

Suppressed Expression of Ascorbate Oxidase Gene Promotes Ascorbic Acid Accumulation in Tomato Fruit

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Published online: 27 November 2010
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Abstract Reduced ascorbic acid (AsA) can be converted into oxidized state by ascorbate oxidase (AO). In present study, *AO* gene was down-regulated by RNA interference in tomato via *Agrobacterium*-mediated transformation. The existence of the RNA interference T-DNA in transformed plants and their progenies were confirmed by polymerase chain reaction amplification and Southern blotting. *AO* expression levels in transgenic lines were monitored by semi-quantitative reverse transcriptase-polymerase chain reaction. The decreased AO enzyme activity and significantly improved AsA content in tomato fruit were observed to be correlated with *AO* gene suppression. Transgenic plants with *AO* suppression and AsA improvement exhibited higher photosynthesis capacity than untransformed control under drought stress. This report demonstrates the AsA oxidation pathway by RNA interference, and provides a feasible approach to improve AsA content in horticultural plants by regulating genes related to AsA metabolism.

Keywords Ascorbate oxidase · RNA interference · Metabolic modification · Abiotic stress · Tomato (*Solanum lycopersicum*)

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Introduction

Ascorbic acid (AsA), also named vitamin C, plays an extensive role in plant and most animals. However, human and some animals like ape and cavy lack the ability to synthesize AsA as a result of a mutation in the last enzyme required for AsA biosynthesis. AsA, therefore, must be acquired regularly from dietary sources (Dowdle et al. 2007). For plants, AsA is involved in the process of cell division, cell expansion and growth. As a necessary antioxidant, AsA protects plant from oxidative damage by scavenging free radicals and reactive oxygen species (ROS) that are generated during photosynthesis, oxidative metabolism and various stresses (Stevens et al. 2007). AsA is a necessary factor of violaxanthin de-epoxidase, which can change violaxanthin into zeaxanthin through xanthophyll cycle on thylakoid membranes, and thus is very important to protect photosynthesis and consume surplus light energy. Increasing AsA content in plants can improve tolerance to various abiotic stresses (Stevens et al. 2008).

Several AsA biosynthesis pathways have been identified in plants including mannose/L-galactose pathway, galacturonic acid pathway, gulose pathway and myo-inositol pathway (Agius et al. 2003; Lorence et al. 2004; Linster and Clarke 2008; Zhang et al. 2009). Following biosynthesis, AsA can be oxidized into monodehydroascorbate (MDHA) by ascorbate oxidase (AO) and ascorbate peroxidase (APX) in plants. MDHA is not stable and converted into AsA by monodehydroascorbate reductase, or converted into dehydroascorbate (DHA). DHA can be hydrolyzed spontaneously into diketogulonic acid, or converted into AsA by dehydroascorbate reductase using reduced glutathione as reducer (Noctor and Foyer 1998) (Fig. 1).

AO is a cell wall-localized enzyme that uses oxygen to catalyze the oxidation of ascorbate to the unstable MDHA

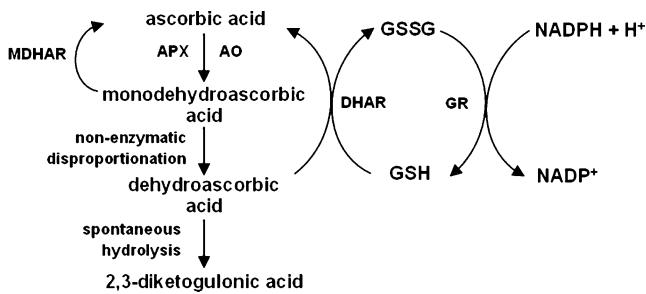


Fig. 1 The ascorbic acid metabolism pathway in tomato

which rapidly disproportionates to yield DHA and AsA, and thus contributes to the modulation of the AsA redox state and perception of external and internal stimuli of plant cells. Regulation of *AO* gene proves its essential role in AsA metabolism and stress responses. Overexpressing cucumber *AO* gene in tobacco leads to significantly increased *AO* enzyme activity and plant sensitivity to ozone (Sanmartin et al. 2003). Both sense and antisense expression of *AO* can improve apoplastic AsA (Pignocchi et al. 2003). Suppressed expression of the apoplastic *AO* gene increases salt tolerance. Under high salt stress, seeds germination ratio, photosynthesis ratio and seeds yield of antisense *AO* transgenic plants were all higher than that of untransformed plants and sense transgenic plants. Even under standard condition, AsA content in apoplast of sense transgenic plants was lower than that of control (Yamamoto et al. 2005). High *AO* activity specifically decreased the AsA content of the apoplast (Pignocchi et al. 2006). Increased *AO* expression in tobacco lowered the apoplast AsA redox state and enhanced sensitivity to various oxidative stresses (Fotopoulos et al. 2006). Leaves of *AO* overexpressing plants exhibited reduced stomatal conductance due to partial stomatal closure. *AO* overexpressing plants also exhibited elevated levels of hydrogen peroxide and a decline in hydrogen peroxide-scavenging enzyme activity (Fotopoulos et al. 2008).

Besides the enzymatic ROS scavenging mechanisms of superoxide dismutase (SOD), catalase (CAT), the water–water cycle (WWC), the ascorbate–glutathione cycle (AGC), and the glutathione peroxidase cycle (GPXC), plant cells have numerous non-enzymatic antioxidant molecules involved in protection against oxidative stress and damage caused by ROS (Mittler et al. 2004). Ascorbate is one of the most important non-enzymatic antioxidant molecules (Linster et al. 2008). *Arabidopsis thaliana* mutant *vtc1* lacking efficient AsA synthesis ability (about 25% AsA content of wild-type) is sensitive to ozone. Exogenous application of AsA can improve *vtc1* tolerance to ozone (Conklin et al. 1999). Thus the metabolic engineering of AsA content could alter plant responses to abiotic stress (Yamamoto et al. 2005). The AsA biosynthesis and metabolism pathways provide a possible

approach for genetic engineering of high AsA accumulation and improved tolerance to abiotic stress through regulating AsA biosynthesis and metabolism related genes. In this study, tomato *AO* was cloned and down-regulated by RNA interference in tomato to investigate the effect of *AO* suppression on AsA accumulation and stress response.

Materials and Methods

Plant Material

Seeds of tomato cultivar Zhongshu No.5 (ZS5) were purchased from Vegetable and Flower Institute of Chinese Agriculture Academy Sciences (Beijing). The ZS5 was used as plant material for gene isolation and genetic transformation.

Gene Cloning and Plasmid Construction

Total RNA was isolated from fruits of tomato ZS5 using Trizol® reagent according to the manufacturer's recommended protocol (Invitrogen, USA). Three micrograms of total RNA was reverse-transcribed using the reverse transcription-polymerase chain reaction (RT-PCR) HIGH kit (Toyobo, Tokyo, Japan). The resultant first-strand cDNAs were diluted into 100 μ l with RNase-free water. *AO* gene was cloned by RT-PCR method from fruit cDNA based on the Unigene SGN-U581990 from Solanaceae Genomics Network (SGN) database and nucleotide sequence (GenBank accession no. AY971876) from National Center for Biotechnology Information (NCBI) under following conditions: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 2 min, and then followed by a 6-min extension at 72°C.

To construct the RNA interference vector, a 573-bp length fragment on 5' end of *AO* gene was amplified using gene specific primer pair (AOF: 5'-TTGCTATTCATTGGCATG GA-3' and AOR: 5'-TTACCACAAATGGCTCCACA-3') (Fig. 2). The amplified fragment was cleaned up by gel extraction kit (Promega, USA) and cloned into the pMD18-T (TaKaRa) vector and then subcloned into pHellsgaet2 using the BP Clonase Reaction Kit (Invitrogen). Positive clones were confirmed by PCR analysis and sequencing. The resulted *AO* RNAi plasmid was electroporated into *Agrobacterium* LBA4404 for tomato transformation.

Molecular Characterization of Transgenic Plants

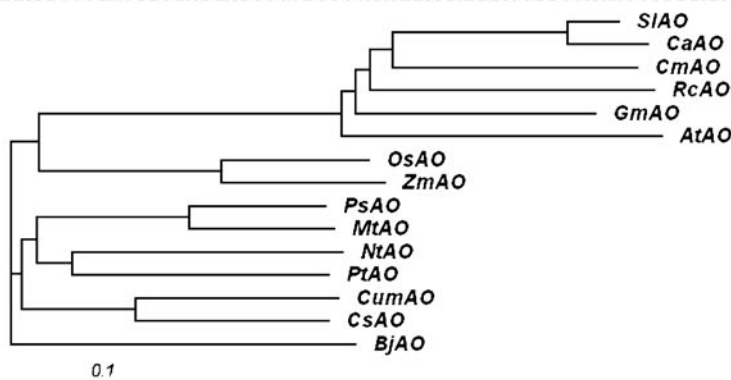
Regenerated transgenic plants were screened for *nptII* gene by PCR and Southern blot. For PCR analysis, each PCR

Fig. 2 Nucleotide and deduced amino acid sequences of tomato *AO* gene, and its phylogenetic relationship with counterparts from other plant species. The amino acids are shown above the corresponding codons. The stop codon is indicated by asterisk (*). Three regions for multicopper oxidase (c106664) are respectively marked by grey boxes. Primer binding sites for RNA interference were underlined. Nucleotide and amino acids are numbered on the left (upper part). Fifteen *AO* genes from tomato (*SIAO*, AY971876), pepper (*CaAO*, AAF33751.1), tobacco (*NtAO*, Q40588.1), *Arabidopsis* (*AtAO*, NP195693.1), mustard (*BjAO*, AF206722_1), rice (*OsAO*, Os06g0567200), melon (*CmAo*, CAA71274.1), pumpkin (*CumAO*, P24792.2), cucumber (*CsAO*, P14133.1), pea (*PsAO*, BAH28261.1), soybean (*GmAo*, BAB86897.1), *Medicago truncatula* (*MtAO*, CAA75577.1), poplar (*PtAO*, XP_002312838.1), maize (*ZmAo*, ACG30138.1) and *Ricinus communis* (*RcAO*, XP_002528975.1) were used for phylogenetic analysis. The scale bar represents the estimated evolutionary distance as 0.1 amino acid substitutions per site (lower part)

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1 M V E H D F H Q Q T Q S L V K L V V L V
1 ATGGTTGAGCATGATTTTCATCAACAACTCAAAGTTTGTAGTTAAGTTGGTGGTACTTGTG
21 C L L F L S A S I S V E A R I R H Y Q W
21 TGTTTGTATTCTATCTAGTATTTTCAGTTGAAGCGGAATTCGTCATTATCAATGG
41 E V K Y E Y K S P D C F K K P S I S I N
121 GAAGTTAAATGAAATATAAATCCCAGATTGTTTCAAAAACCTCTATTAGCATCAAT
61 G K T P G P T I V A Q Q G D T V V V E V
181 GGTAAACTCCAGGACCTACTATTGTTGCTCAACAAGGTGACACTGTTTGTGTTGAAGTT
81 K N S L L T E N L A I H W H G I R Q I G
241 AAAAATAGCTTGTAACTGAAAATCTTGTCTATTTCATTGGCATGGAATTAGACAGATTGGA
101 T P W A D G T E G V T Q C P I V P G D T
301 ACACCATGGGCTGATGGAACAGAGGAGTGACACAATGTCTATTGTGCCAGGGGATACT
121 F I Y K F V V D R A G T Y L Y H A H Y G
361 TTTATATACAAATTTGTTGTTGATAGGGCTGGAACATATTTGTACCATGCTCATTATGGT
141 M Q R Q A G L Q G M M R V S L P D G V L
421 ATGCAAAGACAAGCAGGACTACAAGGAATGATGAGAGTATCATTACCTGATGGAGTTTTA
161 E P F S Y D H D R S I L L T D W Y H K S
481 GAGCCATTGTCATGATGAGGATTTCTTCTCTGATGGTGGTGGTGGTGGTGGTGGTGGTGG
181 T Y E Q A T G L A S L P F S W V G E P Q
541 ACTTATGAACAAGCAACTGGTCTTGCTTTCATTGCCTTTTTCTTGGGTTGGAGAACCTCAG
201 S I L I H G R G R F N C S I P T I D P T
601 TCAATCTTGTATGGAAGAGGAAGATTCAATGTTCTATTCCGACCATAGATCTACTACT
221 L C N A T N P Q C T P Y S M T V V P G K
661 CTTTGTAAATGCTACAAATCCTCAATGTACACCTTATTCAATGACTGTGGTTCCTGGCAAG
241 T Y R L R I G S L T A L S A L S F E I E
721 ACTTATAGGCTTAGAATGGCAGCTTGACTGCTCTTTCAGCTCTAAGTTTTGAAATTGAG
261 G H N M T V V E A D G H Y V E P F V V K
781 GGGCATAACATGACAGTTGTTGAGGCAGCGGTCACTATGTGGAGCCATTTGTGGTAAAA
281 N L F I Y S G E T Y S V L I K A D Q D P
841 AATCTGTTTCATTATTCTGGAGAAACATACTCAGTCCTAATTAAGCTGATCAAGACCCT
301 T R N Y W A S T K V V S R N N T T P N G
901 ACAAGAAATTATTGGGCAAGTACAAAAGTTGTCAGCAGGAATAATACTACTCCAAATGGT
321 L G I F N Y Y P N H P R R Y P P T V P P
961 TTGGGTATTTCAATTATTACCCAAACCATCCTAGGAGATATCCTCCTACTGTACCACCC
341 S G P R W D D V A P R M A Q S V A I K S
1021 TCGGGGCCACGTTGGGATGACGTTGCCCCAGGATGGCTCAGAGTGTGTACTCAAGTCC
361 H R D F I H A P P L T S D R V I V M L N
1081 CACAGAGATTTTCATCCATGCCCCCTCTGACATCGGACAGGGTCATAGTCTGCTCAAC
381 T Q N R V N G Y V R W S V N V S F N M
1141 ACACAAAATAGGGTGAACGGGTATGTACGTTGGTCAGTCAACAACGTCCTCATTCAACATG
401 P H T P Y L I A L K H N L L H A F E Q T
1201 CCCCATACGCCCTTACCTGATCGCCCTTAAACACAACCTTGTACATGCTTTCGAGCAAAC
421 P P P D N Y D Q K N Y D I F N I A P N V
1261 CCTCCTCCGGACAACATATGACCAAAAGAATTACGACATCTTCAATATCGCGCAAATGTG
441 N A T T S N S I Y R L K F N T T V D I I
1321 AATGTACAACACTAGCAACTCGATTTATCGATTGAAGTTCAACAACAACAGTGGACATCATA
461 L Q N A N T M N L N N S E T H P W H L H
1381 TTGCAAAATGCCAACACAATGAACCTAAACAACAGTGAGACACATCCTTGGCATTACAT
481 G H D F W V M G Y G N G K F N Q S I D P
1441 GGACATGACTTTTGGGTCATGGGCTATGGTAATGGCAAGTTTAAACCAATCTATTGATCCA
501 K N Y N F V N P I M K N T I P V H P Y G
1501 AAGAATTACAACCTTGTGAACCAATAATGAAAAACAACATCCAGTCCATCCTTATGGA
521 W T A L R F R A D N P G V W A F H C H I
1561 TGGACCGCATTGCGATTTCGGGCGGATAATCCAGGAGTTTGGGCTTCCATTGCCATATT
541 E S H F F M G M G V V F E E G I E K V G
1621 GAGTCACATTTCTCATGGGAATGGGAGTTGTATTGAAGAAGGAATTGAGAAAGTTGGC
561 K L P T S I M G C G E S K R F H R P *
1681 AAATGCTACTTCTATTATGGGCTGTGGAGAAAGTAAAAGTTCCACAGGCCCTTAA

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reaction mixture contained 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 U *Taq* DNA polymerase (MBI Fermentas), 0.5 μM primer pair, and 100 ng templates. PCR was conducted with an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min, and a final extension at 72°C for 10 min in a PTC-100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). For Southern blot analysis, about 15 μg DNA was digested with *EcoRV* (MBI, Fermentas), and separated on 0.8% (w/v) agarose gel in 1× TAE buffer, followed by blotting onto Amersham Hybond-N⁺ nylon membrane in 0.4 M NaOH. The blots were hybridized at 65°C in a phosphorus buffer (0.5 M) containing 7% SDS, 1% BSA, 1 mM EDTA. *nptII* gene probe was labeled with [³²P]-dCTP (Beijing Furui Company). The membranes were washed with 2× SSC, 0.5% (w/v) SDS at room temperature for 10 min and 0.2× SSC, 0.1% (w/v) SDS under 65°C for 5 min. *nptII* primers used for PCR analysis and probe preparation were *nptII*-F: 5'-AGACAATCGGCTGCTCTGAT-3' and *nptII*-R: 5'-TCATTTCGAACCCCAGAGTC-3'. The transgenic plants with single copy of T-DNA insertion were propagated into T₂ generation as homozygous lines for further analysis.

To determine the expression of *AO* gene in homozygous transgenic plants, semi-quantitative RT-PCR was performed. Total RNA was extracted from ripe fruits and transcribed into cDNA according to gene cloning method above. Semi-quantitative RT-PCR was carried out with gene specific primers AOF and AOR under the under following conditions: denaturation at 94°C for 3 min, followed by 28 cycles of 94°C for 45 min, 55°C for 45 s, and 72°C for 45 min, and then followed by a 6-min extension at 72°C. The *β-actin* gene was used as internal control to keep the same RNA loading. Primers for *β-actin* are *β-actin*-F: 5'-ATGGCAGACGGAGAGGATATTCA-3' and *β-actin*-R: 5'-GCCTTTGCAATCCACATCTGCTG-3'.

AO Activity Determination in Tomato Fruits

AO activity in tomato fruits from homozygous transgenic plants was determined according to Esaka et al. (1990). Ripe fruits from five independent plants as well as untransformed control (ZS5) were harvested and mixed for AO activity assay. Experiment was repeated three times. Five gram of fruit tissue plus 25 ml pre-cold 50 mM phosphate buffer were ground and then centrifuged, the resulting supernatant transferred to fresh tubes and placed on ice. A volume of 3 ml reaction mixture including 2.88 ml phosphate buffer (pH 7.0), 0.5 mM AsA, and 0.12 ml exaction supernatant was used for analysis. Using reaction mixture without exaction supernatant as control, OD₂₆₅ value was recorded on an ultraviolet and visible spectrophotometer (Agilent 8453). One unit is defined as

that 0.01 OD value was changed within 1 min. Results were calculated using the following formula:

$$\text{Enzyme activity} = \frac{\Delta A \times V_T}{W \times 0.01 \times t \times V_S} \text{ (U/ min g FW)}$$

where ΔA denotes the absorption change range during reaction time, W is the tomato fresh weight (g), t denotes the reaction time (min), V_T is the total enzyme extraction volume (ml), V_S is enzyme extraction volume used for assay (ml).

Determination of AsA Content in Tomato Fruits

AsA content in tomato fruits of homozygous plants was analyzed by titration method with 2,6-dichlorophenol indophenol (Li 2000). Ripe fruits from five independent plants as well as untransformed control (ZS5) were harvested and mixed for ascorbate assay. AsA content was calculated by the following formula:

$$\text{AsA content per 100 g fresh weight} = \frac{(Y_0 - Y_1) \times A \times Z \times 100}{B \times X}$$

where Y_1 is the dye volume used for control titration (ml), Y_0 is the dye volume for sample titration (ml), A is the amount of AsA which demands 1 ml dye for titration (mg), B is the sample weight (g), X is the sample solution volume used for titration (ml), and Z is the total volume of sample solution (ml). The experiment was repeated three times.

Drought Treatment of Tomato Plants

Seeds from the homozygous transgenic plants with improved ascorbate content were harvested and pre-germinated and sown on pots with culture substrate (compost/perlite/sand=1:1:1 (v/v/v)) in greenhouse. The seedlings are equally watered until four leaves stage, and then irrigation stopped. When the substrate surface became dry, the seedlings were further subjected to drought treatment for 1 week without irrigation.

After the drought treatment, the photosynthesis rate and stomatal conductance was measured with TPS-1 photosyntometer (PP Systems, UK) at noon of a fine day, under natural illumination at 32±2°C. The electric conductance (EC) of tomato leaves was assayed according to Li (2000). Leaves at the same position of plants were taken and cleaned, placed at 40°C oven for 1 h. Heat treated leaves were rinsed with distilled water two times and blotted dry, and cut into 1-cm segments, and put into a beaker capable of holding electrode of conductivity meter. Twenty milliliter distilled water was added in the beaker immersing leaves at the bottom. The beaker was put in to a vacuum desiccator and pumped to remove air among cells, and then atmo-

sphere was let in gently. The beaker was taken out and placed still for 20 min. The leaves were agitated gently before EC value was measured using DDS-307 digital conductivity meter at 20–25°C.

Statistical Analysis

All data are presented as the mean±SE and treated with ANOVA program of SAS software. Comparisons between transgenic plants and wild-type plants were performed using Duncan's multiple range test at 5%. Values of $P < 0.05$ were considered statistically significant.

Results

Cloning and Analysis of Tomato *AO* Gene

We searched SGN and NCBI for tomato *AO* and identified a Unigene (SGN-U581990) and *AO* gene accession (GenBank accession no. AY971876). The tomato *AO* gene was cloned by RT-PCR method. Tomato *AO* gene contains a 1,737-bp open reading frame encoding 578 amino acid residues. The region from 41 to 158 bp encodes three representative multicopper oxidase motifs, which are characteristic domains of *AO*. The phylogenetic tree based on the deduced amino acid sequence of plant *AO*s demonstrates that tomato *AO* has high identity with counterparts from other plant species. Tomato *AO* gene has the highest identity of 92% with pepper *AO*, with the second highest identity score of 72% with melon *AO*. Interestingly, tomato *AO* gene has a relatively lower identity (49%) with tobacco *AO* (Fig. 2). The high homology of tomato *AO* gene with counterparts from other plant species makes it necessary to characterize *AO* function in ascorbate metabolism in tomato.

RNAi Suppression of *AO* Gene in Tomato

To investigate the effect of *AO* suppression on ascorbate accumulation and stress response, 5' end of *AO* gene sequence was used for RNA interference vector construction based on pHellsgate vector (Fig. 3a). There are only one copy of *AO* gene in tomato genome, as proved by RFLP analysis (Zou et al. 2006), which will permit specially characterizing function of *AO* gene through RNAi suppression.

A total of 10 independent transgenic plants were obtained through *Agrobacterium*-mediated transformation. Using the untransformed plants as negative control and plasmids as positive control, transgenic plants were PCR analyzed by *nptII*-specific primers (data not shown). Genomic DNA was extracted from PCR-positive plants and their progenies, and digested by *EcoRV*, transferred to

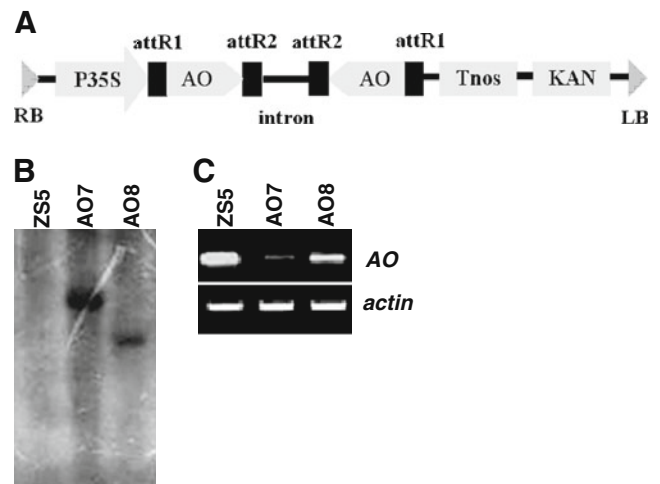


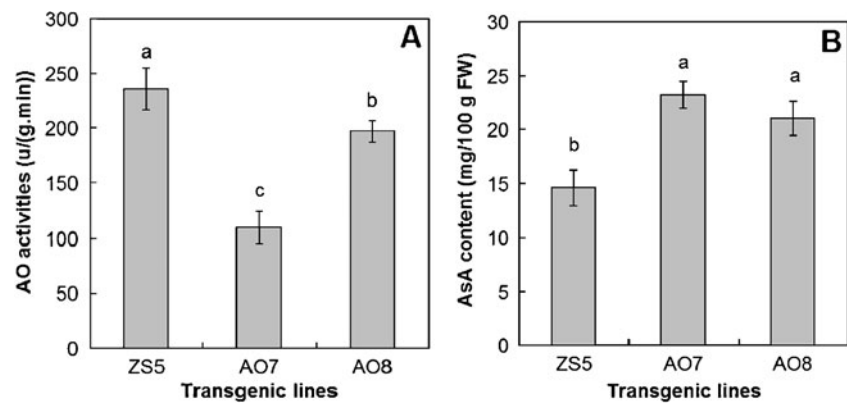
Fig. 3 Schematic structure of T-DNA region of *AO* RNAi transformation vector and molecular characterizations of transgenic plants. *RB* right border, *LB* left border, *P35S* 35S promoter of cauliflower mosaic virus, *intron* *PDK* gene intron, *Tnos* noplone synthesis gene terminator, *KAN* kanamycin resistance coding region (*nptII*) containing its promoter and terminator. attR1, attR2, recombination sites from attB–attP reaction (a). Genomic DNA from young leaves was digested with *EcoRV* and transferred to membrane, and hybridized to *nptII* probe labeled with [α^{32} P]-dCTP (b). Expression of *AO* gene in ripe fruits was investigated by semi-quantitative RT-PCR. *actin* gene was used for internal control (c). Transgenic lines used for Southern blot and semi-quantitative RT-PCR analysis are indicated above lanes. ZS5 is wild-type used as untransformed control (b, c)

nylon membrane by capillary method, and hybridized with *nptII* gene probe labeled with [α^{32} P]dCTP. PCR and Southern blot analysis indicated the T-DNAs were integrated into tomato genome. Two transgenic lines with single copy of T-DNA integration, AO7 and AO8, were propagated to T_2 generations for further characterization (Fig. 3b). In order to investigate expression profile of *AO* gene expression in RNAi transgenic plants, RNA was extracted from ripe fruits and transcribed into cDNA to carry out semi-quantitative RT-PCR. The RT-PCR result indicated that the expression levels of *AO* gene in RNAi transgenic lines, AO7 and AO8, were significantly lower than that of untransformed control (ZS5) (Fig. 3c). The two homozygous lines, AO7 and AO8, with *AO* suppression were selected for further analysis.

AO Enzyme Activity Decreases in Tomato Fruit

In the homozygous lines with obvious *AO* gene suppression, enzyme activity was analyzed to investigate the effect of *AO* expression changing on *AO* enzyme activity. The *AO* activity in transgenic lines of AO7 and AO8 decreased 54% and 17% compared to the untransformed control (Fig. 4a). The decrease in *AO* enzyme activity was found to be correlated with the variation of mRNA expression level as shown in Fig. 3. The suppression of *AO* expression effectively decreased the *AO* enzyme activity in tomato fruit.

Fig. 4 Changes of AO enzyme activity and ascorbic acid content in tomato fruits of AO suppression plants. AO activity was decreased significantly in fruits as compared to untransformed control (ZS5) (a) accompanied by ascorbic acid content improvement in fruits (b). Different letters above columns indicate significant differences by Duncan's multiple range test at 5% level



Improvement of AsA Content in Tomato Fruits by Suppression of *AO* Gene

In order to analyze the effect of *AO* expression suppression and AO activity decrease on AsA accumulation, the AsA content of tomato fruit was determined in ripe stage. The result indicated that the AsA content of tomato fruits of AO7 and AO8 was significantly higher than that of untransformed control. The AsA content in tomato fruits was improved up to 155% and 144% of the wild-type (Fig. 4b). The AsA accumulation was correlated with the suppression of *AO* gene expression and decrease in AO enzyme activity.

Alleviated Damage Under Drought Stress

ROS is frequently generated as by-products in photosynthesis, and is usually promoted to an enhanced production by various abiotic stresses. As ascorbate is an important antioxidant to scavenge ROS, plant photosynthesis indexes and electrolyte leakage were determined to investigate the effect of *AO* suppression and AsA accumulation on stress response. Several indexes including net photosynthetic rate (*Pn*), stomatal conductance (*Gs*) and leaf electrolyte leakage (*EC*) were not significantly different between wild-type and transgenic plants under normal conditions. However, after 1 week drought treatment, the *Pn* and *Gs* value of wild-type plants decreased more than onefold, and the *EC* value of

wild-type plants increased more than twofolds, whereas the transgenic plants suffered a lesser impact under drought stress. After drought treatment, *Pn* and *Gs* of *AO* suppression lines, AO7 and AO8, were significantly higher than that of untransformed control, and *EC* of the *AO* suppression lines was significantly lower than that of untransformed control (Table 1). It could be considered that RNA interference of *AO* gene help the plants better adapt to drought stress with less affected photosynthesis capacity.

Discussion

Ascorbate can be oxidized into MDHA by AO in plants, which makes it possible to promote ascorbate accumulation in plants through suppressing expression of *AO* gene. In this study, the expression level of tomato *AO* was suppressed by RNA interference in tomato, and the AO enzyme activity was decreased and the AsA content was improved. There was a correlation between the *AO* expression suppression and AsA accumulation. Similar results were found in tobacco that AO activity decreased in antisense *AO* transgenic tobacco, leading to increasing content of AsA (Pignocchi et al. 2003). Together with our study, results indicated that suppressing enzyme in AsA metabolism pathway could improve AsA biosynthesis in plants. It should be noted that the reduced ascorbate (AsA)

Table 1 Effect of *AO* suppression on photosynthesis characteristic and leaf electrolyte leakage of tomato plants

Line	<i>Pn</i> ($\text{CO}_2 \mu\text{mol m}^{-2}\text{s}^{-1}$)		<i>Gs</i> ($\text{mol m}^{-2}\text{s}^{-1}$)		<i>EC</i> (S cm^{-1})	
	Control	Drought	Control	Drought	Control	Drought
ZS5	58.6±4.3 a	29.1±3.2 b	1784.6±89.2 a	862.8±49.9 b	22.1±0.6 a	72.6±2.1 a
AO7	59.1±5.8 a	38.2±4.4 a	1806.8±92.8 a	955.7±50.9 a	21.8±0.8 a	63.8±1.0 b
AO8	57.2±7.1 a	37.6±4.9 a	1736.5±86.5 a	946.5±40.4 a	23.3±1.1 a	64.1±2.8 b

Different letters after the numbers in the same column indicate significant differences by Duncan's multiple range test at 5% level

Pn net photosynthetic rate, *Gs* stomatal conductance, *EC* leaf electric conductance

was determined in this study, while the total ascorbate (AsA+DHA) remained to be investigated. On the other hand, in transgenic tomato plants overexpressing ascorbate biosynthesis related genes such as GDP-D-mannose-3',5'-epimerase gene (*GME*) and myo-inositol oxygenase gene (*MIOX*), major changing in ascorbate exists in reduced form of AsA, while the content of oxidized form of DHA remains largely unchanged (unpublished data).

Ascorbate is one of the most important of non-enzymatic antioxidant to scavenge ROS and protect plants from both abiotic and biotic stress damages (Darwish et al. 2008). The *AO* suppression in tomato lead to significantly increased ascorbate content in fruit, and the photosynthetic capacity and leaf electrolyte leakage under drought stress was less affected compared to wild type plants. The main reason probably lies in that the improved accumulation of ascorbate as an antioxidant protects plants from drought damage. Similarly, suppression of *AO* gene by antisense expression results in salt stress tolerance in tobacco, accompanied by low level of hydrogen peroxide accumulation and a high redox state of symplastic and apoplastic ascorbate which, in turn, permits stress tolerance (Yamamoto et al. 2005). Besides ascorbate as a non-enzymatic antioxidant, the enzymatic ROS scavenging mechanism in the ascorbate metabolism pathway includes APX. In *APX* mutant of wheat, both APX activity and photosynthesis efficiency decreased as compared to wild-type (Danna et al. 2003). Regulation of APX at the transcript level affects plant tolerance to drought stress (Fotopoulos et al. 2008). *APX* gene transcription was induced in plants by various abiotic stresses including drought, salt, salt-alkaline, CdCl₂, and abscisic acid (Xu et al. 2009; Gao et al. 2010). In this study, transgenic plants with *AO* suppression has less leaf damage and improved photosynthesis ability compared to untransformed control after drought stress treatment (Table 1).

Ascorbate biosynthesis and metabolism related genes often exist in form of multiple gene family. *AO* gene family of three to four members has been reported in melon and Arabidopsis. RFLP analysis results demonstrated that tomato *AO* enzyme is encoded by a single-copy gene, similar to tobacco and pepper (Zou et al. 2006). The single copy of *AO* gene would permit the specific silence of *AO* gene to investigate gene function.

It is interesting to observe that the leaf morphology of *AO* RNAi lines were totally different from that of untransformed control. The compound leaves of wild type plants were converted to simple leaves in *AO* suppression plants. Besides its role of oxidization of AsA into MDHA, *AO* was frequently reported to be involved in auxin response and plant growth (Pignocchi et al. 2003, 2006). Promoter analysis revealed a *cis*-acting region involved in induction by auxin upstream pumpkin *AO* gene (Kisu et al. 1997). Application of auxin increased *AO* gene expression

in tobacco (Pignocchi et al. 2003). Oxidation of apoplastic AsA in *AO* sense transgenic plants was associated with loss of the auxin response (Pignocchi et al. 2006). All these data indicate that *AO* expression is closely related to auxin response. On the other hand, auxin is a crucial regulator for the morphological patterning of compound leaves (Blein et al. 2008; Koenig et al. 2009). It would be very intriguing to investigate how *AO* is involved in the auxin patterning during leaf morphogenesis.

In conclusion, it is possible to increase AsA content through engineering AsA metabolism related genes. Modifying AsA metabolism in plant is another approach for regulating stress response as well as increasing nutrition quality.

Acknowledgements This research was supported by grants of Major State Basic Research Development Program (2011CB100600), National Natural Science Foundation of China (No. 30800756), Xinjiang Corps Doctoral Fund (2008JC04).

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