnoff, 2000). Most (over 90%) of the AA is localized in the cytoplasm, but unlike other soluble antioxidants, a substantial proportion is exported to the apoplast, where it is present at millimolar concentrations. Apoplastic AA is believed to represent the first line of defense against potentially damaging external oxidants such as ozone, SO_2, and NO_2 (Plöchl et al., 2000; Barnes et al., 2002). In the apoplast, AA is oxidized to monodehydroascorbate (MDHA) by the enzyme ascorbate oxidase (AO). MDHA is an unstable radical and rapidly disproportionates to yield DHA and AA. DHA is then transported into the cytosol through the plasma membrane by a specific carrier that preferentially translocates the oxidized form in exchange for the reduced form, ensuring a continuous flux of reducing power to the cell wall (Horemans et al., 2000). Perhaps the most intriguing and poorly understood of the enzymes involved in AA metabolism in plants is the apoplastic AO. No clear biological functions for AO have been described to date. However, it is widely believed that AO plays a role in cell elongation because of its extracellular localization and its high activity in rapidly expanding tissues (Esaka et al., 1992; Moser and Kanellis, 1994; Ohkawa et al., 1994; Kato and Esaka, 1999). Recent work has shown that tobacco (*Nicotiana tabacum*) Bright Yellow-2 protoplasts overexpressing a pumpkin (*Cucurbita pepo*) AO cDNA elongate more rapidly than untransformed controls (Kato and Esaka, 2000). Several mechanisms whereby AO controls cell growth have been proposed (Esaka, 1998; Smirnoff, 2000). MDHA generated from AA by AO in the apoplast, stimulates cell growth through enhanced vacuolization (Hidalgo et al., 1989) and ion uptake caused by depolarization of the plasma membrane. Moreover, DHA is considered to be responsible for cell enlargement by promoting cell wall loosening (Lin and Varner, 1991). Local auxin-mediated production of free radicals has also been shown to induce cell wall extension (Joo et al., 2001). Because AA is believed to be the most important antioxidant in the apoplast of leaves and stems, its destruction via AO may be important in facilitating cell expansion.

Plant growth is triggered by auxin. Exogenous applications of auxin can trigger a variety of cellular processes such as cell elongation and lateral root growth (Laskowski et al., 1995). Auxin promotes cell enlargement via the enhancement of proton excretion into the cell wall (Cleland, 1987). The transduction process leading to activation of the associated ATPase proton pump localized in the plasma membrane may involve oxidant/antioxidant signals (Mills et al., 1980). The similarities and relationship between auxin and AO action in inducing cell growth are intriguing and merit further study.

Relatively little information is available on other enzymes involved in AA synthesis and metabolism. The last step of the pathway of AA synthesis in plants is relatively well characterized and involves the cytochrome c-dependent oxidation of L-galactono-1, 4-lactone by the mitochondrial enzyme L-galactono-γ-lactone dehydrogenase (GLDH; Ostergaard et al., 1997; Bartoli et al., 2000). Similarly, the AA peroxidase (APX) family that is responsible for the scavenging of H₂O₂ is well characterized. There are at least seven APX isoforms in plants, some of which are encoded by discrete genes with expression localized in different subcellular compartments: cytosol, mitochondria, peroxisomes, apoplast, and chloroplasts (Asada, 1997). Two isoforms are located in the cytosol (cAPX1 and cAPX2), with cAPX2 expression restricted to high photosynthetic photon flux densities in Arabidopsis leaves (Karpinski et al., 1999). In chloroplasts, APX is located in the stroma (sAPX) and is bound to the thylakoids (tAPX).

The present study was undertaken to elucidate the function of AO in tobacco leaves. We show that expression of AO is regulated by light and by plant hormones that modulate growth. Moreover, we demonstrate that enhanced apoplastic AO activities in transformed tobacco plants favor increased growth. Taken together, these observations provide evidence that AO has a role in the control of growth.

RESULTS

Diurnal Changes in Transcript Abundance of Enzymes Involved in the AA Metabolism

The expression of GLDH, sAPX, and tAPX were similar in the dark and in the light (Fig. 1), giving similar values after 16 h of dark or 24 h of light. In contrast, AO transcripts fell to very low levels in the dark and increased only slowly in the light (Fig. 1). The abundance of cAPX1 mRNA was also influenced by light, being minimum in the dark and attaining maximum values in the light (>6 h; Fig. 1). Actin was used as the internal control in these and all of the following experiments after confirmation that the abundance of actin transcripts was unaffected by any of the treatments used. This was achieved by performing actin-specific RT-PCR on RNA samples extracted from leaves subjected to a wide range of light-dark transitions and hormone treatments.

Further controls were performed to rule out the possibility that the observed light-dark changes in AO and cAPX1 transcript levels were due to circadian rhythmicity. No circadian control was found in AO or cAPX1 (data not shown).

Effects of SA and α-Naphthaleneacetic Acid (NAA) on AO Transcript Abundance and Plant Growth

Pathogenesis-related protein (PR) 1a was used as a positive control for the efficacy of the following treat-
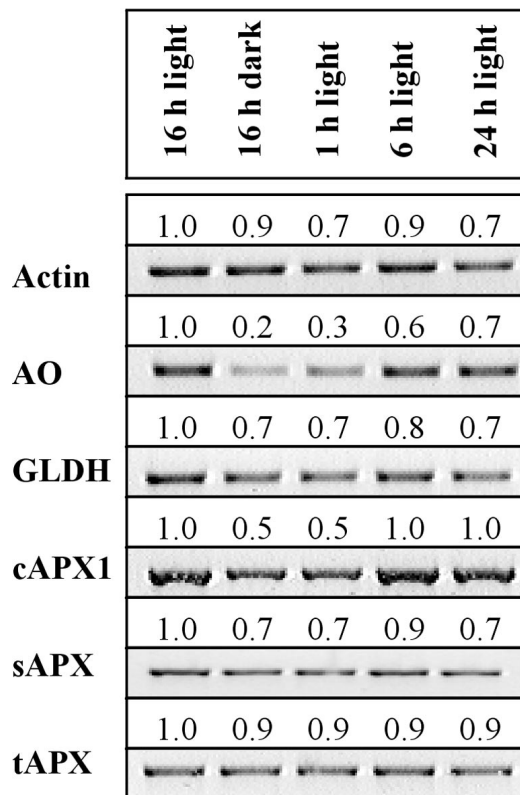

Figure 1. Light-dependent changes in transcripts of genes of AA metabolism. Leaf discs were harvested in plants exposed to 16 h of light or 16 h of dark and then, after 16 h dark, after 1, 6, or 24 h of light. Semiquantitative reverse transcriptase (RT)-PCR was performed on 3-week-old tobacco plants 1, 6, 16, and 24 h after transfer to light (250 μmol m⁻² s⁻¹) or 16 h of dark. Actin was used as the internal control. The relative intensity of each band is expressed as a density number, which can be used as a indication of the relative abundance of transcripts. Values are normalized to the 16-h value. This experiment was repeated three times and similar results were obtained.

ments as its expression has previously been shown to be triggered by SA (Durner et al., 1998) and by auxin (Ohashi and Matsuoka, 1987) in tobacco leaves. Application of 10 μM SA led to transient expression of PR1a, with maximum induction occurring between 1 and 8 h after spraying. The abundance of GLDH, AO, sAPX, tAPX, or cAPX1 transcripts was unaffected by the application of 10 μM SA (Fig. 2A). On the other hand, application of 1 mM SA resulted in a sustained increase in PR1a expression. Although AO transcripts rapidly decreased after application of 1 mM SA, the abundance of GLDH, sAPX, tAPX, or cAPX1 mRNA was unaffected (Fig. 2A).

A marked increase in AO and PR1a expression was observed 8 h after spraying with the auxin, NAA. In contrast, the expression of GLDH, sAPX, tAPX, or cAPX1 mRNA was unchanged by this treatment (Fig. 3A). SA and NAA had inverse effects on plant growth (Figs. 2B and 3B). The growth of plants sprayed with 1 mM SA was much slower than that of
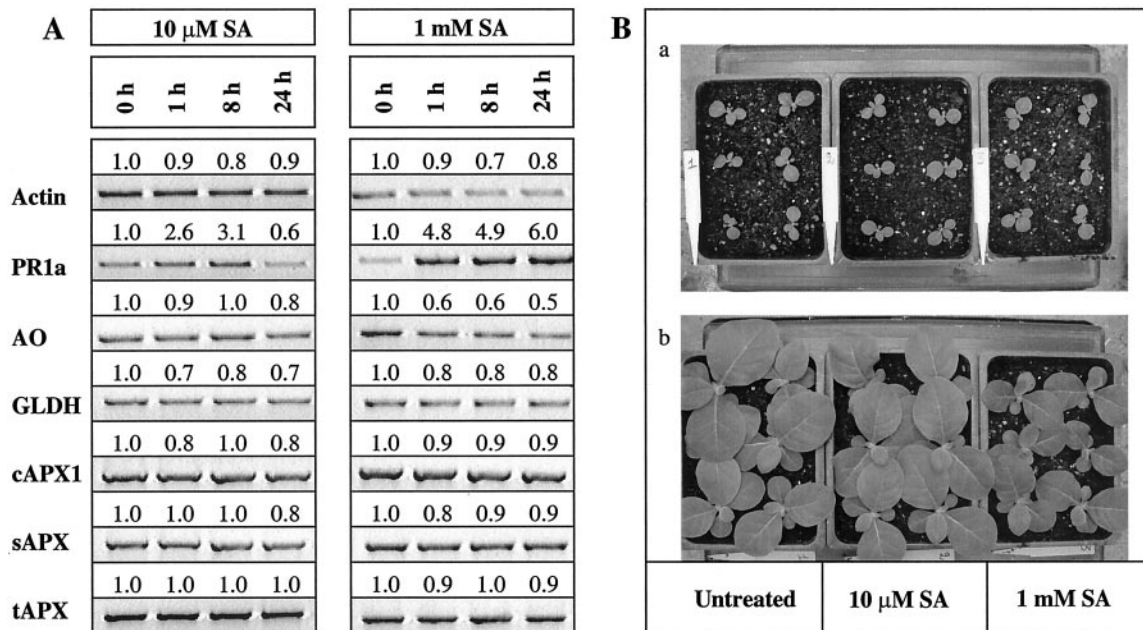
Figure 2. Effects of SA on transcripts abundance and plant growth. A, Transcript abundance; whole shoot samples were harvested 1, 8, and 24 h after spraying, which was performed halfway through the light period. Semiquantitative RT-PCR was performed on 3-week-old tobacco plants sprayed with 10 μM or 1 mM SA and then exposed to an extended photoperiod (24 h at 250 μmol m^{-2} s^{-1}). Actin was used as the internal control. B, Plant growth; phenotype of SA-treated and -untreated plants showing seedlings before treatment (a) and 1 week after the treatment (b). This experiment was repeated three times and similar results were obtained.

control plants sprayed with low SA concentrations (10 μM; Fig. 2B). In contrast, low concentrations (0.5 μM) of NAA stimulated growth (Fig. 3B).

Transgenic Tobacco Overexpressing AO in Sense and Antisense Orientations

To further explore the function of AO in tobacco leaves, two constructs for the expression of AO in the sense and antisense orientations were produced. Six independent primary transformants (T_0 generation) were selected for each construct on the basis of their ability to survive growth on kanamycin, the presence of the transgene, and their leaf AO activity. Leaf AO activity was markedly different in all the transformants compared with the wild type. AO activity was five to 16 times that of *PAO* leaves and 0.3 to 0.5 times that of the *TAO* leaves than the wild type (Fig. 4). Transgene-dependent differences in leaf AO were similar whether plants were grown in tissue culture or in soil (data not shown). Genomic DNA from each of the lines was probed with 2X35S-PAO or 2X35S-TAO DNA fragments to confirm the presence of the transgene and check the copy number of the insertion. All lines tested positive for the transgene (data not shown). Three sense (*PAO*2-2-1, *PAO*1-7-2, *PAO*3-7-2) and two antisense (*TAO*2-7-1, *TAO*1-6-1) lines containing only one copy of the introduced sequence were selected for further analysis. These lines were grown for two additional

generations (T_1 and T_2) and the relationship between AO and plant development was followed in each generation.

The *AO* Over- and Underexpression Phenotypes

Plant growth, measured as biomass accumulation over time, was monitored in T_1 and T_2 populations derived from each selected transgenic line. Each population was composed of 25 individuals germinated on nonselective media, allowing simultaneous measurements of individuals that had passed through the transformation procedure but did not express the transgene and AO-expressing plants. All plants were grown under controlled environment conditions at the same time and shoot fresh weights were recorded weekly from three plants per population. Additional wild-type populations were not necessary in these experiments, given the presence of internal controls within each population. No statistically significant differences in shoot fresh weight were observed in 3-, 4-, or 5-week-old sense (*PAO*) or antisense (*TAO*) plants (Fig. 5A). However, between weeks 6 and 8, plants expressing the AO transgene in the sense orientation (*PAO*) accumulated greater biomass than plants expressing the AO transgene in the antisense orientation (*TAO*; Fig. 5A). By the end of the experiment (8 weeks), AO sense-expressing plants (*PAO*) showed a statistically significant increase in fresh weight ($P < 0.05$ at 7 weeks, and $P < 0.01$ at 8 weeks) compared
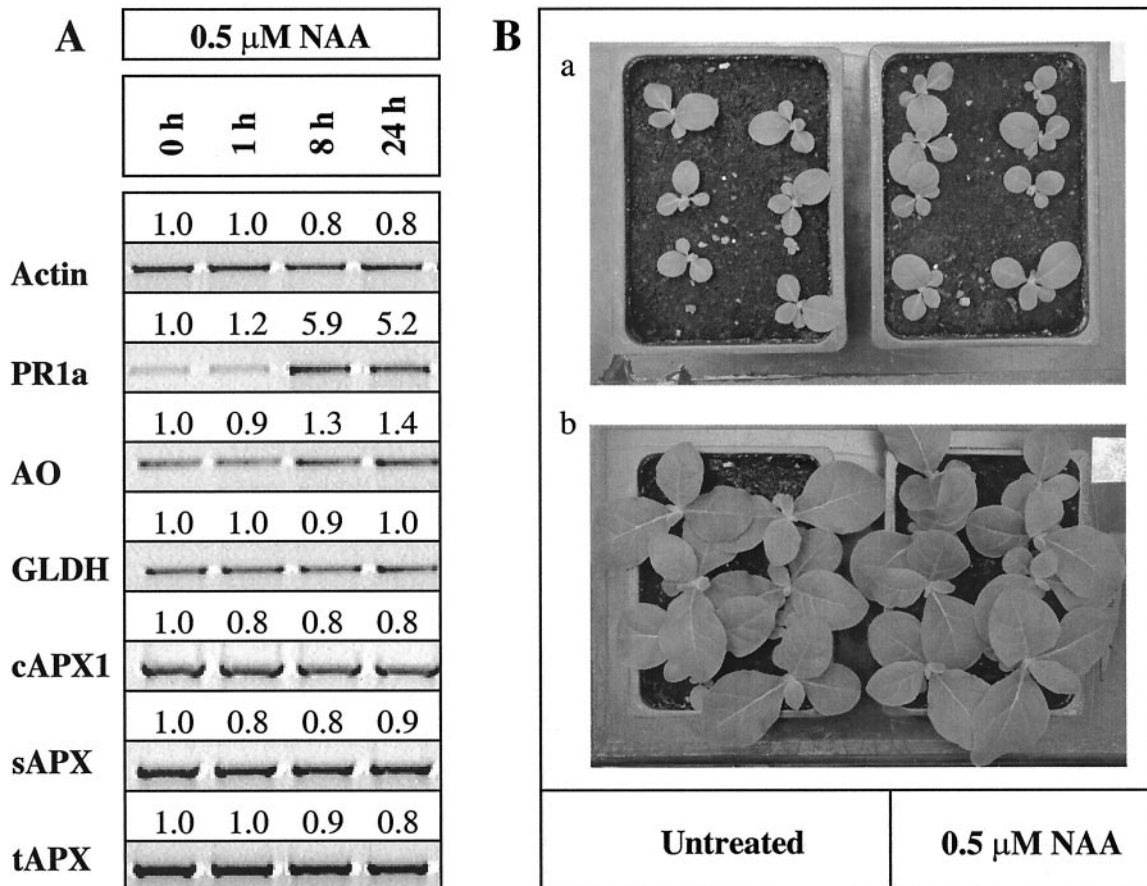
A | 0.5 μM NAA

Figure 3. Effects of auxin on transcript abundance and plant growth. A, Transcript abundance; whole shoot samples were harvested 1, 8, and 24 h after spraying, which was performed halfway through the light period. Semiquantitative RT-PCR was performed on 3-week-old tobacco plants sprayed with 0.5 μM NAA and then exposed to an extended photoperiod (24 h at 250 μmol m^{-2} s^{-1}). B, Plant growth; phenotype of NAA-treated and -untreated plants showing seedlings before treatment (a) and 1 week after the treatment (b). This experiment was repeated three times and similar results were obtained.

with the AO antisense plants (*TAO*; Fig. 5A). The relative growth (measured as weekly biomass accumulation) rate was linear ($r^2 > 0.95$) between weeks 6 and 8. At this point, the growth rate of AO sense plants (*PAO*) was 30% higher than that of AO antisense (*TAO*) plants. Values of 20.8 g fresh weight gain per week were measured in AO sense plants (*PAO*)

compared with 14.1 g fresh weight gain per week in antisense (*TAO*) plants (Fig. 5A).

Once established that AO sense plants (*PAO*) showed an increased growth rate when compared with antisense (*TAO*) plants, growth of sense plants (*PAO*), measured this time as plant height, was compared with that of wild-type plants. This experiment

Figure 4. Characterization of primary transformants. AO activity in leaf samples of *PAO* sense (1) and *TAO* antisense (2) primary transgenic lines. Each value represents the mean ± SD of three independent measurements.
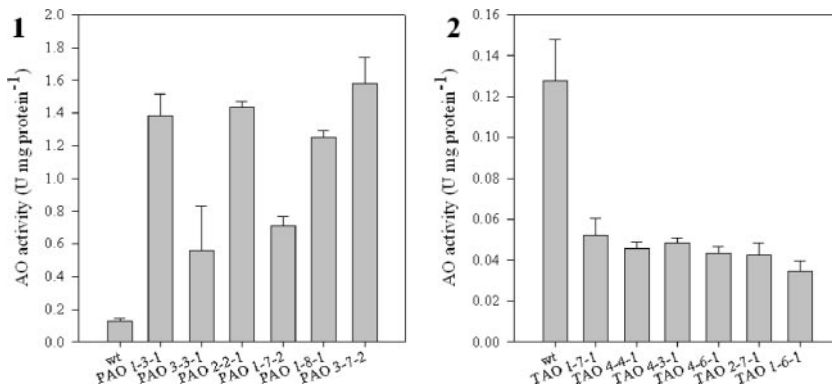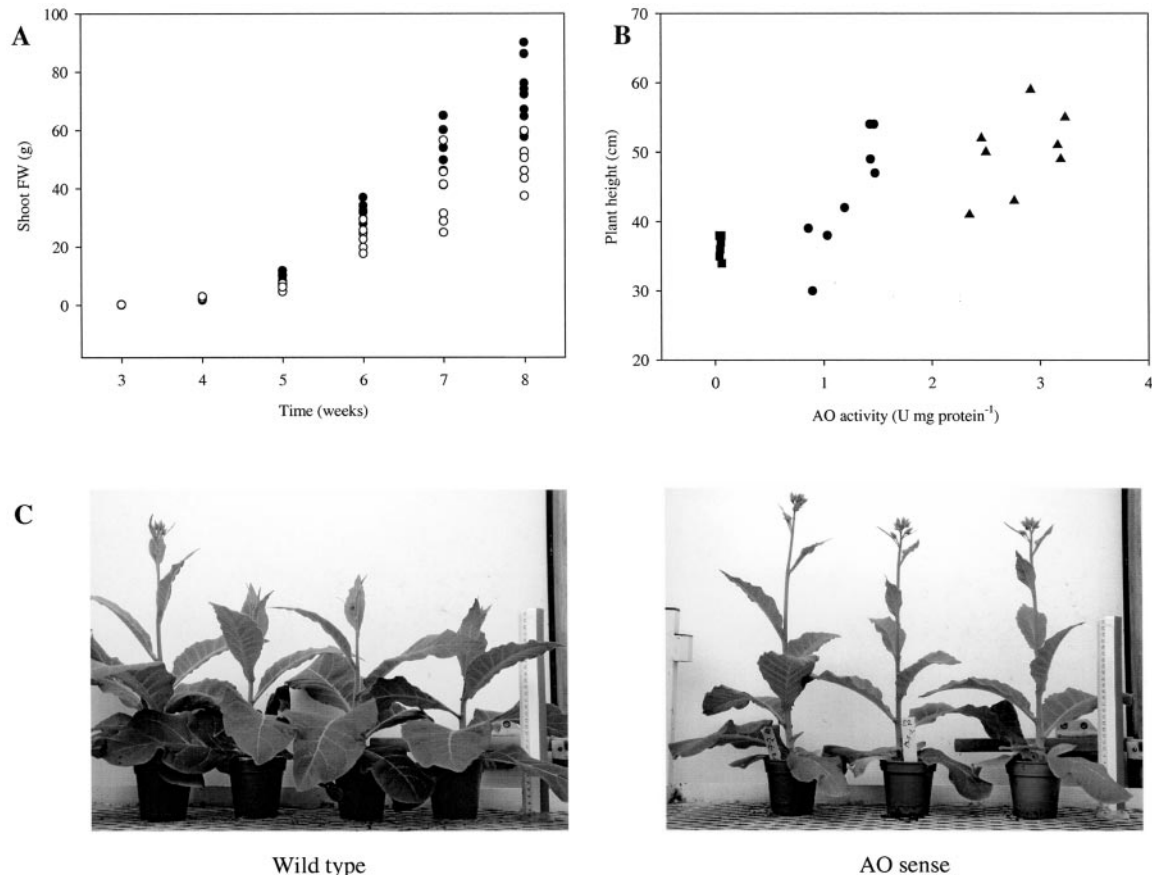
Figure 5. Phenotypes of AO sense and antisense transgenic plants. A, Effect of AO over- and underexpression on growth rates of T_1 populations: three AO sense and two AO antisense. Growth rate of sense (●) and antisense (○) AO transgenic T_1 populations measured as the increase in shoot fresh weight per week. All plants were grown under controlled environment conditions. B, Correlation between AO activity and height in 7-week-old AO sense (*PAO*) and wild-type plants. Squares, wild type; circles, T_1 generation; triangles, T_2 generation. Each activity value represents the mean of three independent measurements. Mean values for AO activities are 0.05 ± 0.00 for wild type; 1.4 ± 0.5 for T_1 generation; 3.0 ± 0.4 for T_2 generation. Mean values for plant heights are 33 ± 5.0 for wild type; 42 ± 9.8 for T_1 generation; 50 ± 8.0 for T_2 generation. This experiment was repeated three times and similar results were obtained. C, Phenotype of wild-type and 7-week-old AO sense tobacco plants (*PAO*).

was conducted to establish whether a correlation between AO activity and plant height could be drawn. T_1 and T_2 seeds from two AO sense lines (*PAO*2-2-1 and *PAO*3-7-2) that had been selected on the basis of high AO activity, were germinated on media containing kanamycin to select for plants containing the transgene. Once this was confirmed by PCR (data not shown), *PAO* plants were transferred to compost and were grown in controlled environment chambers together with wild-type populations. After 6 weeks, AO activities were measured in young fully developed leaves of four to five plants per line, and the height of the corresponding plants was recorded. Overall, T_2 plants exhibited higher AO activity and a taller phenotype than their respective T_1 progenitors (Fig. 5B). A positive correlation was demonstrated between AO activity and plant height (Fig. 5B; $r^2 = 0.85$). Analysis of variance of the data confirmed that T_2 *PAO* lines grew significantly taller

than T_1 *PAO* lines and that T_1 and T_2 *PAO* lines grew taller than the wild type ($P < 0.01$; Fig. 5, B and C).

Relationships between AO Activity and AA in the Leaf Apoplast

Whole leaf AA, DHA contents, and AO activities were measured in extracts from discs excised from young fully expanded leaves of 6-week-old T_1 sense (*PAO* lines *PAO*2-2-1 and *PAO*3-7-2) and antisense (*TAO* lines *TAO*2-7-1 and *TAO*1-6-1) plants. *PAO* plants exhibited about a 40-fold higher leaf AO activity than the wild type (Table I), whereas *TAO* lines exhibited 0.4-fold less leaf AO activity. Apoplastic AO activity was measured in whole leaf extracts, soluble, and ionically bound fractions (Table I). Total AO activity measured in whole leaf extracts was found to be entirely associated with the cell wall (ionically bound) and localized in the apoplast (Table

Table I. *AO activity and ascorbate content in whole leaves and apoplast of T_1 and T_2 PAO sense and TAO antisense transgenic lines and in wild-type plants*[a]

Whole-Leaf Parameters	*PAO* Sense[b]	*TAO* Antisense[c]	Wild Type
AO activity[d]	1.92 ± 0.1	0.02 ± 0.0	0.05 ± 0.0
Total ascorbate[e]	218.7 ± 7.8	282.1 ± 8.6	270.3 ± 34.4
AA[e]	186.6 ± 6.5	258.8 ± 8.1	250.5 ± 30.2
DHA[e]	33.9 ± 3.4	24.1 ± 2.3	20.9 ± 5.8
Apoplastic parameters			
AO activity[d] (ionically bound)	1.92 ± 0.1	0.02 ± 0.0	0.05 ± 0.0
AO activity[d] (soluble)	0.00	0.00	0.00
Total ascorbate[e]	32.6 ± 2.9	26.0 ± 6.2	13.7 ± 3.7
AA[e]	1.1 ± 0.2	17.3 ± 5.4	5.2 ± 0.9
DHA[e]	31.5 ± 2.8	8.6 ± 1.1	8.1 ± 2.9

[a] Leaf samples were taken from 6-week-old-plants grown in controlled environment. Transgenic plants were generated from T_1 seeds germinated in selective medium. Values are the mean of three samples per leaf and 20 leaves per line.   [b] Lines *PAO* 2-2-1 and *PAO* 3-7-2.   [c] Lines *TAO* 2-7-1 and *TAO* 1-6-1.   [d] Given as units per milligram of total protein content.   [e] Given as nanomoles per milligram of total protein content.

I). AO activity was undetectable in soluble fractions of leaf extracts. *TAO* leaves contained similar amounts of AA and DHA to the wild type, but *PAO* leaves contained less AA and total (AA plus DHA) AA (Table I). DHA contents were higher in *PAO* lines, but the leaf ascorbate pool was always largely reduced, values being slightly higher for wild-type (92%) than *PAO* plants (85%). In contrast to whole leaf AA, modified AO expression resulted in dramatic changes in the apoplastic AA pool. The total (AA plus DHA) AA content of the apoplast was at least double that of the wild type in sense and antisense plants (Table I). However, the greatest effect was on the AA redox state (Table I). In the wild type, the AA pool in the apoplast was about 40% reduced, whereas it was 66% reduced in the antisense plants and only 3% reduced in the sense plants. The DHA content in the apoplast of sense plants was 3.5-fold greater than in wild-type or antisense plants. For apoplastic AA and DHA measurements, cytosolic contamination of the apoplast (monitored by measuring Glc-6-P in intercellular washing fluid (IWF) was maintained below 0.1% (data not shown). However, it should be noted that the presence of apoplastic acid

phosphatases may lead to underestimation of the contamination of the apoplastic fraction as well.

Light/Dark Effects on AO Transcript Abundance in AO Transgenic Plants

AO transcript abundance was highest in the light and showed a marked light/dark expression pattern in wild-type plants (Fig. 1). The following experiments were performed to investigate whether such marked diurnal patterns of expression were maintained in the native wild-type AO of *PAO* and *TAO* transgenic plants. Due to the sequence similarity between the tobacco and the pumpkin AO (used for the generation of the AO sense plants), careful design of species-specific primers was necessary to allow expression patterns of native and transgenic AO to be discriminated. No expression patterns in the expression of the transgenic AO (*PAO*) were expected given the presence of the strong constitutive promoter 35S cauliflower mosaic virus (CaMV). The specificity of *TAO* (specific for tobacco AO) and *PAO* (specific for pumpkin AO) primers was confirmed by sequencing of the relative PCR products. They were then used to

Figure 6. Effects of light and dark on native wild-type AO transcript abundance and transgenic pumpkin AO in wild-type and transgenic plants by semiquantitative RT-PCR. Expression analysis of native wild-type tobacco in sense (*PAO*) and antisense (*TAO*) primary transgenic lines. L, after 16 h of light; D, after 16 h of dark. Sense lines: *PAO* 2-2-1, *PAO* 3-7-2, *PAO* 1-7-2; antisense lines: *TAO* 2-7-1, *TAO* 1-6-1. The density numbers above each band represent the relative intensity of each band and can be used as an indication of the relative abundance of transcripts. Actin was used as the internal control. Similar results were obtained in three other independent experiments.
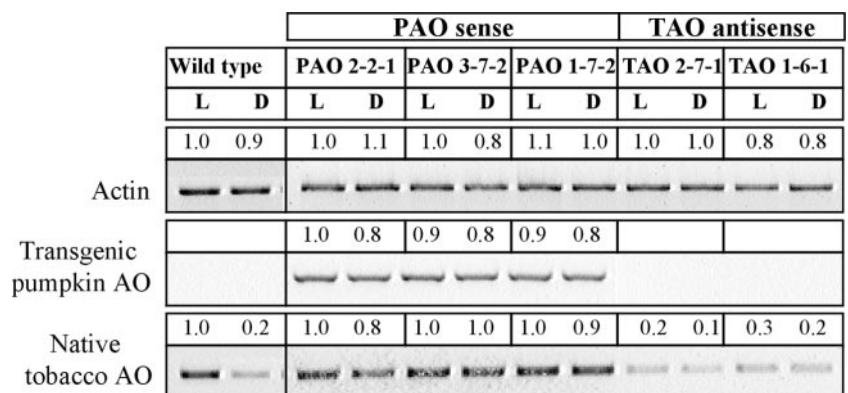
analyze by RT-PCR the expression of the native tobacco *AO* and the introduced pumpkin AO (*PAO*) in the leaves of T_1 and T_2 generations of transformants grown in soil under controlled environment conditions. Samples from wild type, three sense (*PAO*), and two antisense (*TAO*) lines were harvested after 16 h of light or 16 h of dark (Fig. 6). *PAO* was highly and equally expressed in all the sense *PAO* leaves in the light and in the dark. This is consistent with the constitutive expression of *PAO* under the control of the CaMV35S promoter. Moreover, *PAO* plants showed no cosuppression of the native wild-type AO because the transcript abundance of the native wild-type tobacco *AO* transcripts was similar in the sense *PAO* lines and in the wild type. In contrast to the strong dark/light pattern of AO expression observed in wild-type plants, no dark/light effects on native wild-type tobacco AO transcript abundance was detected in the *PAO* sense lines. In this case, the amounts of native AO transcripts were similar in the light and dark (Fig. 6). There was a large overall decrease in native AO transcripts in *TAO* leaves compared with the wild type (Fig. 6), demonstrating the efficacy of the antisense suppression strategy adopted. However, in the same *TAO* plants, there was also a decrease in the observed stimulation of native wild-type AO expression by light (Fig. 6).

DISCUSSION

Although AO activity was first described in plants many years ago, its biological function has remained elusive. Much circumstantial evidence has linked tissue AA contents to growth (Chinoy, 1984), but to date, the mechanism has remained unknown and largely unexplored. Recent evidence has provided an indication of the mechanism whereby AA is involved in the regulation of growth (Pastori et al., 2003). Several lines of evidence also suggest AO may be involved in this process (for example, see Esaka, 1998). The results presented here show that the expression of native AO is regulated by light but that this control is lost when overall AO transcript abundance is manipulated in transgenic plants; AO transcripts are modified by auxin and SA in a manner consistent with effects on growth, and enhanced AO activity oxidizes the apoplastic AA pool and this can stimulate growth in certain conditions. Taken together, these observations demonstrate that AO activity can influence plant growth.

Light Regulation of Gene Expression in Enzymes of the AA Metabolism

We report here the effects of light and dark over a 24-h period on the expression patterns of genes involved in AA metabolism. The expression of cAPX1 is regulated by light in tobacco (Fig. 1), consistent with earlier observations of APX1 mRNA abundance

in tobacco (Tabata et al., 2002), Arabidopsis (Kubo et al., 1995), and APX activity in mustard (*Sinapis alba*; Thomsen et al., 1992). The expression of GLDH, the last enzyme of AA biosynthesis, was not induced by light over a 24-h period in tobacco leaves in the growth conditions used here (Fig. 1). Therefore, we conclude that the light-dependent AA accumulation that we observe under these conditions, increasing from 800 ± 200 nmol g^{-1} fresh weight at the end of a 16-h dark period to 3000 ± 400 nmol g^{-1} fresh weight after 16 h in the light is not the result of modulation of GLDH expression. Therefore, the light dependency of AA accumulation observed over the day/night cycle in leaves must result from other factors such as the requirement for carbon skeletons produced by photosynthesis (Smirnoff and Pallanca, 1996). Several of the genes encoding enzymes of the AA biosynthetic pathway increase in expression after transfer of plants to high light intensity (Tabata et al., 2002). GLDH transcripts were found to decrease after a prolonged (5 d) dark treatment (Tabata et al., 2002). Although a recent study in Arabidopsis leaves has reported that GLDH transcripts are lowest in the morning, increasing during the day, and decreasing in the subsequent night (Tamaoki et al., 2003), this is clearly not the case in tobacco.

Complex transcriptional and translational controls modulate AO expression (Esaka et al., 1992; Kato and Esaka, 1999). Phytochrome-dependent modulation of AO activity by light has been reported (Leaper and Newbury, 1989; Hayashi and Morohashi, 1993). Although our data are not exhaustive in exploring the regulation of AO in response to light, they provide corroborative evidence that AO expression is modulated by light and is repressed in the dark (Fig. 1). Controls showed that this diurnal pattern of regulation was not due to circadian rhythmicity (data not shown). It is surprising that sense and antisense manipulation of *AO* expression led to the elimination of light-induced changes in abundance of native wild-type AO transcripts (Fig. 6). None of the *PAO* lines showed a decrease in native wild-type AO transcript abundance after a 16-h dark period (Fig. 6). It is our hypothesis that the observed perturbation of the redox state of the apoplast, as discussed below, is responsible for the loss the endogenous diurnal regulation of AO.

AO Transcript Abundance Is Regulated by Plant Hormones in Wild-Type Tobacco

An induction of AO by auxin, similar to that observed here, was reported in pumpkin (Esaka et al., 1992). Moreover, the existence of a *cis*-acting region in the AO promoter responsible for auxin regulation has been suggested (Kisu et al., 1997). We demonstrate that AO expression is also induced by auxin in tobacco and that the effect is associated with the promotion of plant growth. AO has been shown to

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catalyze the oxidative decarboxylation of auxin (Kerk et al., 2000), suggesting a role for AO in the regulation of auxin levels within the quiescent centers of maize roots.

High concentrations of SA (1 mM) caused a pronounced inhibition of the growth of tobacco seedlings (Fig. 2B). SA inhibition of plant growth and development is a well-known phenomenon (Petersen et al., 2000). SA can affect cell growth, positively or negatively, depending on the context in which signaling occurs, as studies on Arabidopsis mutants have illustrated (Vanacker et al., 2001). Of the AA-related enzymes analyzed, only the expression of AO was changed by the application of SA and NAA (Figs. 2A and 3A).

Sense and Antisense Manipulation of AO

The modification of *AO* expression in tobacco by sense (*PAO*) and antisense (*TAO*) technologies has allowed us to explore the relationship between AO and growth in planta. To minimize the risk of gene silencing by cosuppression, we used a pumpkin *AO* cDNA for the sense construct, which shares approximately 68% homology with the tobacco sequence. This choice proved to be successful, as no silencing of the native AO was found in the sense (*PAO*) lines. The native AO gene was expressed at wild-type levels in *PAO* lines (Fig. 6). For the antisense suppression of *AO*, we used one-third of the 5′ sequence of the tobacco cDNA, as in recent years, the use of only partial cDNA sequences proved to be very effective in antisense suppression (Bourque, 1995). In antisense (*TAO*) lines, we obtained a dramatic reduction in AO transcript abundance (Fig. 6). However, none of the *TAO* lines exhibited complete suppression of *AO* transcripts, perhaps because of the limited efficiency of the chosen antisense fragment or because of the genomic position of the transgene.

The data presented here demonstrate that our genetic approach was successful in significantly modifying apoplastic AO activity in leaves, as increases of up to 40-fold were measured in *PAO* sense transformed lines, whereas activities were halved in antisense *TAO* lines. We were unable to measure AO protein abundance in this study, as the AO antibodies that we prepared did not have sufficient specificity. Nevertheless, the parallel changes in transcripts and AO activities reported here allow us to assume that our manipulation also modified amounts of AO protein.

AA Content of the Apoplast Was Modulated by AO Activity

PAO sense lines contained less (22%–27%) total AA and AA and more (50%) DHA than *TAO* antisense lines or wild-type plants (Table I). No differences in leaf AA content were detected between *TAO* anti-

sense and wild-type plants. The effect of high AO activity on the whole leaf AA pool is minimal, taking into account the fact that AO activities in sense lines were increased by up to 40-fold. The observation that such large increases in AO activity did not lead to major changes in leaf AA may be explained by differential localization of the two components (Lin and Varner, 1991). AO is located in the apoplast, whereas most of the AA is in the cytoplasm (Table I). MDHA and DHA generated in the apoplast have to be translocated to the cytoplasm where they are efficiently regenerated. The observation that the DHA content was doubled in sense lines suggests that the rate of DHA transport or the rate of AA regeneration (or both) were too slow to compensate for the enhanced AO activity in sense lines. We measured the AA content and redox state in the leaf apoplast in the same plants as those used for total leaf AA determinations. The AA pool was virtually all oxidized (97%) in the *PAO* sense lines and was more reduced than the wild type in *TAO* antisense lines (66% as opposed to 40%; Table I). These data, together with the observation that the sense lines accumulated almost four times more DHA than the wild type in the apoplast, confirmed two points. First, our sense construct successfully targeted AO to the apoplast. Second, manipulation of AO produced a marked localized effect on (apoplastic) AA. It is interesting to note that in *PAO* sense and *TAO* antisense lines, the total AA content of the apoplast was twice that of the wild type. This may suggest that AO-mediated perturbation of the AA and DHA contents of the apoplast influences the transport of reduced and oxidized forms, such that when the apoplastic pool is very reduced or very oxidized, transport processes are increased in an attempt to redress the balance.

AO and Plant Development

In the present study, we demonstrate that increasing AO activity by 40-fold leads to enhanced biomass accumulation and elongation in tobacco plants (Fig. 5). In contrast, reduction of AO activity by antisense technology results in a reduction in growth rate (Fig. 5A). Taken together, the data presented here suggest that enhanced AO activity can have a positive effect on growth (Fig. 5B). Increased biomass production in PAO sense plants appears to be mainly due to enhanced internode elongation. It is possible that AO action alone stimulates growth through the generation of MDHA radicals in the apoplast. This enhanced growth could occur by a chemorheological wall-loosing reaction as described for superoxide- and hydroxyl radical-mediated extension growth (Schopfer et al., 2002), or by enhancing cell enlargement via the polarization of the plasmalemma, leading to expansion by vacuolization as suggested previously (Esaka, 1998; Smirnoff, 2000), or both.

Plant growth is the outcome of various mechanisms of regulation, signaling, and crosstalk. We

have provided in planta evidence that AO is one of these components and therefore participates to this broad dialogue between signaling molecules and environmental cues. It should be noted that in another study on modulation of AO in tobacco, no effects on growth were reported (Sanmartin at al., 2003). In the experimental conditions described here, changes in AO activity alone produced marked effects on plant growth, measured as shoot biomass accumulation and stem height. However, the growth effect was clearly environment dependent as observed in the AA-deficient Arabidopsis vtc 1 mutant (Conklin et al., 1997; Veljovic-Jovanovic et al., 2001). Vtc1 shows a marked conditional phenotype, such that when grown on short days where vegetative growth predominates, vtc1 is much slower growing than the wild type (Col0). Under long days where reproductive growth predominates, there is no significant difference in growth. In this case, AA has been shown to regulate genes controlling plant development through hormone signaling (Pastori et al., 2003). We conducted a large-scale greenhouse experiment in the summer months, with growth conditions markedly different from those described here. In these conditions, where light intensities were higher, the photoperiod was longer, and temperatures were higher, the effects of modified AO activity were much less marked. However, it is interesting to note that although no significant differences in plant height and biomass were recorded between wild-type and transgenic plants, other effects were observed under these conditions. In particular, AO sense plants showed a significantly higher number of smaller flowers compared with wild-type and antisense plants (data not shown). This effect on the flowers of the AO sense lines caused a reduction of 6% to 14% in the weight of seeds. Such differences were not observed when plants were grown in controlled environment chambers, as described here. We conclude that the phenotype associated with increased AO activity in tobacco is conditional and is modulated by environmental cues.

In conclusion, the data presented here suggest that factors such as apoplastic redox state that is dominated by AA and regulated by AO modulate receptor function and signal transduction and that there is scope for modulation and interaction between different signals (hormone, redox, and light) in the apoplast.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tobacco (*Nicotiana tabacum* cv Petit Havana, mutant SRI) seeds were germinated in petri dishes on moistened filter paper. After 10 d, seedlings were transferred to compost (Petersfield Products, Leicester, UK) in pots. Plants were grown at 22°C day/night in controlled environment chambers supplying a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1} at plant height as a 16-h photoperiod. Young fully expanded leaves from 6-week-old plants were used for AA measurements in the light and dark. Three-week-old plants were used for all treatments and RT-PCR analyses.

Hormone Treatments

Three-week-old seedlings were sprayed to run-off (using an aerosol spray bottle supplied by Nalgene, Rochester, NY) with 10 μM and 1 mM SA and 0.5 μM NAA. After treatment, plants were kept in continuous light for 24 h and whole shoots were harvested for RT-PCR analysis. For the evaluation of the effects of hormones on growth, sprayed plants were subsequently returned to "growth conditions" until further analysis 1 week after the initial harvest.

PCR Amplification of *AO* cDNAs for Cloning

Cloning of the genes encoding AO from pumpkin (*Cucurbita pepo*; *PAO*; EMBL GenBank accession no. X55779) and tobacco (*TAO*; accession no. D43624) was achieved from PCR amplification of full-length cDNAs using primers designed to add additional restriction sites to facilitate the cloning. For *PAO* the primers were 5'-ACCA**CTCGAG**ATGCTTCAGATG -3' (nucleotide position 18–29 of cDNA; added *Xho*I site is underlined) and 5'-ACCA**GAGCTC**TTAGGGGGTTATTT-3' (nucleotide position 1,757–1,745; added *Sac*I site is underlined). For *TAO* the primers were 5'-ACCA**GAGCTC**ATGGCTTCCTTA-3' (nucleotide position 88–100 of cDNA; added *Sac*I site is underlined) and 5'-ACCA**CTCGAG**TTTGTGCCACC-3' (nucleotide position 609–559; added *Xho*I site is underlined). The PCR conditions were 10 cycles of 1 min at 94°C, 1 min at 40°C, and 1.5 min at 72°C, and 15 cycles of 1 min at 94°C, 1 min at 61°C, and 1.5 min at 72°C, using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA), 1 ng of cDNA, and 0.2 μM each primer in a 50-μL reaction. At the end of the cycles, the reactions were incubated at 72°C for 10 min. The identity of the 1.7-kb (PAO) and 520-bp (TAO) PCR products was confirmed by single-strand sequencing (ABI PRISM, 310 genetic analyzer; Perkin-Elmer, Warrington, Cheshire, UK).

Construction of Sense and Antisense *AO* in cj102 and Transformation into *Agrobacterium tumefaciens*

PAO and *TAO* PCR products were subcloned as *Xho*I-*Sac*I fragments into the corresponding sites of pp5ln (derived from pUC19; Frenken et al., 1999) by standard in vitro recombination techniques (Sambrook et al., 1989), generating the fusion constructs *PAO*/pp5ln and *TAO*/pp5ln. In *PAO*/pp5ln, the *AO* cDNA is placed in sense orientation under the control of the CaMV35S promoter with duplicated enhancer region (2x35S), originating the 2x35S-*PAO* expression cassette. In *TAO*/pp5ln, the *AO* cDNA is placed in antisense orientation under the control of 2x35S, originating the 2x35S-*TAO* expression cassette. Sense and antisense expression cassettes were ligated into cj102, a derivative plasmid of pGPTV (Becker et al., 1992), as *Hind*III-*Sac*I fragments into the corresponding sites of cj102 in replacement of the 2x35S-*β-glucuronidase* cassette. The cj102-*PAO* and cj102-*TAO* constructs obtained were transformed into *A. tumefaciens* LBA4404 by electroporation (Parry et al., 1998).

Plant Material and Transformation

Sterile cultures of tobacco were transformed with cj102-*PAO* and cj102-*TAO* constructs by *A. tumefaciens* leaf disc infection (Gallois and Marinho, 1995). T_1 seeds from primary transformants were germinated in petri dishes containing 1.5% (w/v) agar in distilled water (nonselective medium), supplied with 100 mg L^{-1} kanamycin (selective medium). T_2 seeds were obtained from individual T_1 plants by self-pollination and were used to generate T_2 transgenic progeny.

Southern-Blotting Analysis

Genomic DNA (5 μg) from *PAO* sense and *TAO* antisense lines was digested with *Hind*III, which cuts once within the transgenic sequence, run on an 0.8% (w/v) agarose gel and blotted onto a nylon membrane (Hybond-NX; Amersham Life Science, Buckinghamshire, UK). The membranes were prehybridized at 55°C for 2 h in hybridization buffer (Amersham Life Science) and were hybridized overnight in the same buffer. The *Hind*III/*Sac*I fragments 2x35S-*PAO* and 2x35S-*TAO* were alkaline phosphatase labeled and were used as probes (AlkPhos Direct; Amersham Life Science).

Hybridization temperatures were 65°C for 2x35S-*PAO* and 68°C for 2x35S-*TAO*. The membranes were washed according to Amersham's recommendations and detection was performed with CDP-*Star* (Amersham Life Science) by exposing Hyperfilm enhanced chemiluminescence film (Amersham Life Science) to the membrane for 1.5 h.

Total RNA Extraction and Gene Expression Analysis by RT-PCR

Total RNA was extracted from using RNeasy plant mini kit (Qiagen, West Sussex, UK) according to the supplier's recommendation. Residual DNA was removed with DNase I, Amp Grade (Invitrogen, Strathclyde, UK). The absence of DNA contamination in the samples was confirmed by a saturating PCR of 40 cycles using actin- (X63603) specific primers (5'-CGCGAAAAGATGACTCAAATC-3' and 5'-AGATCCTTTCTGATATCC-ACG-3'), which give a 687-bp product with genomic DNA and a 533-bp product with cDNA. One microgram total RNA was reverse transcribed using 0.5 μg of Oligo (dT)$_{12-18}$ (Invitrogen) and 200 units of Superscript II (Invitrogen) following the supplier's recommendation. cDNA samples were standardized by PCR for actin content using the gene-specific primers. On the basis of the published sequences, the following gene-specific primers were designed and used for amplification: PR-1a (X12737), 5'-GCC-TTCATTTCTTCTTGTCTC-3' and 5'-TTAGTATGGACTTTCGCCTC-3'; GLDH (AB048530), 5'-TTTTAGGCTTTGACTGTGGTG-3' and 5'-TCAGAT-GAAGAGCTTCTCAAG-3'; cAPX1 (X59600), 5'-CTCAAGCTGTTGAC-AAATG-3' and 5'-AGCTTCAGCAACCAATTC-3'; sAPX (AB02 2274), 5'-TTGT TTCAGTTGGCCAGTGC-3' and 5'-CGCTGCCTTGTGTAGG-3'; tAPX (AB022273), 5'-TGTTTTCTACAGAATGGGC-3' and 5'-GTTGAG-TATTTTG CTGCCAC-3'; *PAO* (X55779), 5'- TTGACCGGAGCAAAAA-CTTC-3' and 5'- AATTCAATGACGACTCCTCC-3'; and *TAO* (D43624), 5'- AACCAAAAACACCTCAAGGC-3' and 5'- GGTGCTTGTTTTAGGA-CATC-3'.

For semiquantitative RT-PCR, the cycle number was kept within the linear range (30 cycles) and the conditions were 3 min at 94°C, cycle of 45 s at 94°C, 30 s at 52°C, and 45 s at 72°C, followed by 10 min at 72°C, using 0.5 μL of the RT reaction and 0.2 μM each oligonucleotide primer in a total volume of 25 μL. The identity of the PCR products was verified by single-strand sequencing (ABI PRISM, 310 genetic analyzer; Perkin-Elmer). RT-PCR products were loaded on 2% (w/v) agarose gel containing 0.5 μg mL^{-1} ethidium bromide and the band intensities were quantified with the Eagle Eye II (Stratagene).

Determination of AA and Glc-6-P Content

Leaf samples were powdered in liquid nitrogen, then 1 mL of ice-cold 1 N HClO$_4$ was added per 0.1-g sample (fresh weight). Extracts were then centrifuged at 14,000 rpm for 5 min at 4°C. HEPES buffer (0.1 M, pH 7.0) was added at a buffer:extract ratio of 1:5 (v/v). K$_2$CO$_3$ (5 M) was added until the extract reached pH 5.6. The extracts were again centrifuged at 14,000 rpm, this time for 2 min, to allow the removal of precipitated K$_2$ClO$_4$, and were then assayed for AA and DHA as described by Foyer and coworkers (1983). Glc-6-P was measured according to Latzko and Gibbs (1972).

Preparation of IWF

IWF was prepared using a method similar to that adopted by Turcsànyi et al. (2000). Young fully expanded leaves were vacuum infiltrated at −70 kPa with chilled 10 mM citrate buffer (pH 3.0) for 5 min. Leaves were then blotted dry, carefully rolled and inserted into a prechilled syringe, and centrifuged at 2,000 rpm for 10 min at 4°C. For ascorbate and Glc-6-P analyses, IWF was collected in empty preweighed Eppendorf tubes. Controls centrifuged into tubes containing cold metaphosphoric acid (2%, w/v) gave similar results.

AO Assay

For total AO activity, leaf tissue was powdered in liquid nitrogen and then homogenized with 0.1 M sodium phosphate, pH 6.5 (1 mL 0.1 g^{-1} fresh weight). The extract was then diluted 10-fold in the same buffer and 50 μL was used in the assay. Measurements of soluble and ionically bound AO

activity were performed according to Sanmartin et al. (2003). Leaf extracts were centrifuged at 15,000g for 10 min at 4°C and soluble AO activity was measured on the supernatant. The pellet (ionically bound fraction) was resuspended in 0.1 M sodium phosphate, pH 6.5, with the addition of 1 M NaCl, vortexed for 10 min at 4°C, and then centrifuged at 15,000g for 10 min at 4°C. AO activity was measured on the supernatant.

AO activity was determined from the decrease in A_{265} at 25°C in a reaction mixture containing 0.1 M sodium phosphate, pH 5.6, 0.5 mM EDTA, and 100 μM AA. One unit of AO activity was defined as the oxidation of 1 μmol AA min^{-1} at 25°C. An extinction coefficient for AA of 14 mM^{-1} cm^{-1} at 265 nm was used in calculations (Nakano and Asada, 1981). Recovery experiments with purified AO were performed to ensure that maximal activity was extracted and maintained during isolation procedures.

Growth Rates of T$_1$ Populations and Phenotypic Analysis

T$_1$ populations were obtained by germinating T$_1$ seeds on nonselective medium. Twenty-five plants per each transgenic line (three AO sense and two AO antisense) were used for growth experiments. Growth curves were drawn by measuring every week shoot fresh weight of three plants per line growing in compost in controlled environment. Shoots were excised at the base of the stem and were weighed for the fresh weight determination. Plant height was measured on 7-week-old plants from the basis of the stem to inflorescence.

Statistical Analyses

Statistical analyses were performed using GENSTAT 5 (Peyne et al., 1993). Data were subjected to analysis of variance and *t* test to investigate the statistical significance of the effects of modified AO activity on growth. Correlations were performed using Sigmaplot 2001 (SPSS, Chicago) using least square linear regression methods to test the goodness of fit.

ACKNOWLEDGMENTS

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