Transformation of β -Lycopene Cyclase Genes from Salicornia europaea and Arabidopsis Conferred Salt Tolerance in Arabidopsis and Tobacco

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Inhibition of lycopene cyclization decreased the salt tolerance of the euhalophyte Salicornia europaea L. We isolated a β -lycopene cyclase gene SeLCY from S. europaea and transformed it into Arabidopsis with stable expression. Transgenic Arabidopsis on post-germination exhibited enhanced tolerance to oxidative and salt stress. After 8 and 21 d recovery from 200 mM NaCl treatment, transgenic lines had a higher survival ratio than wild-type (WT) plants. Three-week-old transgenic plants treated with 200 mM NaCl showed better growth than the WT with higher photosystem activity and less H₂O₂ accumulation. Determination of endogenous pigments of Arabidopsis treated with 200 mM NaCl for 0, 2 or 4 d demonstrated that the transgenic plants retained higher contents of carotenoids than the WT. Furthermore, to compare the difference between SeLCY and AtLCY from Arabidopsis, we used viral vector mediating ectopic expression of SeLCY and AtLCY in Nicotiana benthamiana. Although LCY genes transformation increased the salt tolerance in tobacco, there is no significant difference between SeLCY- and AtLCY-transformed plants. These findings indicate that SeLCY transgenic Arabidopsis improved salt tolerance by increasing synthesis of carotenoids, which impairs reactive oxygen species and protects the photosynthesis system under salt stress, and as a single gene, SeLCY functionally showed no advantage for salt tolerance improvement compared with AtLCY.

Keywords: Carotenoid • β -Lycopene cyclase • Oxidative damage • PEBV • Salt tolerance • SeLCY.

Abbreviations: AM, amitrole; DAB, 3,3'-diaminobenzidine; F_v/F_{mv} Chl fluorescence ratio; H2DCFDA, 2', 7'-dichlorofluorescein diacetate; MDA, malondialdehyde; MS, Murashige and Skoog; NPQ, non-photochemical quenching; ORF, open reading frame; PEBV, pea early browning virus; PQ, paraquat; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; UTR, untranslated region; WT, wild type; YFP, yellow fluorescent protein.

Introduction

Salt stress is one of the major environmental factors limiting the productivity and distribution of major crops worldwide (Tuteja 2007). Under salinity, excessive Na⁺ exerts several detrimental effects on plants, including osmotic stress, ionic toxicity to plants and associated oxidative stress, which causes a reduction in photosynthesis and the production of reactive oxygen species (ROS) (Zhu 2002, Oh et al. 2009).

Carotenoids comprise one of the largest classes of pigments in nature, and are produced from general isoprenoid biosynthesis in the chloroplasts of photosynthetic tissues and chromoplasts of fruits and flowers in plants (Galpaz et al. 2006). Carotenoids serve two major functions, as accessory pigments for light harvesting and as protective agents for scavenging the ROS produced in the photosynthetic apparatus (Tao et al. 2007). These two functions both involve an interaction with Chls but in a different direction of energy usage. Carotenoids help to pass energy into Chls for light collection of photosynthesis, whereas they also assist in transferring excess energy away from Chl for photoprotection and protection of the photosynthetic apparatus from ROS damage (Romer and Fraser 2005, Han et al. 2008). Recent advances in metabolic engineering of carotenoid biosynthesis in plant tolerance to high light, UV, herbicides and salt have been achieved in Arabidopsis and tobacco (Davison et al. 2002, Gotz et al. 2002, Wagner et al. 2002, Han et al. 2008).

As a key branch point in the pathway of carotenoid biosynthesis, two types of cyclic end groups were found in higher plants: the β and ε rings. The β cyclase introduces a ring at both ends of lycopene to form β -carotene, and ε cyclase converts lycopene to δ -carotene. Also, it is reported that the

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 α -branch (from α -carotene to lutein) and the β -branch (from β -carotene to neoxanthin), as shown in **Fig. 1**, have distinct and complementary roles in the mechanisms of photoprotection (Dall'Osto et al. 2007b). To date, β -lycopene cyclase genes have been isolated from many plants including Arabidopsis (Cunningham et al. 1996), tomato (Pecker et al. 1996), maize (Singh et al. 2003), autumn olive (Guo et al. 2009) and citrus (Alquezar et al. 2009), and characterized in regulating the mechanism of carotenoid synthesis, coordinating the expression among carotenogenic genes and accumulation of carotenoids. However, little is known about the other function of the β -lycopene cyclase gene, though β -lycopene cyclase actively takes part in the carotenoid pathway which was widely confirmed to scavenge ROS.

It is suggested that halophytes developed robust mechanisms including enzymatic or non-enzymatic scavenging pathways to counter the deleterious effects of ROS production during salt stress (Jithesh et al. 2006). As a euhalophyte, Salicornia europaea is widely distributed in coastal and inland salt marshes, and regarded as one of most salt-tolerant plants in the world (Davy et al. 2001). For genetic engineering of enhanced stress tolerance in crops, it is interesting to understand the molecular events underlying appropriate genes for oxidative damage defense in S. europaea. In this study, we isolated and characterized the full-length cDNA of the β -lycopene cyclase gene (SeLCY) from S. europaea. Ectopic expression in Arabidopsis was conducted with the aim of understanding the function of SeLCY. In addition, ectopic expression of SeLCY and AtLCY in Nicotiana benthamiana by the pea early browning virus (PEBV) system was carried out to compare



Fig. 1 Partial schematic diagram of the carotenoid biosynthesis pathway in plants.

functional difference between them. These results indicate that *SeLCY* exerts a similar function on salt tolerance as *AtLCY* in transformed tobacco, while the increased synthesis of carotenoids in *LCY* transgenic Arabidopsis and tobacco may contribute to the improvement of salt tolerance by impairing ROS and protecting the photosynthesis system from salt and oxidative damage.

Results

Amitrole (AM) treatment decreased salt tolerance of S. europaea

Salicornia europaea maintains optimal growth under 200–400 mM NaCl conditions. However, after treatment with AM, an inhibitor of lycopene cyclization, S. europaea exhibited salt sensitivity under 200 and 400 mM NaCl condition (**Fig. 2A**). When exposed to 200 mM NaCl, the plants treated with AM displayed decreased height and fresh weight and higher cell death (**Fig. 2B–D**). In addition, S. europaea treated with AM exhibited reduced Chl contents and higher H_2O_2 concentration under 200 and 400 mM NaCl stresses (**Fig. 2E–G**). These results indicate the lycopene cyclization of the carotenoid synthesis pathway plays an important role in salt tolerance of S. europaea.

Isolation of a β -lycopene cyclase gene from S. europaea

A 1,937 bp full-length cDNA of a β -lycopene cyclase was obtained by 3' and 5' rapid amplification of cDNA ends (RACE) from S. *europaea* (GenBank accession No. AY789516). It contains an open reading frame (ORF) of 1,494 bp, with a 294 bp 5'-untranslated region (UTR) and a 120 bp 3'-UTR, respectively. The predicted protein contained 498 amino acids, and the putative transit sequence was predicted to be the N-terminal residues 1–37.

The deduced amino acid sequence of SeLCY was compared with those of known β -lycopene cyclase homologous proteins (Fig. 3A). SeLCY showed high homology with many β -lycopene cyclases in other plants, e.g. 75.7% identity with that from Lycium barbarum (AAW88382.1), 76.5% with that from Gentiana lutea (ABK57115.1), 76.9% with that from Diospyros kaki (ACR25158.1) and 78.6% with that from Citrus sinensis (AAU05146.1). In contrast, a low identity (49%) was observed with Solanum lycopersicum (AAG21133.1). The phylogenetic analysis demonstrated similar patterns among LCYs (Fig. 3B). The homology-based 3D structural modeling of SeLCY was analyzed by Swiss Modeling on the basis of the Escherichia coli 3cgvA crystal structure (np_393992.1) and displayed by Swiss-PdbViewer (Fig. 3C). Escherichia coli 3cgvA was regarded as a geranylgeranyl bacteriochlorophyll reductase-like FixC homolog (Ruepp et al. 2000), which indicates that SeLCY may exert its function on the photosynthetic system.

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Fig. 2 Effect of AM on salt tolerance of S. *europaea*. (A) Phenotypes of S. *europaea* grown without AM (control) or with 200 μ M AM (AM-treated) at different NaCl concentration. Measurements of height (B), fresh weight (C), total Chl (E), relative cell death (D) and H₂O₂ content (F–G). The reduced or increased ratio (%) was calculated as follows: [1 – (data from AM treatment/data of control)] × 100 (%). All data are the mean ± SE of six replicates. The same letter on the top of the bar indicates that the values are not significantly different at $P \le 0.05$.

Molecular characterization of the transgenic Arabidopsis

The ORF of *SeLCY* was constructed into the plant expression vector SN1301 through digestion with *Bam*HI and *SacI* and transferred into Arabidopsis via the *Agrobacterium*-mediated method. The transformed plants were verified by PCR. The positive transgenic lines (L2, L3 and L4) were subjected to Northern blot analysis, and the results indicated that *SeLCY* was expressed in transgenic Arabidopsis (**Fig. 3D**).

Enhanced oxidative tolerance in transgenic plants post-germination

It is suggested that the carotenoids in plants are involved in eliminating ROS (Du et al. 2001). Therefore, whether ectopic expression of *SeLCY* conferred enhanced tolerance to oxidative stress was determined in transgenic Arabidopsis on post-germination. To analyze the effect of oxidative stress on plant growth, 4-day-old seedlings on agar plates were exposed to different concentrations (0, 0.3 and 3 μ M) of PQ (paraquat) as an agent producing O₂⁻, causing oxidative stress under normal light (100 μ E m⁻² s⁻¹) conditions.

As shown in **Fig. 4**, no difference was observed with respect to growth or phenotype between the WT and transgenic plants

under normal conditions. Under PQ stress, all plants displayed inhibited growth of the shoot and root, and remarkablely decreased contents of total Chl. However, compared with the WT, transgenic Arabidopsis showed longer roots and higher contents of total Chls under 0.3 or 3 μ M PQ stress and heavier fresh weight under 3 μ M PQ stress (**Fig. 4A, B**). These results indicate that ectopic expression of *SeLCY* conferred enhanced tolerance to oxidative stress in transgenic Arabidopsis on post-germination.

Salt tolerance of the transgenic plants was improved

Four-day-old seedlings were exposed to different concentrations of NaCl for salt tolerance analysis. As shown in **Fig. 4C**, there were comparable root lengths, fresh weights and total Chl contents between transgenic and WT plants under normal and 150 mM NaCl conditions. However, under 200 mM NaCl stress, transgenic plants exhibited greater fresh weight and more total Chl than the WT, indicating that the salt tolerance of transgenic plants on post-germination was improved.

The performance of the SeLCY transgenic plants was examined on recovery from NaCl treatment. Under normal condition, transgenic lines showed no significant difference from the





Fig. 3 Sequence analysis and characterization of SeLCY. (A) Alignment of the deduced amino acid sequences of SeCLY and LCY genes from other species. The LCYs are as follows: LeLCY, *Lycopersicon esculentum* (Solanum lycopersicum; AAG21133.1); DkLCY, *Diospyros kaki* (ACR25158.1); LbLCY, *Lycium barbarum* (AAW88382.1); GlLCY, *Gentiana lutea* (ABK57115.1); CsLCY, *Citrus sinensis* (AAU05146.1) and SeLCY, *Salicornia europaea* (No. AAX19899.1). (B) Phylogenetic analysis of SeLCY and other representative LCYs by Clustal W2 in the EBI website (http:// www.ebi.ac.uk/Tools/clustalw2/index.html). (C) The homology-based 3D structural modeling of SeLCY. (D) Northern blot analysis of SeLCY transgenic plants. 1, represents the WT; 2, 3, 4 and 5 represent different lines of L2, L1, L3 and L4 transformed with *SeLCY*.

WT plants in terms of the phenotype and the survival rate (Fig. 5A, B). After treatment with 200 mM NaCl, almost 80% of the WT plants were dead whereas 80% of transgenic plants survived (Fig. 5A, B). Then the salt-stressed seedlings on plates (Fig. 5A) were further transferred into pots, and their recovery at 8 and 21d was observed. Whether left to recover for 8 or 21d after 200 mM NaCl, the survival ratio was reduced to 8% for the WT plants while the survival ratios of transgenic lines remained at 80% (Fig. 5C, D). These results indicate that the *SeLCY* transformation conferred salt stress tolerance to these plants.

Also, the salt tolerance of 3-week-old seedlings of WT and transgenic Arabidopsis lines of T_3 progeny was investigated by subjecting them to 200 mM NaCl. No significant difference in phenotype was observed between WT and transgenic plants under normal conditions. In contrast, after 2 and 4 d treatment of 200 mM NaCl treatment, the WT plants displayed more serious chlorosis and wilting compared with the transgenic line L4 (**Fig. 6A**). Also the fresh weight of the WT was reduced more significantly than that of transgenic plants (**Fig. 6C**).

Furthermore, as shown in the upper panel of **Fig. 6B**, the cell death induced by NaCl stress was first detected by trypan blue staining, and the dark blue staining of plant cells was more remarkable in leaves of the WT than those of transgenic plants after 2 and 4 d treatment of 200 mM NaCl. Like trypan blue staining, the WT exhibited a marked increase in cell death by Evans blue staining, while a relatively low level of cell death was observed in the transgenic lines after 200 mM NaCl stress (**Fig. 6B**, lower panel).

The photosynthesis parameters were also determined in WT and transgenic plants under normal and 200 mM NaCl stress conditions, respectively. After 200 mM NaCl treatment for 2 and 4 d, higher contents of total Chl were detected in transgenic plants than in the WT, though total Chl decreased in both WT and transgenic plants (**Fig. 6D**). Similar to total Chl, there was no significant difference in the Chl fluorescence ratio (F_v/F_m) between transgenic and WT plants under normal conditions. However, the F_v/F_m in transgenic lines was higher than that in the WT under 200 mM NaCl stress, which indicates higher energy transferring efficiency and PSII activity in transgenic plants (**Fig. 6E**).

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Fig. 4 Effect of PQ and NaCl on Arabidopsis post-germination. (A) Phenotype changes of transgenic lines (L2, L3 and L4) and the WT after PQ treatment. Four-day-old seedlings were grown on MS medium containing 0, 0.3 or 3 μ M PQ for 10 d. (B) Measurement of root lengths, fresh weights and total Chls between transgenic lines (L2, L3 and L4) and the WT after 0, 0.3 or 3 μ M PQ treatments for 10 d. (C) Measurement of root lengths, fresh weights and total Chls between transgenic lines (L2, L3 and L4) and the WT after 0, 150 or 200 mM NaCl treatments for 10 d. The average content of Chl in each line before treatment represents 100% Chl. All data are the mean ± SE of four replicates. The same letter on the top of the bar indicates that the values are not significantly different at $P \le 0.05$.

These results confirmed that the tolerance to salt stress was improved in *SeLCY*-transformed Arabidopsis plants.

Oxidative damage induced by NaCl stress was alleviated in the transgenic plants

To test whether the alleviation of oxidative damage induced by NaCl stress was contributed by transformation of *SeLCY*, a DAB (3,3'-diaminobenzidine) staining assay was carried out on 3-week-old seedlings of WT and transgenic lines. As shown in **Fig. 7A**, the WT exhibited more marked brown DAB staining than transgenic lines after 2 and 4 d treatment of 200 mM NaCl, indicating more serious oxidative damage in WT plants.

Also, the susceptibility to lipid peroxidation of the membranes was determined by analysis of the malondialdehyde (MDA) content. As shown in **Fig. 7B**, there was no significant difference in MDA content between WT and transgenic lines under normal conditions. However, WT plants exhibited higher contents of MDA than transgenic plants after 2 and 4 d treatment of 200 mM NaCl.

These results suggest that the ability to eliminate oxidative damage induced by NaCl was enhanced in transgenic plants.

The contents of photosynthetic pigments were increased in transgenic plants

As a key enzyme at the branch point in the pathway of carotenoid biosynthesis, β -lycopene cyclase initiates the β -branch and shares the processing of the α -branch (**Fig. 1**). Therefore, many photosynthetic pigments of 3-week-old seedlings involved in the α -branch and β -branch were extracted and determined by HPLC to investigate whether transformation of *SeLCY* into Arabidopsis leads to accumulation of pigments in the α -branch and β -branch under normal condition or 200 mM NaCl stress. The contents of β -carotene were 50 mmol mol⁻¹ Chl *a* in L2, 49 mmol mol⁻¹ Chl *a* in L3 and 57 mmol mol⁻¹ Chl *a* in L4, much higher than the 40 mmol mol⁻¹ Chl *a* in the WT under normal conditions (**Fig. 8A**). Although β -carotene in all 3-week-old plants used in our tests decreased after 200 mM NaCl treatment, the transgenic plants displayed a higher content of β -carotene than WT plants (**Fig. 8A**).

The determination of other photosynthetic pigments, including xanthophyll cycle pool members (zeaxanthin, antheraxanthin and violaxanthin), neoxanthin and lutein, showed that the contents of these components in transgenic plants



Fig. 5 Recovery of the SeLCY transgenic plants from salt stress. (A) Growth of the transgenic seedlings in MS medium with 200 mM NaCl for 16 d. (B) Survival rate of the seedling under 200 mM NaCl stress in A. (C) Comparison of the recovery of the plants in pots after 200 mM NaCl stress. Plants from NaCl treatments in A were transferred into pots and the pictures were taken at 8 and 21 d after transfer. (D) Survival rate of the plants in pots after recovery from (C); the results at 8 and 21 d after recovery are the same. All data are the mean \pm SE of three replicates. The same letter on the top of the bar indicates that the values are not significantly different at $P \le 0.05$.

were significantly higher than in the WT under both normal and 200 mM stress conditions (**Fig. 8B–F**).

Taken together, the transgenic Arabidopsis exhibited increased contents of photosynthetic pigments of carotenoid synthesis, including those of the α -branch and β -branch, which was mirrored by increasing contents of lutein (the final product of the α -branch biosynthetic pathway) and β -carotene, xanthophylls and neoxanthin (the products of the β -branch) under both normal and 200 mM NaCl stress conditions (**Fig. 8**). These results demonstrated that SeLCY enhances carotenoid synthesis in transgenic Arabidopsis.

SeLCY and AtLCY improved salt tolerance in transformed tobaccos

For comparing the difference between *SeLCY* and *AtLCY*, we need to use mutant lines with either T-DNA or transposon insertions in the *AtLCY* gene. There are two transcripts of *AtLCY* in Arabidopsis: *AtLCY-1* (AT3G10230.1) and *AtLCY-2* (AT3G10230.2), while specific knocked down mutants of AtLCY (AtLCY-1/-2) are not available. Therefore, *AtLCY-1*, *AtLCY-2* and *SeLCY* were constructed into a viral vector, as pCAPE2-AtLCY-1 (A1), pCAPE2-AtLCY-2 (A2) and pCAPE2-SeLCY (S) (**Supplementary Fig. S1A**). They were expressed in *N. benthamiana* using the PEBV-mediated ectopic gene expression system for functional comparison of *LCY* genes.

The PEBV system was achieved successfully through agroinfiltration of *N. benthamiana* plants with pCAPE1 and pCAPE2 derivative clones. Reverse transcription–PCR (RT–PCR) detection showed that the infection rate of *N. benthamiana* plants after agroinfiltration of pCAPE1 and pCAPE2 derivative clones was consistently 100% (**Supplementary Fig. S1B**).

The leaf discs detached from transformed tobaccos were immersed in water with or without 200 mM NaCl for 3 d. As shown in **Fig. 9A**, there is no significant difference between *YFP*- (yellow fluorescent protein) and *LCY*-transformed leaf discs under control conditions. However, after 200 mM NaCl treatment for 3 d, the *YFP*-transformed leaf discs exhibited more serious bleaching and wilting (**Fig. 9A**, **C**) as well as higher cell death (**Fig. 9D**) compared with those transformed by *LCY* genes. The generation of ROS probed by 2', 7'-dichlorofluorescin diacetate (H2DCFDA) was only observed around the edges in all leaf discs under control conditions, but the *YFP*-transformed leaf discs exhibited more diffuse ROS fluorescence than those transformed by *LCY* genes (A1, A2 and S in **Fig. 9**) under 200 mM NaCl stress (**Fig. 9B**), which was consistent with quantitative H₂O₂ determination (**Fig. 9E**).

Although transformation of SeLCY, AtLCY-1 and AtLCY-2 conferred enhanced salt tolerance in tobacco, the Chl content, cell death and H_2O_2 concentration among them in transformed leaf discs showed no significant difference under 200 mM NaCl stress.





Fig. 6 Analysis of NaCl tolerance in transgenic plants. (A) Continuously changing phenotypes of transgenic line L4 and the WT under NaCl treatment. Thirty-day-old seedlings were grown under 200 mM NaCl stress for 0, 2 or 4 d. (B) Detection of cell death induced by NaCl in leaves of Arabidopsis. Plant cell death was monitored by staining the leaves of WT and transgenic plants of line L4 with Trypan blue (upper panel). Leaves were harvested 0, 2 and 4 d after 200 mM NaCl stress. Every image is representative of nine stained leaves from three seedlings. The experiment was repeated in other transgenic lines with similar results. Cell death measurement by Evans blue staining of detached leaves from the WT and transgenic plants after 200 mM NaCl treatment for 0, 2 and 4 d (lower panel). The relative cell death was determined by comparison with the value at 0 d, which was indicated as 1.0. All data are the mean ± SE of five replicates. (C–E) Measurement of fresh weights, total ChIs and F_v/F_m between transgenic lines (L2, L3 and L4) and the WT after 200 mM NaCl treatments for 0, 2 and 4 d. The average content of ChI in each line before treatment represents 100% ChI. All data are the mean ± SE of five replicates. The same letter on the top of the bar indicates that the values are not significantly different at $P \le 0.05$.

Discussion

The plant carotenoid biosynthesis pathway has been well documented (Romer and Fraser 2005, Galpaz et al. 2006). Also, it was reported that carotenoid metabolic engineering in plants has been undertaken to enhance the nutritional value of staple crops (Alquezar et al. 2009, Ji et al. 2009), but other functions of carotenoids are poorly understood. Recently, more and more attention has been paid to improving the tolerance to abiotic stress by the strategy of carotenoid metabolic engineering. Overexpression of the ε -lycopene cyclase gene in Arabidopsis increased the lutein level and the rate of NPQ (non-photochemical quenching) induction under high light (Pogson and Rissler 2000). Expression of a bacterial β -carotene hydroxylase gene (*crtZ* or *chyB*) increased xanthophylls and tolerance to UV and high light stress in tobacco and





Fig. 7 Detection of oxidative damage in transgenic lines and the WT after 200 mM NaCl treatment. (A) DAB staining of transgenic lines and the WT on 0, 2 or 4 d after 200 mM NaCl treatment. Every image is representative of nine stained leaves from three seedlings. (B) Measurement of MDA in transgenic lines and the WT. All data are the mean \pm SE of five independent replicates. The same letter on the top of the bar indicates that the values are not significantly different at $P \leq 0.05$.

Arabidopsis, respectively (Davison et al. 2002, Gotz et al. 2002). Expression of the phytone synthase gene *SePSY* conferred increased salt and oxidative tolerance in transgenic Arabidopsis (Han et al. 2008). As shown in **Fig. 2**, inhibition of lycopene cyclization of the carotenoid synthesis pathway affected the salt suitability of *S. europaea*. The effect of AM was known to be an inhibition of carotenoid biosynthesis via tetrapyrrole biosynthesis by blocking lycopene cyclization, and the photodynamic property of the tetrapyrrole precursors accumulated led to photooxidation of plastid components (Dalla Vecchia et al. 2001, La Rocca et al. 2001). That is why the growth inhibition was shown in control plants after AM

treatment (**Fig. 2A**). Comparatively, AM-treated plants of S. *europaea* exhibited more serious damage under 200 and 400 mM NaCl stresses, the optimal growth conditions (**Fig. 2E–G**). These results indicated that the lycopene cyclization of carotenoid synthesis plays an important role in salt tolerance of S. *europaea*. Therefore, we cloned a β -lycopene cyclase gene SeLCY from S. *europaea* and stably expressed it in Arabidopsis to confirm its function (**Fig. 3**). The transgenic plants exhibited enhanced salt tolerance compared with the WT (**Figs. 4–6**), indicating that genetic manipulation of β -lycopene cyclase could generate modified crops with improved salt tolerance.

Salinity is a major environmental stress that limits crop productivity in arid and semi-arid regions of the world (Munns et al. 2006). Many pieces of evidence suggested that protection against oxidative stress improves resistance to many abiotic stresses, including salt (Wu et al. 2008, Luo et al. 2009). Overexpression of antioxidant genes of cytosolic ascorbate peroxidase, dehydroascorbate reductase or copper/zinc superoxide dismutase conferring high salinity tolerance in transgenic Arabidopsis and tobacco has been reported by Lu et al. (2007) and Young-Pyo et al. (2007). As shown in Figs. 4 and 7, we reported that the SeLCY transgenic Arabidopsis displayed enhanced tolerance to oxidative stress induced by PQ on post-germination, and by NaCl in 3-week-old seedlings which were mirrored by less H₂O₂ staining and MDA content, respectively, in transgenic plants. Our conclusion that the alleviation of oxidative damage induced by salt stress contributed by SeLCY transformation in Arabidopsis is consistent with the result of increased salt and oxidative tolerance in transgenic Arabidopsis by overexpression of the phytoene synthase gene (Han et al. 2008).

As major sources of ROS in plants, chloroplasts accumulate an amount of ROS produced in response to many physiological stimuli such as UV stress and high salinity which can cause decreases in photosynthetic activities and pigment degradation (Debez et al. 2008). However, the carotenoids in plants play an important role in harvesting light energy, scavenging the ROS produced in the photosynthetic apparatus and protecting the photosynthesis system from damage (Bode et al. 2008). There is much evidence to prove that increased carotenoids, whose importance in oxygenic photosynthesis is well known, can contribute to maintaining the stability of the photosynthesis system in plants (Hansen et al. 2002). Our results demonstrated that ectopic expression of SeLCY in Arabidopsis conferred increased contents of carotenoid pigments such as lutein, β -carotene, neoxanthin and the xanthophyll cycle pool (violaxanthin + antheraxanthin + zeaxanthin) compared with the WT under normal conditions. Under 200 mM NaCl stress, although the carotenoid photopigments of all tested plants were reduced, the transgenic plants maintained higher contents of carotenoids than the WT (Fig. 8). In higher plants, β -carotene binds to reaction center subunits of both PSI and PSII, while pigments of the xanthophyll cycle pool are both accessory pigments and structural elements of light-harvesting complexes.

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Fig. 8 Contents of carotenoids in transgenic and WT plants. (A–F) Contents of (A) β -carotene, (B) lutein, (C) neoxanthin, (D) antheraxanthin, (E) violaxanthin and (F) the xanthophyll cycle pool (Z, A and V indicate zeaxanthin, antheraxanthin and violaxanthin, respectively) were measured in transgenic and WT plants treated with 200 mM NaCl for 0, 2 and 4 d. All data are the mean ± SE of five independent replicates. The same letter on the top of the bar indicates that the values are not significantly different at $P \leq 0.05$.

The xanthophylls (violaxanthin, antheraxanthin and zeaxanthin) with β -carotene as chromophore exert functions in photosynthetic electron transport, and as photoprotectants of the photosynthetic apparatus from ROS (Dall'Osto et al. 2007b). The neoxanthin plays an important part in preserving PSII from photoinactivation and protecting membrane lipids from photooxidation by ROS under abiotic stress conditions (Dall'Osto et al. 2007a). Also, it is reported that the α -branch (lutein) of carotenoid metabolism has distinct and complementary roles in antenna protein assembly and in the mechanisms of photoprotection (Dall'Osto et al. 2007b). Thus, it is understandable that the increased endogenous contents of carotenoids in the transgenic plants results from higher PSII activity and less ROS damage than in the WT under 200 mM NaCl stress (**Figs. 7, 8**).

There are two transcripts of *AtLCY* in Arabidopsis; however, no mutant with a single transcript is available. The PEBV virus belongs to the same genus as tobacco ratlle virus (TRV) with the ability to silence genes efficiently in *N. benthamiana* (Constantin et al. 2004). Therefore, in this study, we compared the functional difference between *SeLCY* and *AtLCY* using the PEBV system in *N. benthamiana*. As shown in **Fig. 9**, there is no significant difference between *LCY* genes from the halophyte and glycophyte under salt stress. The functional fulfillment of the gene involves initiation of signal transduction, mRNA

transcription and translation, protein modification and later phenotype performance related to metabolic processes (Barton and Scheres 2000). In addition, much evidence shows that the difference between the halophyte and glycophyte could not be just contributed by a single gene, but the complex pathway comprised of a series of functional genes responsive to salt stress (Taji et al. 2004, Oh et al. 2010). Therefore, further work to understand the high salt tolerance in *S. europaea* should focus on the underlying responses of the pathway and the complex network in this plant.

Materials and Methods

AM treatment

Seedlings of S. *europaea* were grown in a greenhouse maintained at a thermoperiod of day/night temperature of $25/20^{\circ}$ C, photoperiod 16 h and a relative humidity of $50 \pm 10\%$, and irrigated weekly with 1/2 Hoagland nutrient solution. Twenty days later, the plants were divided into two groups. One group was treated with 200 μ M AM, while the other group was not (Dalla Vecchia et al. 2001). NaCl concentrations of 0, 200 and 400 mM in irrigation solutions were applied for different treatments. The leaf discs were detached for analysis after AM treatment for 15 d.





Fig. 9 The effects of ectopic expression *LCY* genes on salt tolerance in transformed tobacco. (A) Phenotypes of leaf discs transformed by YFP (YFP), *AtLCY-1* (A1), *AtLCY-2* (A2) and *SeLCY* (S) in normal and 200 mM NaCl conditions. (B) Production of ROS in leaf discs in normal and 200 mM NaCl conditions was assayed by an ROS-sensitive probe H2DCFDA. The left side of each column is the visible light field and the right side is the UV light field. The scale bar = 500 μ m. Measurement of total Chl (C), cell death (D) and H₂O₂ content (E) under normal and 200 mM NaCl conditions. The average content of Chl in each line before treatment represents 100% Chl. All data are the mean ± SE of six independent replicates. The same letter on the top of the bar indicates that the values are not significantly different at $P \le 0.05$.

Isolation and sequence analysis of SeLCY

Total RNA was extracted from young shoots of *S. europaea* using Trizol reagent (Gibco-BRL). The first-strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen). Single-stranded cDNA was subjected to PCR amplification with the following degenerate primers: 5'-TGTTTGGG T(G/T)GATGA(A/G)TT(T/C)G-3' and 5'-AA(C/A)CCATGCCA ATAA(T/C)G(T/A)GG-3'. The RACE approach was used to isolate the 5' and 3' ends of the *SeLCY* gene using a kit from

Invitrogen according to the manufacturer's instructions. A putative full-length *SeLCY* cDNA was obtained by PCR using a specific pair of primers at the 5' end, 5'-CAC TCA GCC ACA ACA ACC ATT-3', and 3' end, 5'-ACG TAT CAA CAG AGT GTA TTG-3'. The cDNA product was cloned into pEasy-T vector (TransGen) and sequenced. Multiple sequence alignment and phylogenetic analysis were performed on the website: http:// www.ebi.ac.uk/Tools/clustalw2/index.html. The 3D structures of SeLCY and the chloroplastic transit peptide were predicted

LCYs improve salt tolerance in transgenic plants

on the websites of http://swissmodel.expasy.org/ and http:// www.cbs.dtu.dk/services/ChloroP/, respectively.

Plasmid construction and plant transformation

A plasmid was constructed for sense expression of the *SeLCY* gene by cloning it at *Bam*HI sites into SN1301, a plant expression vector carrying a cauliflower mosaic virus 35S (CaMV35S) promoter and nos terminator. The resultant plasmid was introduced into *Arabidopsis thaliana* (ecotype: Columbia-0) by *Agrobacterium tumefaciens-* (strain LBA4404) mediated transformation using the floral dip method (Clough and Bent 1998). The seeds collected were screened in Murashige and Skoog (MS) medium supplemented with 20 mg l⁻¹ hygromycin, and transgenic seedlings were identified by PCR. Total RNA was extracted from the seedling of the T₂ generation, and Northern blot was carried out following the method described by Sambrook et al. (1989).

Root and shoot growth assay post-germination

Four-day-old seedlings of the WT and three lines of transgenic Arabidopsis plants were placed on 1.5% agar plates containing PQ at different concentrations (0, 0.3 and 3 μ M) or NaCl (0, 150 and 200 mM). Plates were maintained vertically under normal conditions. The increased root length and shoot weight were measured on the 10th day after the seedlings were transferred to plates.

Experiments for salt tolerance analysis

Seeds from A. *thaliana* Columbia (Col-0) ecotype and SeLCY transgenic lines were sown on MS medium, stratified at 4° C for 3 d and incubated at 22° C under continuous light. Seven-day-old seedlings were transferred onto MS medium containing 200 mM NaCl and maintained for 16 d. These plants were further transferred into pots containing a commercial soil mix and grown under normal conditions for 8 and 21 d. Pictures were taken and the survival rates of these plants were evaluated at different periods.

Homozygous T_3 plants were grown in plastic pots containing a mixture of vermiculite, turf and humus (1:1:1, by vol.), and grown in a growth chamber at $23 \pm 1^{\circ}$ C under a 12 h photoperiod. Three-week-old seedlings of WT plants and three transgenic lines were treated with 200 mM NaCl. After 0, 2 and 4 d salt treatment, the fresh weight, MDA and photosynthesis parameters were determined, respectively.

The measurement of MDA content on the fourth to seventh rosette leaves was based on the method of Parida et al. (2004). The PSII activity was determined on the fourth to seventh rosette leaves with a portable modulated fluorometer (PAM-2000, Walz), and expressed as F_v/F_m to assess damage to the leaf photosynthetic apparatus.

The leaf discs from transformed tobacco were detached by a plastic borer for salt tolerance analysis. Leaf discs immersed in 200 mM NaCl solution were vacuum infiltrated for 30 min, and then maintained at room temperature. After 3 d NaCl

treatment, the Chl content, cell death and $\rm H_2O_2$ concentration were determined.

Measurement of Chl content

Chl was extracted at 4° C using acetone, and determined by the method of Arnon (1949).

H₂O₂ determination

 $\rm H_2O_2$ was observed with a fluorescence microscope (AXIOSKOP40, Zeiss) using $10\,\mu M$ H2DCFDA as a probe (Molecular Probes/Invitrogen). The dye was loaded for 30 min, and analyzed by 485 nm excitation and 535 nm emission. In addition, H_2O_2 was detected by the DAB (1 mg ml^{-1}) uptake method (Hernandez et al. 2004). The content of H_2O_2 was determined as described by Bellincampi et al. (2000).

Cell death determination

Cell death was examined by Evans blue staining as described by Shi et al. (2007) with minor modifications. Briefly, the fourth to seventh rosette leaves were vacuum infiltrated in 0.1% Evans blue (w/v) for 15 min and then maintained for 8 h under vacuum. After staining, unbound dye was removed by extensive washing. The dye bound to dead cells was solubilized in 50% (v/v) methanol, 1% SDS for 30 min and quantified by A_{600}/A_{680} .

Cell death was examined by Trypan blue staining as described by Felix Mauch's Group protocols (http://common-web.unifr.ch/biol/pub/mauchgroup/staining.html).

Measurement of pigments

After 0, 2 and 4 d salt treatment, leaf samples (the fourth to seventh rosette leaves) were taken at midday. Samples were extracted in ice-cold 100% acetone and the pigment extracts were filtered through a 0.45 μ m membrane filter (Lu 2003). Pigments were separated and quantified by HPLC as described by Thayer and Bjorkman (1990).

PEBV-mediated ectopic gene expression

PEBV, an RNA virus, has a genome consisting of RNA1 and RNA2. RNA1 encodes all viral proteins required for replication and movement of the virus and can produce infection, while RNA2 encodes the coat protein and proteins needed for nematode transmission (Constantin et al. 2004). The PEBV system consists of two binary vectors, pCAPE1 and pCAPE2, with pCAPE2 adapted for insertion of heterologous sequences to mediate ectopic gene expression in agroinfiltrated plants (Constantin et al. 2004). Three new pCAPE2 derivative clones were prepared using pCAPE2-YFP as the cloning vector (Qian et al. 2007). AtLCY-1 and AtLCY-2 were cloned into pCAPE2 at the Ncol site, and SeLCY at the Sacl site (Supplementary Fig. S1A). Agroinfiltration of N. benthamiana plants (4 weeks old) with pCAPE1 was in combination with pCAPE2-AtLCY-1 (A1), pCAPE2- AtLCY-2 (A2) and pCAPE2-SeLCY (S). After 3 weeks agroinfiltration, the plants infected with PEBV:YFP, PEBV:AtLCY-1, PEBV:AtLCY-2 and PEBV:SeLCY did not differ



significantly from the uninfected plants in either height or numbers of leaves. PEBV-mediated YFP expression in *N. benthamiana* was observed using a fluorescence microscope, and ectopic expression of *LCY* genes in *N. benthamiana* was confirmed by RT–PCR (**Supplementary Fig. S1B**).

Statistical analysis

The SPSS 13.0 statistical package was used for the statistical analysis. The significance was tested using the least significant difference (LSD) at the 5% level and in the figures the same letter on the top of a bar indicates that the values are not significantly different at $P \le 0.05$.

Supplementary data

Supplementary data are available at PCP online.

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