Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and Arabidopsis plants

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Abstract

Transgenic tobacco plants expressing the ascorbate oxidase (AAO) gene in sense and antisense orientations, and an Arabidopsis mutant in which the T-DNA was inserted into a putative AAO gene, were used to examine the potential roles of AAO for salt-stress tolerance in plants. AAO activities in the transgenic tobacco plants expressing the gene in sense and antisense orientations were, respectively, about 16-fold and 0.2-fold of those in the wild type. Under normal growth conditions, no significant differences in phenotypes were observed, except for a delay in flowering time in the antisense plants. However, at high salinity, the percentage germination, photosynthetic activity, and seed yields were higher in antisense plants, with progressively lower levels in the wild type and the sense plants. The redox state of apoplastic ascorbate in sense plants was very low even under normal growth conditions. Upon salt stress, the redox state of symplastic and apoplastic ascorbate decreased among the three types of plants, but was lowest in the sense plants. The hydrogen peroxide contents in the symplastic and apoplastic spaces were higher in sense plants, with progressively lower levels in the wild type and the sense plants. The hydrogen peroxide contents in the symplastic and apoplastic spaces were higher in sense plants, with progressively lower levels in the wild type, followed by the antisense plants. The Arabidopsis T-DNA inserted mutant exhibited very low ascorbate oxidase activity, and its phenotype was similar to that of antisense tobacco plants. These results suggest that the suppressed expression of apoplastic AAO under salt-stress conditions leads to a relatively low level of hydrogen peroxide accumulation and a high redox state of symplastic and apoplastic ascorbate which, in turn, permits a higher seed yield.

Key words: Abiotic stress, antisense expression, apoplast, ascorbate oxidase, hydrogen peroxide, salt stress.

Introduction

Ascorbate oxidase (AAO) (EC 1.10.3.3) is an apoplastic enzyme in plants that catalyses the oxidation of ascorbate (AsA) to monodehydroascorbate (MDHA) using oxygen (Avigliano and Finazzi-Agro, 1997). AAO is highly expressed in expanding fruits such as zucchini (Lin and Varner, 1989), pumpkin (Esaka et al., 1990), cucumber (Ohkawa et al., 1989), and melon (Diallinas et al., 1997). However, it is not clear why AAO is highly expressed in these plants. Recent genome analyses suggest the presence of AAO genes in all higher plants, although their expression levels were relatively low compared with those of the above plants. The activity and expression of AAO are induced by auxin (Lin and Varner, 1991; Esaka et al., 1992) and closely correlated with cell expansion (Kato and Esaka, 1999). These findings imply a role for AAO in hormone-mediated
cell wall loosening. In the quiescent centre (QC) of root tips, a group of mitotically inactive cells, the levels of AAO mRNA, protein, and enzyme activity are very high, causing the decrease in AsA levels within the QC (Kerk and Feldman, 1995). Moreover, AAO has been shown to catalyse the oxidative decarboxylation of auxin, which may have some effects for root development (Kerk and Feldman, 2000).

Another putative function of AAO is to participate in the redox system of AsA (Weis, 1975). The apoplastic space might play an important role for abiotic stresses because the initial events most likely occur at the apoplasm-cell membrane space (Pignocchi and Foyer, 2003). Shalata and Neumann (2001) showed that application of exogenous AsA leads to increased NaCl resistance in tomato. Gossett et al. (1996) reported that a NaCl-tolerant line of cotton had a lower DHA/AsA ratio. In pea (Herandez et al., 2001), the apoplastic DHA/AsA ratio increased with NaCl stress. In transgenic tobacco overexpressing human DHAR, Kwon et al. (2001) showed that application of exogenous AsA leads to increased NaCl resistance in tomato. Gossett et al. (2001) showed that the oxidative decarboxylation of auxin, which may have some effects for root development (Kerk and Feldman, 2000).

The resulting vector was used for the sense transgenic tobacco plants. For the sense transgenic tobacco plants expressing the AAO gene in pBSK- was digested by SacI sites of the pBI121 binary vector. The resulting fragment was ligated into the ECO RI site of pBSK- (Stratagene, CA). The resulting vector was used for the sense transgenic tobacco plants. For the antisense construct, the tobacco AAO cDNA in the pBI121 vector was digested by SmaI and ligated into the BamHI site of pBSK-.

Materials and methods

Transformation of tobacco plants

A plant binary vector pBI121, which contains the cauliflower mosaic virus 35S (35S CaMV) promoter, was used. For the sense transgenic tobacco plants, the tobacco AAO cDNA encoding precursor AAO in the 35S CaMV promoter isolated from tobacco BY-2 cells (Kato and Esaka, 1996) was ligated into the EcoRI site of pBSK- (Stratagene, CA). The AAO gene in pBSK- was digested by EcoRI and SacI, and then ligated into the SmaI and SacI sites of the pBI121 binary vector. For the antisense construct, the tobacco AAO cDNA in the pBI121 vector was digested by BamHI, and ligated into the BamHI site of pBSK-.

One of the correctly orientated plasmid was digested with the SmaI and SacI. The resulting fragment was ligated into the SmaI and SacI digested sites of the pBI121 vector. The resulting vector was used for the antisense transgenic tobacco plants. Tobacco plants (Nicotiana tobacum) were transformed with the sense and antisense AAO genes by an Agrobacterium-mediated method.

A total of 200 plants, 100 sense and 100 antisense plants, were grown aseptically on Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962) supplemented with 3% sucrose containing 50 μg ml⁻¹ kanamycin. Kanamycin-resistant transformants were selected. Twenty-two sense primary transformants (T₅) showing high levels of AAO proteins and activities and 17 antisense T₅ showing low levels of AAO proteins and activities were allowed to flower and set seeds. Of these five sense T₁ (s₁, s₄, s₆, s₈, and s₉) and five antisense T₁ (a₄, a₇, a₃₆, a₅₆, and a₆₇) plants were allowed to flower and set seeds. Four T₅ homozygous transformants (s₁, s₆, a₇, and a₅₆) were used for the detailed analysis of transgenic plants. Two or three wild-type plants were used, and their average values were shown.

Growth conditions

Tobacco seeds were grown aseptically on MS agar medium containing 3% sucrose with a 14 h light (200 μm ² s⁻¹/h) dark cycle and 60% relative humidity. The incubation temperature during the light and dark periods was 27 °C and 25 °C, respectively. Seedlings were then removed and grown hydroponically in 2-fold-diluted MS medium in a growth chamber (Sanyo MLR-350HT, Japan) with the same light conditions. Arabidopsis plants (ecotype Columbia) were incubated with the Murashige and Skoog medium in a growth chamber (Sanyo MLR-350HT, Japan) at 22 °C with a 16 h light (150 μm ² s⁻¹/h) dark cycle and 60% relative humidity.

The standard procedure for germination was as follows. Seeds were surface-sterilized with 2.5% NaClO and 0.002% Triton X-100 for 5 min at 22 °C. After washing with distilled water, seeds were incubated with distilled water overnight at 4 °C. Then seeds were placed in Petri dishes that contained MS agar medium (0.8% agar). The plates were incubated in a growth chamber under the conditions described above. For salt-stress treatment, the growth medium or agar medium contained the indicated concentrations of NaCl. For salt-stress treatment during the reproductive stage, the plants were grown under normal conditions as described above. When bud formation started, plants were transferred to high-salinity growth medium.

Analysis of stress tolerance

The photosynthetic electron transport activity was used as a measure of the effects of salt stress. The quantum yield of photosystem II, (Fₙₒ − F)/Fₙₒ, was measured with a Mini-PAM chlorophyll fluorescence system (Walz, Germany) as previously described (Hoshida et al., 2000). Here, F and Fₙₒ represent the fluorescence levels under irradiation before and after a saturating flash, respectively.

Determination of AsA and DHA contents

Tobacco or Arabidopsis plants (0.1 g fresh weight) were homogenized in 1.0 ml of 6% metaphosphoric acid. After centrifugation at 20 000 g for 10 min, the supernatant was used for the determination of AsA and DHA. AsA and DHA were separated by a Shim-pack SCR-102H column (8 mm×30 cm) using 2 mM perchloric acid aqueous solution and detected by absorbance at 300 nm after reaction with 100 mM NaOH containing 100 mM NaBH₄ (Shimadzu, Kyoto, Japan) (Yasui and Hayashi, 1991).

Apoplastic-washed fluid was prepared using a method similar to that reported by Turcsanyi et al. (2000). Tobacco leaves were vacuum infiltrated with chilled 66 mM potassium phosphate buffer (pH 3.0) and 0.2 mM diethylenetriaminepentaacetic acid for 1 min. Leaves were then blotted dry, inserted into a syringe, and centrifuged at 400 g for 10 min at 4 °C. Apoplastic-washed fluid was collected into Eppendorf tubes containing 6% metaphosphoric acid. Symplastic contamination of the apoplastic-washed fluid measured by the content of glucose-6-phosphate was less than 7% (Latzko and Gibbs, 1972).
Assays of enzyme activities

For the assay of AAO, tobacco or Arabidopsis plants (1 g fresh weight) were homogenized in 3.0 ml of 50 mM potassium phosphate (pH 7.0) containing protease inhibitor (Complete Mini, Roche, Germany) and 30 mM β-mercaptoethanol. After centrifugation at 10 000 g for 10 min at 4 °C, the supernatant was collected by filtration through Sephadex G-25 equilibrated with 10 mM potassium phosphate (pH 7.0). AAO activity was determined by measuring the decrease in absorbance at 265 nm due to AsA oxidation in the reaction mixture containing 66 mM potassium phosphate (pH 6.0) and 170 μM ascorbate. The contents of H2O2 and enzyme activities of ascorbate peroxidase (APX), catalase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase were measured according to the methods of Alia et al. (1999), for which extraction buffers did not contain β-mercaptoethanol. For the APX activity, ascorbate (5 mM) was included in the extraction buffer.

Electrophoresis and immunoblotting

Proteins (25 μg per sample) were separated on SDS-PAGE with 12.5% acrylamide according to Laemmli as previously described by Hibino et al. (2002). Immunoblotting analysis was carried out as previously described (Hibino et al., 2002). An antiserum raised against the cucumber AAO (Esaka et al., 1998) was used. Northern blotting was carried out essentially as previously described by Esaka et al. (1992).

Results

Antisense suppression of the AAO gene enhanced percentage germination at high salinity

The AAO gene from tobacco BY-2 cells, with sense and antisense orientations and under the control of the cauliflower mosaic virus (CaMV) 35S promoter, was introduced into tobacco plants (Fig. 1A). A total of 200 plants, 100 sense and 100 antisense plants, were screened by measuring the levels of AsA and AAO protein. The levels of AsA, AAO protein, and AAO mRNA in most sense and antisense plants were lower or higher than those observed in wild-type plants, respectively (data not shown). Consistent with previous papers (Esaka et al., 1990; Kato and Esaka, 1999), AAO protein was detected in the apoplastic space of transgenic tobacco plants (data not shown). T3 transformants expressing relatively high levels of AAO (s1, s6) and low levels of AAO (a7 and a56) (Fig. 1B) were used for further analysis.

The effects of AAO expression on germination were examined. As shown in Fig. 1C, under normal conditions, almost all tobacco seeds germinated although the percentage germination of sense seeds (s1 and s6) were lower than in antisense and wild-type plants. Upon the increase of NaCl in the growth medium, the germination was delayed in all three kinds of seeds. However, the percentage germination was more severely inhibited in sense plants (s1 and s6), with the antisense plants (a7 and a56) being the least affected.

It is worthwhile mentioning that plant size, especially root length, was significantly different among the three types of plants at high salinity. After 14 d of growth in the presence of 0.1 M NaCl, the root length of the antisense plants (a7 and a56) was more than 2-fold longer than that of the sense plants (s1 and s6) (data not shown).

Levels of AsA and DHA during germination at high salinity

Figure 2A shows that the tobacco seeds contain DHA but no AsA, and their levels were almost the same among the three types of plants. Thus the transformation of tobacco with sense or antisense AAO genes did not change the levels of the ascorbate pool (AsA+DHA) in seeds. Upon imbibition, the accumulation of AsA started whereas DHA remained at relatively low levels (Fig. 2A). After 14 d of germination under normal conditions, the ascorbate pool and the AsA/DHA ratio in the whole-seedlings of sense plants (s1 and s6) were statistically lower than in the wild type and antisense plants (a7 and a56) (Fig. 2B). Under high-salinity conditions for 14 d, the accumulation of AsA was severely inhibited in the whole-seedlings of sense plants (s1 and s6), and as a consequence their AsA/DHA ratio was lower than in wild-type and antisense seedlings (Fig. 2C). It is worth noting that the AsA/DHA ratio in antisense seedlings (a7 and a56) was higher that that of wild-type seedlings, both in the absence and in the presence of NaCl (Fig. 2).

Antisense suppression of the AAO gene increased tolerance for high salinity during vegetative growth

Figure 3A shows the AAO activities of wild-type and transgenic plants grown for 5 weeks under normal conditions. The AAO activity of wild-type, sense (s1 and s6), and antisense (a7 and a56) plants was higher that that of wild-type plants (s1 and s6) were about 15-fold higher than that of wild-type plants, whereas the AAO activities of antisense (a7 and a56) plants were about 20% of the wild type.

The effects of AAO expression on stress tolerance for salt were examined next. Five-week-old plants were transferred to growth medium containing 0.4 M NaCl for 14 d. AAO activities did not change with salt stress (Fig. 3B). Figure 3C shows that after salt stress for 14 d, the quantum yield of photosystem II in wild-type, sense (s1 and s6), and antisense (a7 and a56) plants decreased to about 84%, 50%, and 89% in relation to time zero. The photograph in Fig. 3D also shows that the damage upon salt stress increases in the following order, a56, wild-type, and s1. These data indicate that high AAO activity in sense plants increased the sensitivity to salt stress during vegetative growth, whereas suppressed expression of the AAO gene increased the tolerance for salt stress.
Effects of salt stress on the symplastic and apoplastic ascorbate pool

The changes in the apoplastic and symplastic ascorbate pools during salt stress are shown in Fig. 4. Under normal growth conditions, the redox states of whole-leaf (apoplastic+symplastic) ascorbate were similar among the three types of plants (Fig. 4A), but the redox states of apoplastic ascorbate in the sense plants was significantly lower (Fig. 4B). The levels of whole-leaf AsA decreased upon salt stress (Fig. 4A), to the greatest extent in sense plants.

Fig. 1. Effects of NaCl on the germination of wild-type, sense, and antisense seeds. (A) Schematic structures of binary vectors for sense and antisense plants. NOS-Ter: nopaline synthase promoter and terminator, NPT-II: neomycin phosphotransferase II gene. (B) Western blot of AAO-protein in the sense and antisense T3 plants. In (B), 2-month-old plants were used. (C) Effects of NaCl on the germination of wild-type, sense, and antisense seeds. (A) Germination was under normal conditions. The growth medium contains 0, 50, 100, and 200 mM NaCl, respectively. WT, wild-type plant; s1 and s6, sense plants; a7 and a56, antisense plants. Each value shows the average of three independent measurements.
(s1 and s6), then the wild-type and antisense plants (a7 and a56) followed. The levels of whole-leaf DHA in antisense (a7 and a56) and wild-type plants were retained at relatively low values, whereas those in sense plants (s1 and s6) increased significantly during the salt stress (Fig. 4A).

Measured at 14 d, the apoplastic ascorbate pools (AsA+DHA) did not change very much among the three types of plants in both non-stressed and salt-stressed conditions (Fig. 4B). Upon salt stress, the levels of apoplastic AsA decreased whereas those of DHA increased in all three types of plants (Fig. 4B). Figure 4B shows that the apoplast from the sense plants had the lowest AsA contents and the DHA level was higher in the apoplast from sense plants than in the other types of plants, both in the absence and in the presence of NaCl. Therefore, the redox states of apoplastic ascorbate decreased during salt stress in the following order, antisense (a7 and a56), wild-type, and sense (s1 and s6) plants. These results indicate that the apoplastic AAO changes both symplastic and apoplastic AsA/DHA ratios.

### Sense and antisense expression of the AAO gene altered the flowering time

The effects of AAO gene expression on flower induction were also examined. Flower buds appeared at 18.7 weeks after germination in wild-type plants, and at about 18 weeks in sense (s1 and s6) plants (Fig. 5A). By contrast, the bud formation was delayed by about 3 weeks in antisense (a7 and a56) plants.

Antisense suppression of the AAO gene increased seed yield at high salinity

When buds appeared, some of the plants were transferred to growth medium containing 0.4 M NaCl until harvesting of the seeds. Under normal conditions, the numbers of buds, ovaries, and seeds per plant were the same for the three types of plants. However, in those on the saline medium, during the flowering stage, the numbers of buds, ovaries, and seeds per plants decreased, this drop being statistically higher in sense plants (s1 and s6), whereas in antisense plants (a7 and a56) these values decreased less than in the other types of plants. Under saline conditions, the number of seeds in antisense plants was about 10-fold lower than in sense plants (Fig. 5D), although the weight per seed was similar in all cases (Fig. 5E).

### T-DNA inserted Arabidopsis mutant has a low AAO activity

Genome sequencing of Arabidopsis suggests that Arabidopsis has three putative AAO genes, At5g21100, At5g21105, and AT4g39830. The homology of amino acid sequences of AAOs from cucumber, pumpkin, and melon was very high, 79–93% identity among them, but decreased to 60–67% when compared with putative Arabidopsis and tobacco AAO (Fig. 6A). The deduced amino acid sequences of At5g21105, At5g21100, and AT4g39830 were 64, 60, and 50% identical to that of cucumber AAO, respectively. Functional properties of the putative Arabidopsis AAO have not been studied. Among two highly homologous genes (At5g21100, At5g21105), the T-DNA-inserted mutants were available for At5g21100 (SALK_108854). A mutant in which T-DNA was inserted into exon 3 of At5g21100 was used for analysis (Fig. 6B). The homozygosity of T-DNA insertion mutants was confirmed by PCR experiments (data not shown). T4 T-DNA mutants were used. As shown in Fig. 7A, the AAO activity of T-DNA mutant was about 13% of that of the wild type under normal growth conditions (Fig. 7B) and decreased to about 7% at high salinity (0.1 M NaCl). The AsA contents in whole-leaf and apoplastic fractions of T-DNA mutant were slightly higher than the wild type, but the DHA contents were lower in T-DNA mutants than in
wild-type plants, indicating higher AsA/DHA ratio in T-DNA mutant (Fig. 7B, C).

When the wild-type and T-DNA mutant Arabidopsis plants were grown under normal conditions, both plants germinated with similar rates. However, when the growth medium contained 0.1 M NaCl, the root length of the T-DNA mutant was longer than that of the wild-type plants as shown in Fig. 7D. These results are similar to those observed in the antisense AAO tobacco plants.

T-DNA mutant exhibited short stem and late flowering

As is the case of antisense AAO tobacco plants, the flowering time of the T-DNA mutant was delayed (Fig. 8A). In addition, the stem length of the T-DNA mutant was shorter during the vegetative growth stage (data not shown). During the reproductive stage, the height of the wild type and the T-DNA mutant became similar.

T-DNA mutant exhibited increased seed yield at high salinity

When flower buds appeared, the T-DNA mutant and wild-type plants were transferred to the growth medium containing 0.3 M NaCl until harvesting of seeds. Under normal growth conditions, the numbers of siliques and seeds per plant were similar (Fig. 8B). However, under salt stress, the numbers of siliques and seeds per plant decreased, but significantly higher levels were retained in the T-DNA mutant, t-test (P <0.05) (Fig. 8B). Interestingly, the weight per seed was higher in the T-DNA mutant than that in the wild type at high salinity (data not shown). As shown in Fig. 8C, the rosette leaves in the T-DNA mutant were larger than those of the wild type and retained the green colour after 10 d of NaCl treatment.

Antisense AAO tobacco and Arabidopsis T-DNA mutant exhibited increased stress tolerance for methyl viologen and H$_2$O$_2$

Since it has been reported that high salinity also induces oxidative stress (Hernandez et al., 1993, 1995, 2000), the effects of AAO expression on the oxidative stresses caused by methyl viologen (MV) and H$_2$O$_2$ were examined. When 2-month-old tobacco leaves were detached and incubated for 1 d in the light, wild-type, and both sense and antisense tobacco leaves appeared to be
healthy (Fig. 9A). However, when incubated with MV, bleaching occurred. The sense tobacco leaves (s1 and s6) photobleached more severely than wild-type and antisense leaves, these leaves being the least affected (Fig. 9B). Germination of tobacco seeds on medium containing 30 mM H$_2$O$_2$ for 10 d, produced essentially similar results (Fig. 9C). Bleaching of wild-type Arabidopsis was observed after incubation for 1 d with 0.1 M H$_2$O$_2$, whereas the T-DNA mutant retained the green colour (Fig. 9D, E).

**Levels of H$_2$O$_2$ and ascorbate peroxidase were lower in antisense AAO tobacco and Arabidopsis T-DNA mutant plants**

The effects of AAO expression on the levels of active oxygen species and their quenching enzymes were examined next. Under normal conditions, the level of H$_2$O$_2$ in whole-leaf and apoplastic fractions was high in the following order, antisense (a7 and a56), wild-type, and sense (s1 and s6) plants (Fig. 10A, C). Upon salt stress, the levels of apoplastic H$_2$O$_2$ of wild-type and sense plants significantly increased, whereas the levels of apoplastic H$_2$O$_2$ of antisense plants and of whole-leaf-H$_2$O$_2$ of the three types of plants increased slightly. The activities of APX were fairly similar among the three types of plants under normal conditions, but increased most significantly upon salt stress in antisense (a7 and a56) plants, whereas wild-type and sense (s1 and s6) plants followed (Fig. 10B, D).

The whole-leaf activities of catalase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase were also altered, but their changes were smaller than APX (data not shown). Essentially, similar results were observed for Arabidopsis T-DNA mutant (data not shown).
The results presented here show the importance of AAO during salt stress. This conclusion was obtained based on the data that antisense tobacco plants exhibited a higher percentage germination (Fig. 1C), longer root lengths (see text), higher photosynthetic activities (Fig. 3), and

Fig. 6. Homology of the deduced amino acid sequences of AAO and schematic diagram of T-DNA mutant of At5g21100. (A) Homology of deduced amino acid sequences among five AAO genes. The sequences of Arabidopsis (At5g21100), tobacco AAO (Kato and Esaka, 1996), pumpkin (Esaka et al., 1990), cucumber (Ohkawa et al., 1989), and melon (accession number, AF233593) (Diallinas et al., 1997) were compared. (B) Schematic diagram of T-DNA insertion mutant of At5g21100.

Fig. 7. Effects of salt stress on the AAO activity, ascorbate contents, and root length during seedling growth of wild-type and T-DNA mutant of Arabidopsis. (A) AAO activity, (B) whole-leaf AsA and DHA, (C) apoplastic AsA and DHA, and (D) root length in wild-type and T-DNA insertion mutant. Two-week-old plants were used. In (D), wild-type and T-DNA mutant plants were grown for 7 d or 14 d in the medium containing 0.0 M and 0.1 M NaCl. Each value shows the average of three independent measurements. Different letters denote significant differences (P < 0.05) from wild-type plants.

Fig. 8. Effects of salt stress on seed yield of the wild type and the T-DNA mutant. (A) Bud appearing time. (B) Numbers of silique and seeds per plant. When bud formation was started, plants were transferred to the growth medium containing 0.0 M or 0.3 M NaCl. (C, D) Photographs of wild type and T-DNA mutant during flowering. Photographs were taken 10 d after bud formation. Each value shows the average of three independent measurements.

Discussion
The results presented here show the importance of AAO during salt stress. This conclusion was obtained based on the data that antisense tobacco plants exhibited a higher percentage germination (Fig. 1C), longer root lengths (see text), higher photosynthetic activities (Fig. 3), and
higher seed yields than the wild type under salt-stress conditions (Fig. 5). By contrast, the sense plants exhibited greater damage to these parameters than the wild type under salt-stress conditions. Under normal growth conditions, no significant differences of phenotypes, except flowering time, could be observed. In addition, a mutant in which T-DNA was inserted to a putative AAO gene in *Arabidopsis* showed the similar phenotypes observed in the antisense tobacco plants. Namely, the T-DNA mutant exhibited longer root length (Fig. 7) and higher seed yield (Fig. 8) than those of wild-type *Arabidopsis* plants under salt-stress conditions. Furthermore, it was shown that suppressed expression of AAO also conferred resistance to oxidative stresses brought on by MV or H_{2}O_{2} (Fig. 9). These data demonstrate that the decreased AsA/DHA ratio in the apoplast (and cytoplasm) can lead to changes in root growth, seed yield, and seed germination upon exposure to NaCl, which has not been reported previously.

The reason for these observations might be due to the decrease of both symplastic and apoplastic AsA/DHA ratios under salt-stress conditions, as shown in Figs 2, 4, and 7. The AsA/DHA ratios were higher in antisense tobacco plants than those of wild-type plants during germination (Fig. 2) and growth (Fig. 4) under salt-stress conditions. Similar observations were obtained for the *Arabidopsis* T-DNA mutants (Fig. 7). Under normal growth conditions, the symplastic AsA/DHA ratios were similar between the antisense and wild-type tobacco plants (Fig. 4). These are consistent with the results recently reported by Sanmartin *et al.* (2003) which showed that overexpression of cucumber
AAO gene in tobacco caused a decrease of AsA/DHA ratios and increased-sensitivity to ozone.

Hitherto extensive studies have been carried out to study the relationship between symplastic redox state and cellular defence reactions. Several studies with various tolerant and sensitive genotypes demonstrated a coincidental relationship between the symplastic redox state of ascorbate and/or glutathione and plant sensitivity to a wide range of stress factors (Noctor and Foyer, 1998; Turcsanyi et al., 2000). However, few studies have been reported on the role of apoplastic ascorbate for plant stress tolerances, especially for salt tolerance. There have been no previous reports on the role of AAO expression for salt tolerance in plants.

How can these data be explained on a molecular level? Upon salt stress, the levels of H$_2$O$_2$ in the apoplastic space increases (Fig. 10C) which causes the decrease of the AsA/DHA ratio in the apoplastic space (Fig. 4B). Perhaps it is this altered redox status that is being sensed by the plant leading to the induction of APX (Fig. 10D). High concentrations of H$_2$O$_2$ in the apoplastic space of sense plants would cause the increase in symplastic H$_2$O$_2$ (Fig. 10A) which in turn decreases the AsA/DHA ratio (Figs 2, 4A, 7B). Since H$_2$O$_2$ can pass from the cytosol to the cell wall rapidly (Allan and Fluhr, 1997), a cytoplasmic origin of released H$_2$O$_2$ in the apoplast cannot be ruled out. Since salt stress produces an increase in activated oxygen species in chloroplasts and mitochondria (Hernandez et al., 1993, 1995), it would induce the activity of activated oxygen quenching enzymes in whole-leaf (Fig. 10; text). These interpretations are consistent with the results of a comparison between salt-tolerant and salt-sensitive pea plants (Hernandez et al., 2001).

The Blast search suggested that Arabidopsis has three AAO genes (Fig. 6). None of them was characterized. It was found that the inactivation of one putative AAO gene (At5g21100) decreased the AAO activity by about 90% (Fig. 7) which suggests that the At5g21100 gene is the major one in Arabidopsis. The Arabidopsis T-DNA inserted mutant exhibited similar phenotypes to those of antisense tobacco plants, including the production of more seeds than the wild type at high salinity. These results also indicate the importance of suppression of apoplastic AAO under salt-stress conditions.

Antisense suppression of the AAO gene and T-DNA insertion mutation of AAO enzyme enhanced the root growth of seedlings at high salinity (Fig. 7). The inhibition of root growth in sense plants was probably due to the low AsA/DHA ratios, as seen in Figs 2 and 7. It is well known that AsA is necessary for the transition from G$_1$ to S in the cell cycle (Kato and Esaka, 1999; Horemans et al., 2003). It has also been reported that AsA stimulates root elongation by inhibiting the cell-wall peroxidase (Cordoba-Pedregosa et al., 1996). The quiescent centre cells of maize roots, where the cell cycle is arrested in the G$_1$ phase, has been shown to contain very high AAO mRNA, AAO protein, and DHA, but no AsA (Kerk and Feldman, 1995). All these data are compatible with the above viewpoint.

The present observations do not obscure the role of the AAO gene in plants. Although no clear biological functions for AAO have been described to date, it is widely believed that AAO plays a role in cell elongation (Esaka et al., 1990, 1992; Kato and Esaka, 2000; Potters et al., 2000). AAO might be indirectly involved in sensing the stresses in apoplastic space as AAS molecule (Pignocchi and Foyer, 2003; Pignocchi et al., 2003). The present study also suggests that AAO is involved in time to flowering. Early flowering was observed when the AAO gene was overexpressed (Fig. 5A) whereas late flowering was observed when the AAO gene was suppressed (Figs 5A, 8A). Moreover, a short stem was observed in a T-DNA mutant. Thus AAO is required in least the above phenomena.

Finally, one of important findings in this study is the effect of AAO expression on seed yield at high salinity (Figs 5, 8). Under salt-stress conditions, the number of seeds in the antisense tobacco plants was about 10-fold larger than that in the sense plants (Fig. 5). The previous observations that vitamin C-deficient mutations caused sensitivity to environmental stresses as well as slow growth, and late flowering (Pastori et al., 2003; Conklin et al., 1996), also suggested the importance of high redox ratios of ascorbate for seed yield. Since NaCl stress is a limiting factor to decrease the crop yield, it would be interesting to test whether co-expression of the antisense AAO gene, betaine synthesis gene (Waditee et al., 2003), and Na$^+/H^+$ antiporter gene (Waditee et al., 2002) produce additive effects.
effects on salt tolerance in plants. These genes have been shown to affect salt tolerance if expressed individually.

In conclusion, it is shown in this paper that the suppressed expression of apoplastic AAO under salt stress conditions leads to a relatively low level of hydrogen peroxide accumulation and a high redox state of symplastic and apoplastic ascorbate which, in turn, permit a higher seed yield.

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