Function of *SlTILs* and *SlCHL* under heat and oxidative stresses in tomato

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Received March 29, 2020; accepted April 22, 2020 (Edited by S. Nonaka)

**Abstract** Lipocalins are very important proteins for stress resistance in plants. To better understand the function of tomato lipocalins, we observed responses to oxidative stress using over-expressed *SlTIL1, SlTIL2, SlCHL*, and silenced-plants. Significant differences in reactive oxygen species accumulation (oxidative damage) were observed in all tested plants under heat stress. Plants with over-expressed *SlTIL1, SlTIL2*, and *SlCHL* showed less oxidative damage compared with wild-type plants under heat stress. The expression of *SISO* was induced in over-expressed *SlTIL1, SlTIL2*, and *SlCHL* plants under normal and heat stress conditions. Furthermore, silenced *PDS, SlTILs*, and *SlCHL* plants showed slightly increasing oxidative damage under heat stress alongside with lower *SISO* under normal and stress conditions. These results suggest that *SlTIL1, SlTIL2*, and *SlCHL* were involved in antioxidant defense by eliminating ROS in tomato plants.

**Key words:** gene silencing, lipocalins, *SlCHL, SlTILs*, tomato.

Increasing temperatures are known to play an important role in yield reduction (Lipiec et al. 2013), suggesting that climate change and a rise in global temperatures will significantly impact global food security. Comprehensive research on plant heat tolerance, stress response, and adaptive capacity is essential (Buchner et al. 2013). Heat stress causes the formation of reactive oxygen species (ROS) in plants (Munne Bosch and Pennelus 2003; Pucciariello et al. 2012). Excessive ROS production can irreversibly damage plant cells through the oxidation of cellular components, such as lipids, proteins, and DNA (Apel and Hirt 2004). Therefore, excessive ROS needs to be removed as soon as possible. Several studies have examined the various ROS prevention and repair mechanisms, which exist to protect plants against heat stress (Larkindale and Knight 2002; Suzuki and Mittler 2006). To prevent ROS accumulation, plants have developed detoxification enzymes (e.g., superoxide dismutase, catalase, peroxidase, glutathione reductase, ascorbate peroxidase) and non-enzymatic antioxidants (e.g., ascorbate, glutathione, carotene, and tocopherol; Gill and Tuteja 2010; Suzuki et al. 2012). Anthocyanins are a class of flavonoids with antioxidative properties, due to the presence of hydroxyl groups in aglycon moiety (Yokozawa et al. 1998). In tropical countries such as Indonesia, heat stress is a major environmental factor that limits wheat crop productivity (Althuaish et al. 2014).

Temperature-induced lipocalin (TIL) proteins have been identified from wheat and Arabidopsis plants (Charron et al. 2002). Lipocalins are a group of proteins found in bacteria, invertebrates, and vertebrate animals, which play a major role in response to environmental stress, membrane formation and fixation, apoptotic induction, regulation of immune response, cell growth, and metabolic adjustment. Recent studies related to lipocalin show that it is involved in the tolerance response to abiotic oxidative stress (He et al. 2015; Levesque-Tremblay et al. 2009). However, this protein and its various functions remain mostly unknown. According to Charron et al. (2005), the tissue specifications of *Triticum aestivum* TIL (TaTIL), *Triticum aestivum* CHL (TaCHL) and their accumulated transcripts protect plants against stress damage. Lipocalin chloroplastic in Arabidopsis (AtCHL) is involved in the antioxidative response of Arabidopsis leaves against light-induced oxidative stress. Some lipocalin members are thought to be found in chloroplasts (Levesque-Tremblay et al. 2009). The cell structure of vascular bundles in over-expressed *SlTILs* and *SlCHL* consists of wider (tenuous) cells compared to wild-type vascular bundle cells. These findings indicate that cells

Abbreviations: CHL, chloroplastic lipocalin; PDS, phytoenedesaturase; ROS, reactive oxygen species; SOD, superoxide dismutase; TIL, temperature-induced lipocalin; VIGS, virus-induced gene silencing.

This article can be found at http://www.jspcmb.jp/

Published online July 30, 2020
in over-expressed \textit{SITILs} and \textit{SICHL} grow more rapidly (Wahyudi et al. 2019). Lipocalins in Arabidopsis (AtTIL and AtCHL) play a major role in lipid protection, which is essential for stress resistance and survival (Boca et al. 2014).

In this study, we report that tomato lipocalins are involved in heat stress and oxidative tolerance using over-expressed and silenced \textit{SITIL1}, \textit{SITIL2}, \textit{SICHL} plants.

This study used planting material: the “Micro-Tom” plant (\textit{Solanum lycopersicum} cv “Micro-Tom”). “Micro-Tom” seeds were obtained from University of Tsukuba, Japan. The fruit ripening process was divided into four stages: the green fruit stage (30–33 DPA), the yellow fruit stage (32–33 DPA), the orange fruit stage (33–35 DPA), and the red fruit stage (41–45 DPA) (Suzuki et al. 2015).

Leaves, flowers, roots, and fruits from each stage were frozen in liquid nitrogen, then stored at −80°C until further analysis. The samples were then planted under 24–28°C, 60±10% relative humidity, and light: dark cycle of 16:8 h. Their cultivation was carried out using a pot with sterile soil media and a hydroponic system using half-aqueous Enshi formula [808 mg l−1 KNO₃, 492 mg l−1 MgSO₄.7H₂O, 944 mg l−1 Ca(NO₃)₂.4H₂O, 152 mg l−1 NH₄H₂PO₄, and 50 mg l−1 Otsuka house 5 (Otsuka Agri Techno. Co., Ltd.,)]. The heat treatment was carried out under the above conditions after heating at 37°C for 5 days.

Qualitatively, the level hydrogen peroxide (H₂O₂) was determined using a 2,7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) assay. The H₂DCFDA test to detect ROS (predominantly H₂O₂) was carried out according to the Kaur method (Kaur et al. 2016; www.bio-protocol.org/e2061). For the detection of O₂ and H₂O₂, over-expressed and silenced \textit{SITILs} and \textit{SICHL} plant leaf samples were cut, then immediately immersed in a 2-mL 6 mM NBT solution prepared in sodium citrate (pH 6) in a petri dish (35 mm) using tweezers. Samples were then infiltrated for 10 min at a pressure of 60 kPa, then incubated at room temperature for 10 min under ambient light. After incubation, samples were dipped in absolute ethanol, then stored in a water bath (100°C) until chlorophyll was completely removed from the cells. Samples were dipped to be cooled in 20% glycerol, then observed using a stereomicroscope under magnification up to 40× objective.

In the H₂DCFDA assay, samples were observed under a confocal microscope (LSM 700, Carl Zeiss) using a laser beam of a 488-nm wavelength. Green fluorescence indicated the presence of H₂O₂, while red was the autofluorescence of chlorophyll.

In this study, the expression of \textit{superoxide dismutases} (SODs) genes was detected using the RT-PCR method. Total RNA was isolated from the leaves and fruits using the RNeasy mini kit (Qiagen, Germany) following the manufacturer’s instructions, then treated extensively with RNase-free DNase I. First-strand cDNA was synthesized from 1 µg total RNA using PrimeScript™ first-strand cDNA synthesis kit (TaKaRa, Japan). PCR was carried out under standard conditions: 30 cycles of 10 s at 98°C, 15 s at 55°C, and 1 min at 68°C, using the primers SI SOD1-F (5'-TCT GCC CTA AAA CCT GGA CT-3'), SISOD1-R (5'-ACC AGT GAG AGG AAT CTG CT-3'), SISOD3-F (5'-CTC CTG GAC TTC ACC GGT TT-3'), SISOD3-R (5'-CAC AAG TGTC TCC AAC AA-3'), SISOD6-F (5'-AGG ACA GCC ATC TGG TGA AC-3'), and SISOD6-R (5'-TGG CGA GTA ATC CCA AAC GA-3'). ACTIN cDNA (425 bp) as an internal standard of gene expression was amplified using Actin-F (5'-AGA TGG TGT CAG CCA CAC AG-3') and Actin-R (5'-ACC ACC ACT GAG GAC GAT GT-3'). RT-PCR analyses were performed in triplicates.

The results of RT-PCR were quantified using the ImageJ software and normalized with the value of ACTIN. The gene expression of different tissues in wild-type plants was used as calibrators (value = 1). The data shown are the mean values for the three separate experiments ± standard deviation. * and ** indicate significant differences from the wild-type at the p < 0.05 and p < 0.01 levels, respectively, as calculated by the Student’s t-test.

When the morphological phenotypes were compared with over-expressed \textit{SITIL1}, \textit{SITIL2}, \textit{SICHL}, and wild-type plants under heat stress, over-expressed \textit{SITILs} and \textit{SICHL} plants showed leaves curling but no wilting. Their over-expressed plants had bigger flowers and fruits, with fruits and seeds emerging earlier after 5 days of heat stress. The wild-type phenotype showed leaf wilting after heat stress treatment (Figure 1). Furthermore, we used the pTR-based VIGS system in these studies to silence \textit{PDS}, \textit{SITIL1}, \textit{SITIL2}, and \textit{SICHL}. The silenced \textit{PDS} plant was used as a positive control for the VIGS system, inhibiting carotenoid biosynthesis and in turn causing a photo-bleached phenotype in leaves, flowers, and fruits in both conditions (Figure 2). The white-colored regions on the leaves and petals showed that \textit{PDS} was silenced by VIGS at least until the flowering stage. The silenced \textit{SITIL1}, \textit{SITIL2}, and \textit{SICHL} plants showed different phenotypes compared with wild-type plants used as control under heat stress (Figure 2). Silenced \textit{SITIL1}, \textit{SITIL2}, and \textit{SICHL} led to delayed fruit ripening (Figure 2). \textit{SITIL1}, \textit{SITIL2}, \textit{SICHL} and \textit{PDS} silenced-plants showed leaves with curling, wilting and shorter leaves compared to wild-type plants under heat stress (Figure 2B, D). Silenced plant growth was slower compared than that in wild-type plants (Figure 2).

In a previous study, over-expressed \textit{SITILs} and \textit{SICHL} plants subjected to light stress showed early flowering and fruit set, an increased number of flowers, inflorescences, and fruits, as well as larger peduncle,
flower, fruit, and leaves with curling, longer terminal leaflet, bullwhip phenotype compared to the wild-type plants (Wahyudi et al. 2018). The over-expressed \(\text{SlTIL1}\) showed the longest leaves with specific curling. The over-expressed \(\text{SlTIL2}\) flowered earlier and the over-expressed \(\text{SlCHL}\) ripened earlier (Wahyudi et al. 2018).

In this study, the phenotypes of over-expressed \(\text{SlTIL1}\), \(\text{SlTIL2}\), and \(\text{SlCHL}\) plants showed curling — but not wilting — leaves, bigger flower and fruits, early fruit setting, and making seed under heat stress for 5 days (Figure 1). The silenced \(\text{SlTIL1}\), \(\text{SlTIL2}\), and \(\text{SlCHL}\) under high light stress in Wahyudi et al. (2018) had patchy fruit coloring with areas exhibiting different shades of yellow and red color. The lipocalins-silenced-plants exhibited symptoms of the stress condition (e.g., leaf curling and faster leaf senescence). Moreover, silenced-plants grew slower than wild-type plants (Figure 2), implying that the \(\text{SlTIL1}\), \(\text{SlTIL2}\), and \(\text{SlCHL}\) genes may play an important role in the oxidative stress response and Arabidopsis chloroplastic lipocalin AtCHL (Abo-Ogiala et al. 2014; Levesque-Tremblay et al. 2009).

Superoxide ions (\(\text{O}_2^-\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)) are vital ROS molecules involved in plant growth and development, including abiotic stress tolerance (Kaur et al. 2016). To investigate the effects of lipocalins on ROS, the results of NBT staining and H\(_2\)DCFDA assay showed that the levels of ROS in over-expressed \(\text{SlTILs}\) and \(\text{SlCHL}\) plants under heat stress were lower than wild-type plants (Figures 3, 4), suggesting that the over-expressed \(\text{SlTILs}\) and \(\text{SlCHL}\) plants had a higher tolerance for ROS under heat stress. On the other hand, NBT staining and H\(_2\)DCFDA assays in \(\text{SlTILs}\) and \(\text{SlCHL}\) silenced-plants showed that their ROS levels were slightly higher than wild-type plants under heat stress (Figures 3, 4). The superoxide ions were accumulated at the lamina and top of leaves in wild-type plants under heat stress, but they were accumulated at all part of leaves (petiole, vein and lamina) in the silenced-plants (Figure 3). These results

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**Figure 1.** Phenotypes of over-expressed \(\text{SlTIL1}\), \(\text{SlTIL2}\), and \(\text{SlCHL}\) plants under normal (24°C) and heat stress conditions (37°C for 5 days). (A, D) Phenotypes of over-expressed plants grown for 2 weeks after transplantation; (B, E) phenotypes of over-expressed plants after 5 days of heat treatment; (C, F) expanded leaves of over-expressed plants.

**Figure 2.** Phenotypes of silenced-plants grown in soil under normal (24°C) and heat stress conditions (37°C for 5 days). (A, C) Phenotypes of silenced-plants grown for two weeks after infiltration; (B, D) expanded leaves of silenced-plants.
suggested that increasing expression of SlTILs and SlCHL was effective in removing ROS.

To better understand the relation of lipocalins and superoxide dismutase (SOD), the expression of SISODs was determined in over-expressed and silenced SITIL1, SITIL2, and SlCHL plants by RT-PCR. In over-expressed SITIL1, SITIL2, and SlCHL plants, SISOD1, SISOD3, and SISOD6 were highly expressed in the leaves under heat stress (Figure 5). Furthermore, the expression of SISOD1, SISOD3, and SISOD6 dramatically decreased in the leaves of silenced SITIL1, SITIL2, and SlCHL plants (Figure 6). The expression of SISOD1, SISOD3, and SISOD6 also decreased in silenced PDS plants. Silenced PDS plants, used as a positive control for the VIGS system, did not accumulate carotenoids and exhibited a photo-breaching phenotype. Carotenoids are known to prevent singlet
oxygen and lipid peroxidation and affect redox status (Cao et al. 2015; Shimizu et al. 1996). SODs are critical antioxidant enzymes which protect organisms from ROS produced under adverse conditions (i.e., stress-induced) and are widely found in the cytoplasm, chloroplast, and mitochondria of eukaryotic and prokaryotic cells (Feng et al. 2016). In recent years, studies have reported that SODs can protect plants against abiotic and biotic stress such as heat, cold, drought, salinity, abscisic acid, and ethylene (Feng et al. 2016). In this study, the expression analyses of SlSODs (SlSOD1, SlSOD3, and SlSOD6) showed high expression in the leaves of over-expressed SlTIL1, SlTIL2, and SlCHL plants (Figure 5), but low expression in the silenced SlTIL1, SlTIL2, and SlCHL plants (Figure 6).

The generation of ROS is a common cellular response under stressful conditions. H2O2 is a central signaling molecule in the stress-induced response in plants (Neill et al. 2002). On the other hand, the over-production of ROS might cause serious oxidative damage, which can alter the normal functioning of plant metabolism (Jaspers and Kangasjarvi 2010). In this study, over-expressed SlTIL1, SlTIL2, and SlCHL plants showed lower oxidative damage under heat stress, similarly to findings reported in the literature. The over-expressed plants did not accumulate ROS under normal conditions or heat stress (Figures 3, 4) and increased the expression of SlSODs (Figure 5). Silenced SlTIL1, SlTIL2, and SlCHL plants increased oxidative damage, ROS under normal condition and heat stress (Figures 3, 4) and decreased the expression of SlSODs (Figure 6). These results of SlSODs expression indicated that over-expressed SlTILs and SlCHL plants could induce the antioxidant defense system to eliminate ROS, suggesting that lipocalins play an important role in the abiotic oxidative tolerance in tomato. These findings contribute to our understanding of the functional analyses of lipocalin proteins in tomato and provide clues for the study of abiotic stress response such as heat stress and oxidative tolerance in tomato.
Acknowledgements

We would like to thank Prof. Nakagawa (Shimane University) for providing pGWB8 vectors to make over-expressed tomato plants. This work was also 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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