Plant Biotechnology 35, 303–312 (2018)
DOI: 10.5511/plantbiotechnology.18.0620a

Original Paper

Functional analyses of lipocalin proteins in tomato

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Received May 31, 2018; accepted June 20, 2018 (Edited by T. Mizoguchi)

Abstract In this study, two temperature-induced lipocalin genes *SlTIL1* and *SlTIL2*, and a chloroplastic lipocalin gene *SlCHL* were isolated from 'Micro-Tom' tomato. The coding sequences of *SlTIL1*, *SlTIL2* and *SlCHL* were 558, 558, and 1002 bp, respectively. By TargetP analysis, no characteristic transit peptides were predicted in the proteins of SlTIL1 and SlTIL2, while a chloroplastic transit peptide was predicted in the protein of SlCHL. The subcellular localization results indicated that SlTIL1 and SlTIL2 proteins were major localized in the plasma membrane, while SlCHL was localized in chloroplast. To understand the function of lipocalins, transgenic tomato over-expressed *SlTIL1*, *SlTIL2* and *SlCHL* and their virus-induced gene silencing (VIGS) plants were generated. The phenotypes were significantly affected when the *SlTIL1*, *SlTIL2* and *SlCHL* were over-expressed or silenced by VIGS, which suggested that the three lipocalins played important roles in regulating the growth and development of tomato. In addition, the level of ROS (O_2^- and H_2O_2) was low in *SlTIL1*, *SlTIL2* and *SlCHL* over-expressed plants, while it was high in their silenced plants. The changes in the expression of *SOD*s were consistent with the accumulations of ROS, which indicated that lipocalins might have an important role in abiotic oxidative stress tolerance in tomato plants. Especially SlTIL1 and SlTIL2 are localized around their membranes and protect them from ROS. The results will contribute to elucidating the functions of lipocalin in plants, and provide new strategies to improve the tolerance to abiotic stress in tomato plants.

Key words: lipocalins, *SlCHL*, *SlTIL*, tomato, virus-induced gene silencing.

Introduction

Lipocalins are a large family of ligand-binding proteins widely distributed in bacteria, invertebrate and vertebrate, as well as plants. Members of the lipocalin protein family exhibit great sequence diversity at the amino acid level; however, they are highly conserved in the crystal structure. The common crystal structure of lipocalins is a symmetrical all-β protein with an eight-stranded anti-parallel β-sheet that folds back on itself to form a hydrogen-bonded β-barrel. The β-barrel is in a flattened or elliptical shape and encloses an internal ligand-binding site, which enables the lipocalins to bind to the small hydrophobic molecules and cell-surface receptors, and form complexes with soluble macromolecules (Flower 1996). Because of the molecular-recognition properties, the lipocalins fulfill a variety of biological functions, including transport of small lipophilic molecules, immunomodulation, the mediation of cell homoeostasis, signal transduction, and responses to stress (Bishop 2000; Flower et al. 2000; Grzyb et al. 2006; Malnoë et al. 2018). Recently, a growing number of studies suggested that lipocalins played important roles in human diseases, and they were potential a biomarker and a modulator of human cancers (Xu and Venge 2000).

To date, although significant progresses have been made in understanding the functions of lipocalins in humans and animals, the studies on plant lipocalins are still limited. In plants, data mining of genomic databases suggested that two types of lipocalins, temperature-induced lipocalins (TIL) and chloroplastic lipocalin (CHL) were existed. Phylogenetic analyses showed that plant lipocalins exhibited homology with the bacterial outer membrane lipoprotein (Blc), the insect protein Lazarillo and the mammalian apolipoprotein D (ApoD),

Abbreviations: ApoD, apolipoprotein D; Blc, bacterial outer membrane lipoprotein; CHL, chloroplastic lipocalin; PDS, phytoene desaturase; ROS, reactive oxygen species; sGFP, synthetic green fluorescent protein; SOD, superoxide dismutase; TIL, temperature-induced lipocalin; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing.

This article can be found at http://www.jspcmb.jp/
Published online October 25, 2018

which indicated that these proteins might evolve from a common origin (Charron et al. 2005). In plant cells, the subcellular localization is different between the TIL and CHL. TIL is a plasma membrane protein, while CHL is localized in the thylakoid lumen. Although localized in different compartments, both TIL and CHL are stress-inducible proteins, and they play an important role in the protection of plants from the abiotic stresses (Boca et al. 2014; Chi et al. 2009; Levesque-Tremblay et al. 2009). In Arabidopsis, AtTIL is constitutively expressed in all tissues except for dry seeds, and its expression was further increased by heat shock treatment (Charron et al. 2008; Chi et al. 2009). Different from AtTIL, AtCHL is not sensitive to low and high temperature conditions, but it is significantly induced by high light and drought stresses. AtCHL represents a rapid response to the stresses, and its protection effect against high light mainly occurred at the early stages of stress conditions in Arabidopsis (Levesque-Tremblay et al. 2009). In addition, AtTIL and AtCHL have overlapping functions in decreasing lipid peroxidation, which is a key mechanism that lipocalins protect plants from the abiotic stresses (Boca et al. 2014).

Tomato (*Solanum lycopersicum*) is an economically important food worldwide. In tomato, two lipocalins (GenBank DQ222988 and DQ222981) were identified on the basis of their homology to the wheat TaTIL-1 and Arabidopsis AtTIL proteins (Charron et al. 2005). To date, however, the functions of the lipocalins in tomato were still unclear. The tomato cultivar 'Micro-Tom,' which was produced by crossing Florida Basket and Ohio 4013-3 cultivars, is not only an ideal house plant for home gardening, but also a good model cultivar for tomato research with some advantages, such as small size, short life cycle, easy fruit setting, easy to grow and capacity to grow under fluorescent lights at a high density.

To better understand the mechanism of plastid differentiation from chloroplast to chromoplast, we analyzed and compared plastid proteome and plastid morphologies with 'Micro-Tom' and two other varieties, 'Black' and 'White Beauty.' When we compared plastid proteome of 'Micro-Tom' with 'Black' and 'White Beauty' using the two-dimensional gel electrophoresis, we detected the differences of spot number and isoelectric points of TIL in 'Black' and 'White Beauty' (Suzuki et al. 2015). In this study, to investigate the lipocalins in tomato, we isolated two temperature-induced lipocalins (*SlTIL1* and *SlTIL2*) and one chloroplastic lipocalin (*SlCHL*) from 'Micro-Tom,' and their characterization and expression patterns were analyzed in 'Micro-Tom.' In addition, to further elucidate the roles of the lipocalins in tomato, the functions of *SlTIL1*, *SlTIL2*, and *SlCHL* were investigated using the transgenic tomatoes that over-expressed *SlTIL1*, *SlTIL2*, and *SlCHL* and tomatoes in which *SlTIL1*, *SlTIL2*, and *SlCHL* were silenced using virus induced gene silencing (VIGS) in this study.

Materials and methods

Plant materials

'Micro-Tom' (*Solanum lycopersicum* cv 'Micro-Tom') was used in this study. 'Micro-Tom' seeds were provided by Professor Hiroshi Ezura and Professor Tsuyoshi Mizoguchi (Tsukuba University, Japan). According to the days post anthesis (DPA), the ripening process of 'Micro-Tom' fruit is divided into four ripening stages: green fruit stage (30–33 DPA); yellow fruit stage (32–33 DPA); orange fruit stage (33–35 DPA); and red fruit stage (41–45 DPA) (Suzuki et al. 2015). The leaves, flowers, roots, and fruits of different ripening stages were obtained and immediately frozen in liquid nitrogen, and kept at −80°C until used.

Nicotiana tabacum (SR1) and *Nicotiana benthamiana* were used for analyzing the subcellular localization of SlTIL1, SlTIL2 and SlCHL. All plants ('Micro-Tom,' *N. tabacum*, and *N benthamiana*) were grown under at 24–28°C with 60±10% relative humidity under a 16 h light/8 h dark cycle. Plants were grown at pot soil and hydroponic system using half diluted Enshi formula (808 mg l^{-1} KNO_3, 492 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 944 mg l^{-1} $Ca(NO_3) \cdot 4H_2O$, 152 mg l^{-1} $NH_4H_2PO_4$, and 50 mg l^{-1} Otsuka house 5 (Otsuka AgriTechno. Co., Ltd, Japan).

For study plants light response, 1 week after transplanted (WAT) transgenic tomato plants were moved into a growth chamber with temperature 24–28°C with 60±10% relative humidity under light condition 1 (405 μmol $m^{-2} s^{-1}$) and light condition 2 (200 μmol $m^{-2} s^{-1}$). Photoperiodic lighting (16 h light/8 h dark cycle) programs was used in this study.

RNA extraction and RT-PCR analyses

Total RNA was isolated from leaves, roots, flowers, and fruits of 'Micro-Tom' using a RNeasy mini kit (QIAGEN Co., Ltd., Germany) following the manufacturer's instructions and treated extensively with DNase I (TAKARA Co., Ltd., Japan). First strand cDNA was synthesized from 1 μg purified RNA using PrimeScript™ first-strand cDNA synthesis kit (TAKARA Co., Ltd.). PCR was carried out under standard conditions included 30 cycles of 10 s at 98°C, 15 s at 56°C, and 1 min at 72°C, using the lipocalin primers as shown in Table 1. In the present study, *ACTIN* was used as a reference gene in order to normalize the gene expression results, using *ACTIN* primers as shown in Table 1. RT-PCR analyses were performed in three replicates.

Constructs for the expressing fusion proteins of SlTIL1, SlTIL2 and SlCHL and synthetic green fluorescent protein (sGFP)

The full length cDNA clones of *SlTIL*s (LEFL2018O11 and LEFL1002CA07) and *SlCHL* (LEFL2044D22) were provided by Professor Koh Aoki (KAZUSA DNA Institute, Japan) (Aoki et al. 2010). The coding regions of *SlTIL1* (LEFL2018O11), *SlTIL2* (LEFL1002CA07) and *SlCHL* (LEFL2044D22) were

added to a 4-bp sequence (CACC) on the N terminus and cloned into pENTR/D-TOPO vector (Invitrogen, USA). The coding regions of *SlTIL1*, *SlTIL2*, and *SlCHL* were amplified using the primers as shown in Table 2. The sequences were subcloned into pUGW5 and pUGW6 vectors consisting of a synthetic GFP (sGFP) by using the Gateway LR recombination reaction system (Invitrogen). The pUGW5 and pUGW6 vectors were provided by Professor Tsuyoshi Nakagawa (Center for Integrated Research in Science, Shimane University, Japan).

Observation of subcellular localization of SlTIL1, SlTIL2 and SlCHL

The plasmid DNA of 35Sp::sGFP-SlTIL1, 35Sp::sGFP-SlTIL2, 35Sp::sGFP-SlCHL, 35Sp::SlTIL1-sGFP, 35Sp::SlTIL2-sGFP, and 35Sp::SlCHL-sGFP were introduced into onion (*Allium cepa*) epidermal cells, *N. tabacum* (SR1) and *N. benthamiana* leaf cells by a particle bombardment PDS-1000 System (Bio-Rad, Hercules, CA, USA). Transient expression of the sGFP-SlTIL1, sGFP-SlTIL2, sGFP-SlCHL, SlTIL1-sGFP, SlTIL2-sGFP, and SlCHL-sGFP fusion proteins in *N. tabacum* (SR1) and *N. benthamiana* leaf cells was observed by a confocal laser scanning microscopy (Leica SP, Solms, Germany and LSM 700, Carl Zeiss, Germany). The methods of particle bombardment and observation of sGFP signals were carried out as described by Motohashi et al. (2001).

Generation of over-expression of SlTIL1, SlTIL2 and SlCHL transgenic tomato plants

The coding regions of *SlTIL1*, *SlTIL2* and *SlCHL* in pENTR/D-TOPO vector were subcloned into pGWB8 vector by using the Gateway LR recombination reaction system (Invitrogen), to construct an expression plasmid pGWB8-*SlTIL1*, pGWB8-*SlTIL2* and pGWB8-*SlCHL*. pGWB8 binary vector was provided by Professor Tsuyoshi Nakagawa (Center for Integrated Research in Science, Shimane University). Transgenic tomato plants (35Sp::*SlTIL1*, 35Sp::*SlTIL2* and 35Sp::*SlCHL*) were generated using *Agrobacterium tumefaciens* strain GV 3101 harboring pGWB8-35Sp::*SlTIL1*, pGWB8-35Sp::*SlTIL2*, and pGWB8-35Sp::*SlCHL*. The construct and transformation protocol of transgenic over-expressed *SlTIL1*, *SlTIL2* and *SlCHL* were described previously (Sun et al. 2006).

Genomic DNAs was extracted with DNeasy Plant Mini Kit (QIAGEN Co., Ltd.) from the leaves of the non-transgenic and transgenic plants and then were used in PCR analyses. PCR amplification was done with the forward primers of *SlTIL1*, *SlTIL2* and *SlCHL* as shown in Table 1 and reverse primer of 6xHis (5′-ATG ATG ATG ATG ATG-3′). RT-PCR analyses were done to confirm transgenic plants using the primers as shown in Table 1. The ploidy level of regenerated plants was determined by flow cytometry (Beckman Coulter EPICS XL-MCL, USA).

Generation of SlTIL1, SlTIL2 and SlCHL silenced tomato plants

TRV-based vectors pTRV1, pTRV2 and pTRV2-*SlPDS* were provided by Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org/abrc/catalog/individ_cloned_gene_1.html). pTRV2-VIGS recombinant plasmids were

Table 1. Primers used for RT-PCR.

Gene name	Forward	Reverse
SlTIL1	ATGGCTACAAAAGTAATGGAAGTG	CTATTTTCCAAGGATTGATTTG
SlTIL2	ATGACCACAAAAGAGATGGAAGTA	CTATTTTCCCAATATTGATTTGATCC
SlCHL	CACCATGGTTTGCTACAATTTGGTGGCCC	CTTGAAATATAGTTCAGCAAGTTTC
SlSOD1	TCTGGCCTAAAACCTGGACT	ACCAGTGAGAGGAATCTGCT
SlSOD3	CTCCTGGACTTCACGGGTTT	CACAAGTGCTCGTCCAACAA
SlSOD6	AGGACAGCCATCTGGTGAAC	TGGCGAGTAATCCCAAACGA
SlPDS	TAACTGCCAAACCACCACAA	ACAGGTTCTGAATATTTGGGTAAGC
ACTIN	AGATGGTGTCAGCCACACAG	ACCACCACTGAGGACGATGT

Table 2. Primers used for making constructs of the expressing fusion proteins.

Gene name	Forward	Reverse
SlTIL1	CACCATGGCTACAAAAGTAATGGAAG	*(CTA)TTTTCCAAGGATTGATTTGAT
SlTIL2	CACCATGACCACAAAAGAGATGGAAGTA	*(CTA)TTTTCCCAATATTGATTTGATCCACC
SlCHL	CACCATGGTTTGCTACAATTTGGTGGCCC	*(CTA)CTTGAAATATAGTTCAGCAAGTTTC

Note: *(CTA) means the stop codon. The primers without stop codon were used for pUGW5 vector, and the primers with stop codon were used for pUGW6 vector.

Table 3. Primers used for making silenced tomato plants.

Gene name	Forward	Reverse
SlTIL1 (505 bp)	GAATTCGGAAGTGGTGAAAAATCTTGA	GGATCCTTTGGTGTCTTTGGGGCTATC
SlTIL2 (326 bp)	GAATTCTGAAGGGACTGCCTATAAAGCTG	GGATCCCTTTAGTGTCCTTTGGGGCATC
SlCHL (360 bp)	GAATTCTTGCGGCATCAATCCTATT	GGATCCTGCCTGTGATGTAGCCATCAG
SlPDS (286 bp)	TAACTGCCAAACCACCACAA	ACCCATTGATTCGCTACCAG

constructed by inserting fragments of *SlTIL1*, *SlTIL2*, and *SlCHL* cDNA into pTRV2 vector. Fragments of these cDNA were amplified using the primers as shown in Table 3. Each PCR fragment was inserted into the digested pTRV2-vector resulting in pTRV2-*SlTIL1*, pTRV2-*SlTIL2* and pTRV2-*SlCHL* vectors. pTRV2-*SlPDS* was used as a positive control for VIGS. PDS is a rate-limiting enzyme in the carotenoid biosynthetic pathway (Fu et al. 2005; Orzaez et al. 2006), when we silenced an endogenous *PDS* in tomato using pTRV1 and pTRV2-*SlPDS*, tomato plants showed photobleached phenotypes.

pTRV1, pTRV2-*SlPDS*, pTRV2-*SlTIL1*, pTRV2-*SlTIL2*, and pTRV2-*SlCHL* were transferred into *Agrobacterium* strain GV3101. These transferred *Agrobacterium* were grown overnight at 28°C in 5 ml L-broth (LB) medium containing 50 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin for preculture. The precultures were cultured in 50 ml of LB medium containing the same antibiotics for two days at 28°C. The cells were harvested by centrifugation and resuspended in an infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 200 mM acetosyringone), adjusted to an optical density at $O.D_{600}$ 2.0 and shaker slowly at room temperature for 3 h. Agro-infiltration on cotyledons: the *Agrobacterium* suspensions of pTRV2, pTRV2-*SlPDS*, pTRV2-*SlTIL1*, pTRV2-*SlTIL2*, and pTRV2-*SlCHL* were mixed with pTRV1 at a ratio of 1 : 1 and were injected into the bottom of cotyledons in 3-week-old-seedlings using a 1 ml syringe without a needle. The infiltrated plants were observed for 12 weeks. Agro-infiltration on fruits: the 1 ml syringes with needle were used for each fruit agro-injection. The *Agrobacterium* suspensions of pTRV2, pTRV2-*SlPDS*, pTRV2-*SlTIL1*, pTRV2-*SlTIL2*, and pTRV2-*SlCHL* were mixed with pTRV1 at a ratio of 1 : 1 and were injected into mature green fruits (30–35 DPA). The needle was introduced 3 to 4 mm in depth into the fruit tissue through the stylar apex. The phenotypes were observed 10 days after the agro-injection.

Detection of reactive oxygen species (ROS)

The level of superoxide ions (O_2^-) ions is determined qualitatively using nitrobluetetrazolium (NBT) assay while the hydrogen peroxide (H_2O_2) is qualitatively estimated using 3,3-diaminobenzidine (DAB). For detection O_2^- and H_2O_2, the leaves were cut from the plants and then dipped immediately into 6 mM NBT solution (2 ml) prepared in sodium citrate (pH 6.0) or DAB solution (1 mg ml^{-1}) prepared in double distilled water (2 ml) (pH 3.8) in a petri dish (35 mm) using tweezers. The dipped leaves were vacuum infiltrated for 10 min at 60 kPa pressure and then incubated at room temperature for 10 min under room light. After incubation, the samples were dipped in the absolute ethanol and then kept them in a water bath (100°C) till the chlorophyll was removed from the samples completely. The samples were dipped in 20% glycerol for cooling then captured the images using a stereo microscope by keeping the samples on a slide.

Results

Characterization of SlTIL1, SlTIL2, and SlCHL

In this study, two temperature-induced lipocalin genes *SlTIL1* and *SlTIL2*, and a chloroplastic lipocalin gene *SlCHL*, were isolated from 'Micro-Tom' tomato. The coding sequences of *SlTIL1* and *SlTIL2* contained 558 bp, and encoded a putative protein of 186 amino acids with a predicted molecular weight of 21.47 kDa and 21.26 kDa, respectively. The identity of SlTIL1 and SlTIL2 was 84.4% at the amino acid level (Supplementary Figure S1). Phylogenetic analyses showed that SlTIL1 and SlTIL2 belonged to TIL family, and SlTIL1 exhibited high identity (96.8%) with potato StTIL (Figure 1A). In N-terminal region of the proteins encoded by SlTIL1 and SlTIL2, no characteristic transit peptides were detected

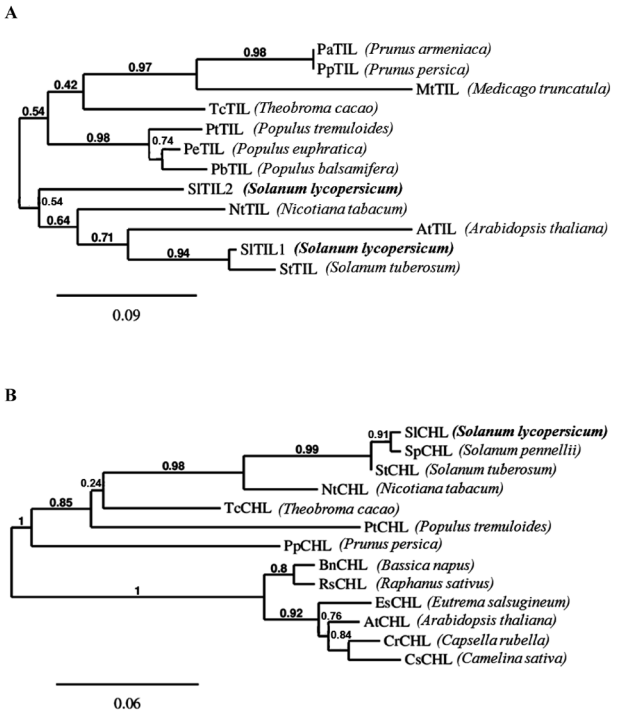

Figure 1. Phylogenetic trees of TIL proteins and CHL proteins in plants. The values near branch represent bootstrap value, and the scale bar indicates 0.09 (TILs) and 0.06 (CHL) amino acid substitutions per site. (A) A phylogenetic tree of TIL proteins in plants. The GenBank accession numbers of the published *TIL* genes are as follows: *AtTIL* (NM_125192.4), *MtTIL* (XM_003610323), *NtTIL* (XP_016489430.1), *PaTIL* (DQ222998.1), *PbTIL* (DQ223002.1), *PeTIL* (FJ238513.1), *PpTIL* (DQ222997.1), *PtTIL* (DQ223003.1), *StTIL* (XP_006350080.1), *TcTIL* (XM_007011912). (B) A phylogenetic tree of CHL proteins in plants. The GenBank accession numbers of the published *CHL* genes are as follows: *AtCHL* (NM_114656.4), *BnCHL* (XM_022710322.1), *CrCHL* (XM_006291292.2), *CsCHL* (XM_010505102.2), *EsCHL* (XM_024158068.1), *NtCHL* (XM_016581925.13), *PpCHL* (XM_020562672.1), *PtCHL* (XM_006370603.2), *RsCHL* (XM_018633289.1), *SpCHL* (XM_015203764.1), *StCHL* (DQ223008.1), *TcCHL* (XM_007020875.2). The sequences of *SlTIL1* (LEFL2018O11), *SlTIL2* (LEFL1002CA07), *SlCHL* (LEFL2044D22) were obtained from 'Micro-Tom' Database (http://www.pgb.kazusa.or.jp/mibase/index.html).

by TargetP. The genomic DNA sequences of *SlTIL1* and *SlTIL2* contained an intron, which was 2202 bp and 259 bp in length, respectively. In the meanwhile, a 5′UTR and a 3′UTR were predicted in *SlTIL2* (Supplementary Figure S2).

The coding sequences of chloroplastic lipocalin (*SlCHL*) was 1002 bp in length, and encoded a putative protein of 334 amino acids with a predicted molecular of 37.34 kDa. In the N-terminal region of SlCHL, a chloroplastic transit peptide of 28 amino acids was predicted by TargetP. Phylogenetic analyses showed that SlCHL belonged to CHL family, and exhibited high identity with that of SpCHL (*Solanum pennellii* CHL) and StCHL (*Solanum tuberosum* CHL), which was 98.2% and 96.4%, respectively (Figure 1B). In the gene structure of *SlCHL*, there were four introns and five exons (Supplementary Figure S2).

Expression of SlTIL1, SlTIL2, and SlCHL in different tissues

In this study, we investigated the expression of *SlTIL1*, *SlTIL2*, and *SlCHL* in leaves, roots, flowers and the fruits of 'Micro-Tom.' The results showed that *SlTIL1* was expressed in all the tissues, and its expression levels in roots and fruits were much higher than that in leaves. Similar to *SlTIL1*, *SlTIL2* was expressed in all the tissues. The expression level of *SlTIL2* was high in young roots and leaves (3 weeks plants), flowers, and all stages of fruits, but low in old roots and leaves (6 weeks plants). *SlCHL* was detected with a similar expression level in leaves and flowers, while its expression was lower in roots. In the fruits, the expression of *SlCHL* decreased significantly from orange fruit stage (Figure 2 and Supplementary Figure S3).

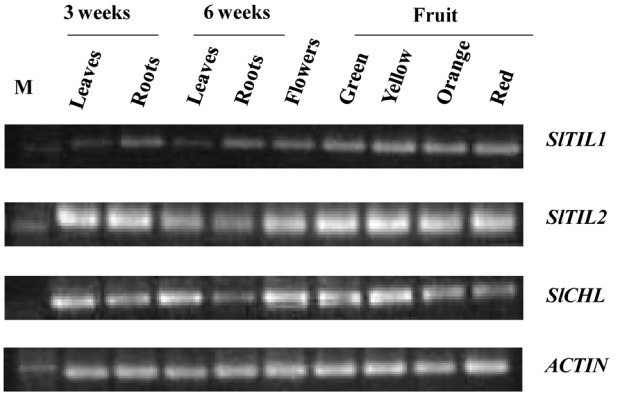

Figure 2. Gene expression analyses of *SlTIL1*, *SlTIL2*, and *SlCHL* in different tissues of 'Micro-Tom' tomatoes analyzed by RT-PCR. Green: green fruit stage, Yellow: yellow fruit stage, Orange: orange fruit stage, Red: red fruit stage. M: marker. *ACTIN* was used as a reference gene in order to normalize the gene expression results. Three experimental data were shown in Supplementary Figure S3.

Observation of subcellular localization of SlTIL1, SlTIL2, and SlCHL

To investigate the subcellular localization of SlTIL1, SlTIL2, and SlCHL, the cDNAs of *SlTIL1*, *SlTIL2*, and *SlCHL* were fused with GFP reporter gene under the control of *Cauliflower mosaic virus* 35S promoter and bombarded into tobacco leaves and onion epidermal cells. Confocal imaging of GFP fluorescence showed that SlTIL1 and SlTIL2 fusion proteins were mainly localized in the plasma membrane, around the plastids, and nuclear in the tobacco leaves and onion epidermal cells. The fusion protein of SlCHL was localized in the chloroplasts of the tobacco leaves (Figure 3 and Supplementary Figure S4).

The phenotypes of over-expressed and silenced SlTIL1, SlTIL2, and SlCHL plants

To elucidate the biological functions of *SlTIL1*, *SlTIL2*, and *SlCHL*, transgenic tomatoes that over-expressed *SlTIL1*, *SlTIL2*, and *SlCHL* and tomatoes in which *SlTIL1*, *SlTIL2*, and *SlCHL* were silenced using VIGS were generated in this study (Figures 4, 5). Compared with the wild-type plants, transgenic tomatoes over-expressed *SlTIL1*, *SlTIL2*, and *SlCHL* exhibited a bullwhip phenotype in leaves. The number of curling leaves increased, and the terminal leaflets became longer in the transgenic tomatoes over-expressed *SlTIL1*, *SlTIL2*, and *SlCHL* (Figure 4B). In the meanwhile, the over-expression of *SlTIL1*, *SlTIL2*, and *SlCHL* also caused early flowering, increases in the numbers of flowers, inflorescences, and fruits (Supplementary Figure S7A), and bigger peduncle and fruits in tomato (Figure 4). In the transgenic tomato over-expressed *SlCHL*, seedling and mesocarp of mature green fruits were dark green in color, and the fruits ripened earlier as compared with others (Figure 4). Gene expression results showed that the over-expressing *SlTIL1*, *SlTIL2*, and *SlCHL* driven by 35S promoter significantly increased their expression in their leaves of 'Micro-Tom' (Supplementary Figure S5).

Moreover, we silenced the expression of *SlTIL1*, *SlTIL2*, and *SlCHL* in 'Micro-Tom' using VIGS method (Figure 5). As a positive control, silencing of *SlPDS* was also conducted using the VIGS method. Gene expression results showed that the silencing of *SlPDS*, *SlTIL1*, *SlTIL2*, and *SlCHL* by VIGS significantly decreased their expression in their leaves of 'Micro-Tom' (Supplementary Figure S6). In the tomato silenced *SlPDS*, white color regions in leaves and petals were observed (Figure 5B, C). The phenotypes in the *SlPDS* silenced 'Micro-Tom' was consistent with those reported in the study of Orzaez et al. (2006), which indicated that the gene expression could be effectively silenced by VIGS in 'Micro-Tom' (data not shown). In this study, the suppressed expression of *SlTIL1* and *SlTIL2* caused an aberrant shape of leaves, such as yellowing and curling

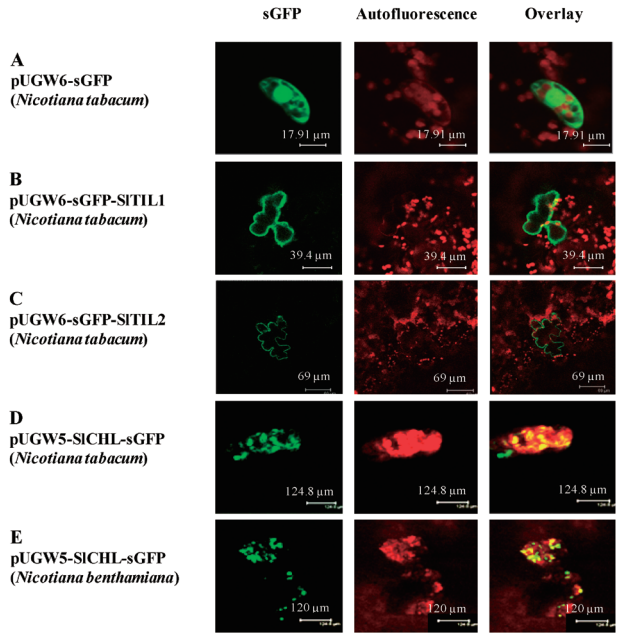

Figure 3. Subcellular localization of SlTIL1, SlTIL2, SlCHL in *Nicotiana tabacum* and *N. bentamiana* leaf cells. (A) Fluorescent microscopic images of pUGW6-sGFP fusion protein in *N. tabacum* leaf cells. (B) Fluorescent microscopic images of pUGW6-sGFP-SlTIL1 fusion protein in *N. tabacum* leaf cells. (C) Fluorescent microscopic images of pUGW6-sGFP-SlTIL2 fusion protein in *N. tabacum* leaf cells. (D) Fluorescent microscopic images of pUGW5-SlCHL-sGFP fusion protein in *N. tabacum* leaf cells. (E) Fluorescent microscopic images of pUGW5-SlCHL-sGFP fusion protein in *N. benthamiana* leaf cells.

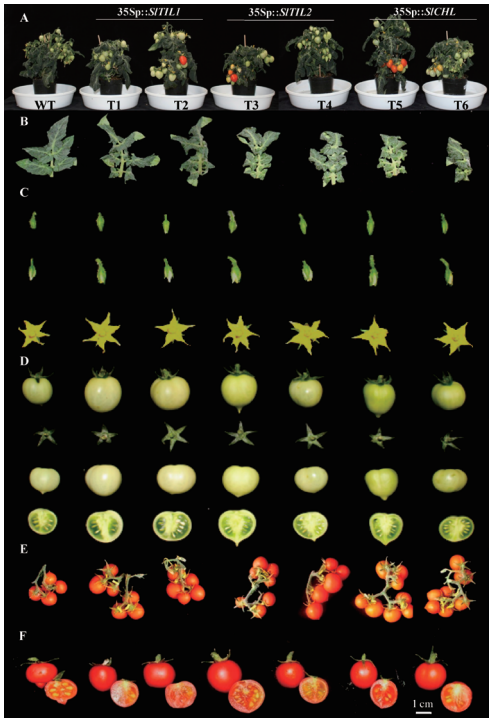

Figure 4. Phenotypes of *SlTIL1*, *SlTIL2*, and *SlCHL* over-expressed tomato plants. (A) Phenotypes of transgenic tomato plants were grown for 5 weeks after transplanted. Phenotypes of old leaves (B), flowers (C), mature green fruits (D), inflorescences (E), and red fruits (F) in transgenic tomato plants.

(Figure 5A, B), and yellowing in pericarp and mesocarp of fruits (Figure 5F). The suppressed expression of *SlCHL* caused yellowing in leaves edges but not curling (Figure 5B), little yellowing in pericarp and mesocarp and darkening in endocarp of fruits (Figure 5F). In addition, the silencing of *SlTIL1*, *SlTIL2*, and *SlCHL* led to 10–15 days delaying on flowering and fruit ripening and decreasing in the fruit numbers (Figure 5A, F and Supplementary Figure S7B).

Analyses of ROS accumulation in SlTIL1, SlTIL2, and SlCHL over-expressed or silenced plants

H_2O_2 and O_2^- were at a low level in leaves under light condition 2 (200 μmol $m^{-2}s^{-1}$). They were greatly accumulated in the wild-type plants treated with light condition 1 (405 μmol $m^{-2}s^{-1}$), while less O_2^- and H_2O_2 were accumulated in *SlTIL1*, *SlTIL2*, and *SlCHL* over-expressed plants (Figure 6). On the other hand, to further understand the functions of lipocalin with ROS, the levels of O_2^- and H_2O_2 were analyzed in the *SlTIL1*, *SlTIL2*, and *SlCHL* silenced plants (Figure 7). The results showed that under light condition 1 (405 μmol $m^{-2}s^{-1}$), the higher levels of O_2^- and H_2O_2 were accumulated in leaves of *SlTIL1*, *SlTIL2*, and *SlCHL* silenced plants, and the levels of O_2^- were much higher than those of over-

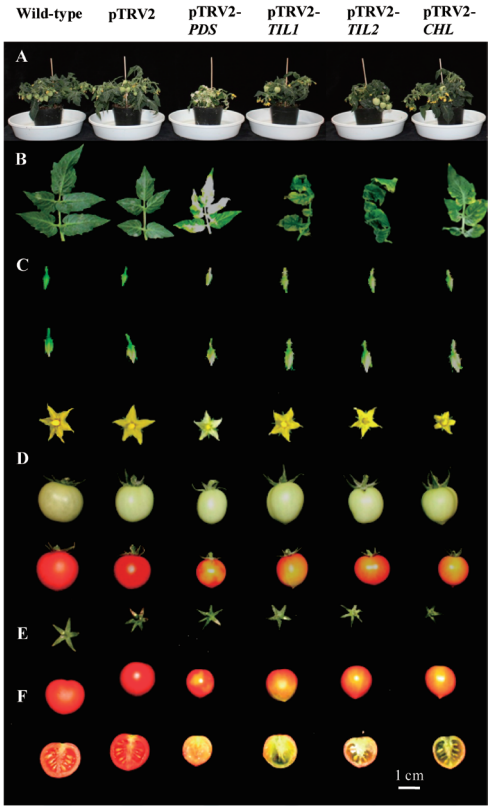

Figure 5. Phenotypes of *SlTIL1*, *SlTIL2*, and *SlCHL* silenced plants. (A) Phenotypes of their silenced plants were grown for 5 weeks after infiltration. Phenotypes of leaves (B), flowers (C), fruits (D), calyxes (E) and mature tomato fruits (F) in their silenced plants.

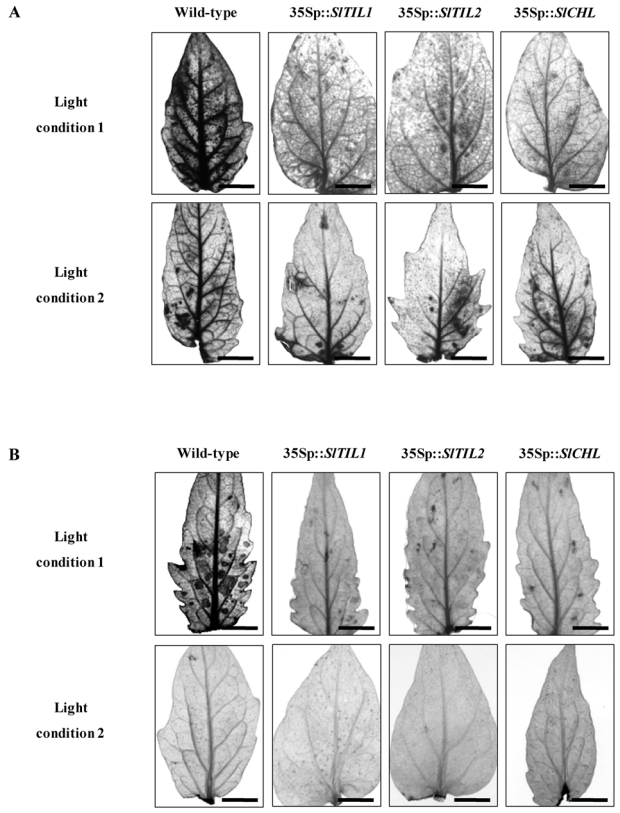

Figure 6. ROS accumulation in *SlTILs* and *SlCHL* over-expressed plants and wild-type plants under light condition 1 (405 μmol $m^{-2} s^{-1}$) and light condition 2 (200 μmol $m^{-2} s^{-1}$) after 2 weeks. (A) Superoxide ions (O^{2-}) accumulation detected by NBT staining; (B) Hydrogen peroxide (H_2O_2) accumulation detected by DAB staining. Scale bar shows 5 mm.

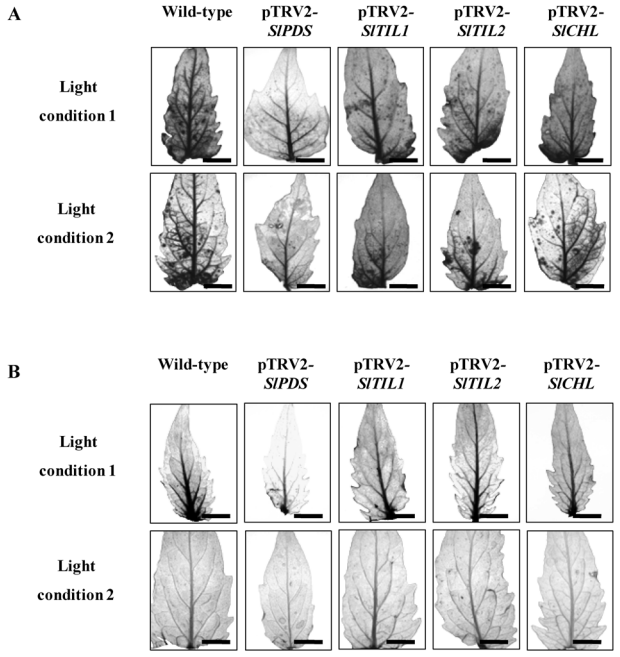

Figure 7. ROS accumulation in *SlTILs* and *SlCHL* silenced plants and wild-type plants under light condition 1 (405 μmol $m^{-2} s^{-1}$) and light condition 2 (200 μmol $m^{-2} s^{-1}$) after 2 weeks. (A) Superoxide ions (O^{2-}) accumulation detected by NBT staining; (B) Hydrogen peroxide (H_2O_2) accumulation detected by DAB staining. Scale bar shows 5 mm.

expressed plants (Figures 6, 7). These results indicated that *SlTIL1*, *SlTIL2*, and *SlCHL* over-expressed tomato plants increased ROS-scavenging capacity, while *SlTIL1*, *SlTIL2*, and *SlCHL* silenced tomato plants decreased it.

Analyses of the expression of SODs in SlTIL1, SlTIL2, and SlCHL over-expressed or silenced plants

To understand if the elevated ROS-scavenging capacity was associated with plant antioxidants, the expression of three superoxide dismutase (*SOD*) genes were analyzed in *SlTIL1*, *SlTIL2*, and *SlCHL* over-expressed and silenced plants. As shown in Figure 8A, the expression of *SlSOD1*, *SlSOD3*, and *SlSOD6* was enhanced in leaves of *SlTIL1*, *SlTIL2*, and *SlCHL* over-expressed plants. In flowers, the expression of *SlSOD1* and *SlSOD6* was enhanced in *SlTIL2* and *SlCHL* over-expressed plants, and the expression of *SlSOD3* was enhanced in *SlCHL* over-expressed plants (Figure 8B and Supplementary Figure S8). In fruits, the expression of *SlSOD3* was enhanced in *SlTIL1*, *SlTIL2*, and *SlCHL* over-expressed plants. In contrast, the expression of *SlSOD1*, *SlSOD3*, and *SlSOD6* in leaves, flowers and fruits was decreased when *SlTIL1*, *SlTIL2*, and *SlCHL* were silenced by VIGS, although in some tissues it was not significant (Figure 8D and Supplementary Figure S9).

Discussion

Identification and characterization of lipocalins in tomato

In the present study, two types of lipocalins, temperature-induced lipocalins (TIL) and chloroplastic lipocalin (CHL), were isolated and characterized in tomato. In plants, Charron et al. (2005) reported that monocotyledonous species, such as wheat, possessed two different members of TIL, *TIL-1* and *TIL-2*. On the other hand, dicotyledonous species Arabidopsis possessed only one TIL, and there was no conclusive evidence of the existence of two forms of TIL in dicotyledonous plants (Charron et al. 2005). Tomato is a typical dicotyledonous plant. In our study, the results showed that two different *TILs*, *SlTIL1* and *SlTIL2*, were existed in tomato cultivar 'Micro-Tom,' which shared 84% identity at the amino acid level. Phylogenetic analyses showed that *SlTIL1* and *SlTIL2* belonged to TIL family, and they exhibited 78% and 76% identities with Arabidopsis AtTIL, respectively. In the present study, no characteristic transit peptides were detected in N-terminal region of SlTIL1 and SlTIL2 by TargetP. Subcellular localization results showed that fusion proteins of SlTIL1-sGFP, sGFP-SlTIL1, SlTIL2-sGFP, and sGFP-SlTIL2 mainly accumulated in the plasma membrane of the onion epidermal cells. In

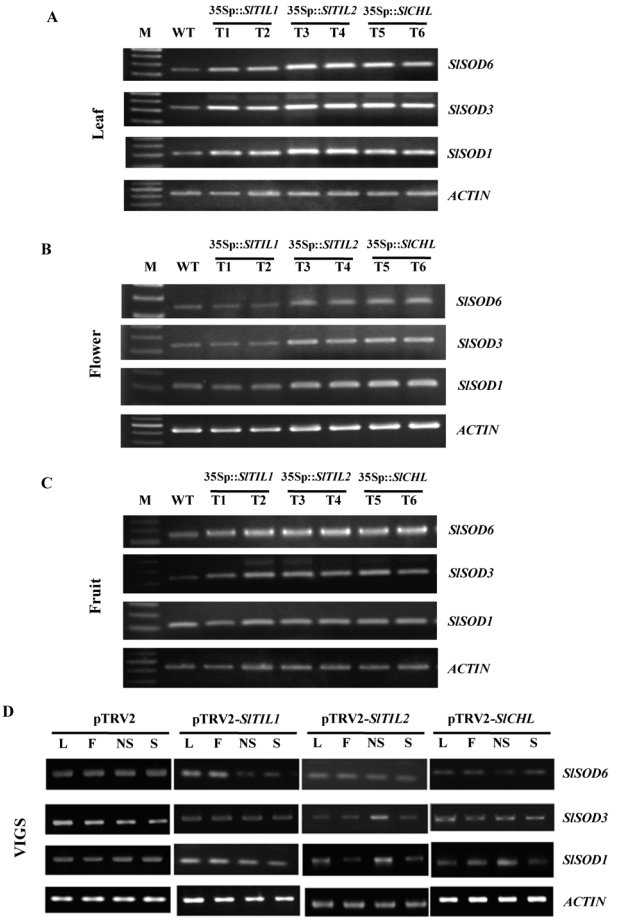

Figure 8. The expression of *SOD*s (*SlSOD1*, *SlSOD3* and *SlSOD6*) in leaves (A), flowers (B), and fruits (C) of *SlTIL1*, *SlTIL2* and *SlCHL* over-expressed plants. (D) The expression of *SOD*s (*SlSOD1*, *SlSOD3* and *SlSOD6*) in leaves, flowers, and fruits of *SlTIL1*, *SlTIL2* and *SlCHL* silenced plants. M: marker; L: leaves, F: flowers, NS: non-silenced fruits, S: silenced fruits. The experiment was performed at least three times with similar results. Three experimental data were shown in Supplementary Figure S8 and S9.

the case of tobacco leaf cells, subcellular localization of fusion proteins SlTIL1 and SlTIL2 (SlTIL1-sGFP, SlTIL2-sGFP, sGFP-SlTIL1 and sGFP-SlTIL2) were detected not only in the plasma membrane but also around plastids, nuclear and in reticulate structures (Figure 3). The subcellular localization of SlTIL1 and SlTIL2 was in agreement with that of AtTIL, which was reported be targeted to different cell membranes and organelles (Brugiere et al. 2004; Dunkley et al. 2006, Eubel et al. 2008; Nikolovski et al. 2012; Parsons et al. 2012). Hernández-Gras and Boronat (2015) reported that the AtTIL did not contain any recognizable signal for membrane targeting or transmembrane motifs, and it interacted with the plasma membrane by means of a short hydrophobic sequence present in a loop located between β-strands 5 and 6, which was similar with ApoD (Bishop 2000; Charron and Sarhan 2005; Charron et al. 2005). Moreover, previous report showed that subcellular localization of YFP-PeuTIL (*Populus euphratica*) was localized to the plasma membrane but was re-translocated to the symplast under salt stress (Abo-Ogiala et al. 2014). SlTIL1 and SlTIL2 might be localized mainly around the plasma membrane and re-translocate to plastids, nuclear and mitochondria under some environmental stresses.

CHL is a chloroplast-associated lipocalin. To date, CHL has been identified in wheat and Arabidopsis, however, the information on CHL in tomato was completely unknown (Levesque-Tremblay et al. 2009). In this study, we isolated one CHL (SlCHL) from tomato cultivar 'Micro-Tom,' which shared 98.2% identity with that of SpCHL. Sequence analyses showed that SlCHL possessed the three lipocalin structure conserved regions (SCRs), which were also detected in SlTIL1 and SlTIL2. In tomato, the SlCHL had 27% and 26% identities with SlTIL1 and SlTIL2 at the amino acid level, respectively. Although SlCHL shared low identities with SlTIL1 and SlTIL2, the three tomato lipocalins exhibited common three-dimensional structure features with a large cup-shaped cavity and a loop scaffold for ligand binding. In addition, different from SlTIL1 and SlTIL2, SlCHL had a chloroplastic transit peptide of 28 amino acids in the N-terminal region of SlCHL, and the fusion protein of SlCHL-GFP was localized in the chloroplasts of the tobacco leaves (Figure 3D, E). In Arabidopsis, immunoblot analysis showed that AtCHL was specifically localized in the thylakoid lumen, and the location of AtCHL in chloroplast contributed to protecting thylakoid membranes against lipid peroxidative damage in Arabidopsis (Levesque-Tremblay et al. 2009). To date, however, the mechanism that AtCHL imports into the lumen is still unknown.

The roles of lipocalins in tomato

In the present study, *SlTIL1*, *SlTIL2*, and *SlCHL* were detected in all tissues, including leaves, roots, flowers, as well as fruits (Figure 2 and Supplementary Figure S3). However the expression levels of *SlTIL1*, *SlTIL2*, and *SlCHL* were significantly different among the tissues (Figure 2). *SlTIL1* was highly expressed in the fruits during the ripening process, while it was lowly expressed in leaves. The expression level of *SlTIL2* was high in young leaves, young roots, and all stages of fruits, but low in the leaves and roots of 6-week-old-plants. In wheat, it was reported that *TaCHL* was highly expressed in leaves, while it was not detected in roots (Charron et al. 2005). In the present study, the gene expression results showed that *SlCHL* was expressed in roots of tomato, but its expression level in roots was lower than that in other tissues (Figure 2). The tissue-specific expression of *SlTIL1*, *SlTIL2*, and *SlCHL* indicated that the three lipocalins might have different functions in tomato.

Virus-induced gene silencing (VIGS) is an effective tool to down-regulate gene expression and was useful

for gene functional characterization in tomato fruits (Fu et al. 2005; Senthil-Kumar and Mysore 2011), which is an easy, rapid, reliable and transformation-free method (Lange et al. 2013). In the present study, to elucidate the roles of lipocalins in tomato, the functions of *SlTIL1*, *SlTIL1*, and *SlCHL* were investigated using the transgenic tomatoes that over-expressed *SlTIL1*, *SlTIL2*, and *SlCHL* and tomatoes in which *SlTIL1*, *SlTIL2*, and *SlCHL* were silenced using VIGS. The results showed that the phenotypes in tomato were significantly affected when the *SlTIL1*, *SlTIL2*, and *SlCHL* were over-expressed or silenced by VIGS. The over-expression of *SlTIL1*, *SlTIL2*, and *SlCHL* caused early flowering, increases in the numbers of flowers, inflorescences, and fruits (Figure 4 and Supplementary Figure S7A), and bigger peduncle and fruits in tomato (Figure 4E, F). In contrast, the silencing of *SlTIL1*, *SlTIL2* and *SlCHL* led to 10–15 days delaying on flowering and fruit ripening and decreasing in the fruit numbers (Figure 5A, F and Supplementary Figure S7B). The significant phenotypes changes in the *SlTIL1*, *SlTIL1*, and *SlCHL* over-expressed and silenced plants suggested that the three lipocalins played important roles in regulating the growth and development of tomato. In addition, the lipocalins (*SlTIL1*, *SlTIL2*, and *SlCHL*)-silenced plants showed the stress condition, such as yellowing and curling on leaves, etiolation in stem and more fast in senescence of leaves and plant growth. This result implied that *SlTIL1*, *SlTIL2*, and *SlCHL* might have a role in oxidative stress response.

In Arabidopsis, it was reported that the lipocalins were stress-inducible proteins, and played an important role in the protection of plants from the abiotic stresses. To date, the research on the molecular mechanism of the plant lipocalin functions is still limited. In the study of Boca et al. (2014), it was reported that AtTIL and AtCHL protected plants against the abiotic stresses probably by decreasing lipid peroxidation, which is a key mechanism that lipocalins protect plants from the abiotic stresses. ROS generation is regarded as a common cellular response during stress condition. ROS overproduction might cause serious oxidation damage which could influence the ordinary operation of plant metabolism (Jaspers and Kangasjarvi 2010; Neill et al. 2002). Thus, the reduction of ROS generation is essential for stress resistance and survival. In the present study, we investigated the effects of light intensity on the tolerance of the *SlTILs* and *SlCHL* over-expressed and silenced plants. Under high light condition ($405\,\mu mol\,m^{-2}\,s^{-1}$), low levels of ROS were detected in the *SlTILs* and *SlCHL* over-expressed plants. In contrast, the levels of ROS were high in the *SlTILs* and *SlCHL* silenced plants under the high light condition. In the meanwhile, we also found that the expression of *SOD* genes was high in the *SlTILs* and *SlCHL* over-expressed plants, while it was low in the *SlTILs* and *SlCHL* silenced plants. The high expression levels of *SODs* might contribute to scavenging ROS in the *SlTILs* and *SlCHL* over-expressed plants under high light stress. Thus, these results suggested that lipocalins might play an important role for high light stress tolerance in tomato plants by increasing the ROS scavenging activity. This result contributes to understanding the functions of lipocalin proteins in tomato and provides clues for the study of abiotic stress responses in plants.

Acknowledgements

We gratefully acknowledge Prof. Tsuyoshi Nakagawa (Shimane University) for providing pUGW5, pUGW6, and pGWB8 vectors to analyze the localization of proteins and to make over-expressed plants. We thank Prof. Yoshikazu Kiriiwa (Shizuoka University), Prof. Mitsuo Omura (Shizuoka University) and Prof. Takashi Nakatsuka (Shizuoka University) for valuable discussion on the technical aspects of this study. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (Japan) [Grants-in-Aid for Scientific Research (No. 23580039 to R M)] and Ministry of Research, Technology and Higher Education of the Republic of Indonesia, Directorate General of Resources for Research, Technology, and Higher Education.

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