

Functional analyses of lipocalin proteins in tomato

メタデータ	言語: en 出版者: Shizuoka University 公開日: 2018-12-04 キーワード (Ja): キーワード (En): 作成者: Wahyudi, Anung メールアドレス: 所属:
URL	https://doi.org/10.14945/00026082

Functional Analyses of Lipocalin Proteins In Tomato

2018

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DOCTORAL DISSERTATION

**Functional Analyses of Lipocalin Proteins
In Tomato**

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June, 2018

PREFACE

Special thanks to Allah SWT, for the blessing and grace that the author can completed the study, research and writing of the doctoral dissertation entitled “Functional analyses of lipocalin proteins in tomato”. The authors would like to gratefully acknowledge to:

1. Prof. Reiko Motohashi and Prof. Yuichi Takikawa as a main supervisors during the authors conducted a study at Graduated School of Science and Technology of Shizuoka University-Japan for teach, guidance, direction, support, suggestion, helpfully and research opportunities that have been provided.
2. Prof. Nakagawa (Shimane University) for providing pUGW5, pUGW6, and pGWB8 vectors to analyze the localization of proteins. Prof. Yoshikazu Kiriwa (Shizuoka University), Prof. Mitsuo Omura (Shizuoka University), Prof. Takashi Nakatsuka (Shizuoka University), Prof. Katsumi Suzuki (Shizuoka University) and Dr. Gang Ma (Shizuoka University) for valuable discussion on the technical aspects of this study.
3. My affiliation of Politeknik Negeri Lampung (State Polytechnic of Lampung) Indonesia, for supported my study.
4. My family (mother, wife and sons) for patience, support and do’a during completing my doctoral degree at Shizuoka University, and all members of molecular breeding laboratory (Motohashi Lab.) of Shizuoka University for great cooperation during research and 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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LIST OF ABBREVIATIONS

SITIL	:	<i>Solanum lycopersicum</i> temperature-induced lipocalin
SITIL1	:	<i>Solanum lycopersicum</i> temperature-induced lipocalin-1
SITIL2	:	<i>Solanum lycopersicum</i> temperature-induced lipocalin-2
SICHL	:	<i>Solanum lycopersicum</i> chloroplastic lipocalin
CHRC	:	Plastid lipid associated protein
HrBP1	:	Hairpin binding protein-1
VDS	:	Violaxanthin de-epoxidase
ZEP	:	Zeaxanthinepoxidase
Blc	:	Bacterial outer membrane lipoprotein
sGFP	:	Synthetic green fluorescent protein
VIGS	:	Virus-induced gene silencing
TRV	:	Tobacco rattle virus
PDS	:	Phytoenedesaturase
SODs	:	Superoxide dismutases
ROS	:	Reactive oxygen species
2D-PAGE:		Two dimensional polyacrylamide gel electrophoresis

SUMMARY

Plastid proteomic data showed that lipocalins are related to differentiation of chromoplasts and ripening in tomato fruits. Lipocalins are a group of proteins and distributed in bacteria, invertebrate, and vertebrate animals. Lipocalin has various functions including those related to environmental stress response, apoptosis induction, membrane formation and fixation, regulation of immune response, cell growth, and metabolism adjustment. However, very little is known about plant lipocalins. To better understand the function of lipocalins, we made over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced tomato plants. The over-expressed *SITILs* and *SICHL* plants compared with wild-type plants under light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) showed their leaves with curling, longer terminal leaflet, bullwhip phenotype in leaves, early flowering, increasing number of flowers and inflorescences, and bigger peduncle and fruits. The over-expressed *SITIL1* showed longer of leaves with curling, over-expressed *SITIL2* showed earlier flowering, and over-expressed *SICHL* showed dark greening in seedling and mesocarp of mature green fruit and earlier ripening compared others. Moreover pericarp transversal cell structure was expanded in over-expressed *SITIL1*, *SITIL2* and *SICHL* fruits. We also suppressed *lipocalins* expression in tomato using VIGS-system to observe their phenotype. The suppressed expression of *SITILs* induced aberrant shapes of leaves, such as yellowing and curling, and yellowing in pericarps and mesocarps of fruits. The suppressed expression of *SICHL* induced yellowing in pericarps and mesocarps, and greening in endocarps of fruits. Moreover subcellular localization analyses of *SITILs* and *SICHL* using a particle bombardment showed that the fusion proteins of *SITILs*-sGFP and sGFP-*SITILs* were located around the plasma membrane, plastid, nuclear and in reticulate structure. *SICHL*-sGFP was localized in the chloroplast. Their promoters that 1000 bp upstream from the start codons of lipocalins are included many light-responsive *cis*-elements in promoters. *SISODs* (*SISOD1*, *SISOD3* and *SISOD6*) were highly expressed in seedling, leaf, flower and fruit of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants, on the other hand very low expression of them in their gene silenced plants. I found that O_2^- and H_2O_2 also participates in over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced plants under light ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress (37°C ; 5 days) treatments. Over-expressed *SITIL1*, *SITIL2* and *SICHL* under light stress ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress (37°C ; 5 days) showed lower oxidative damage (O_2^- and H_2O_2) compared control (wild-type) plants and increased level activities of *SISODs*. Furthermore, we used pTRV-based VIGS-system in these study to silence *PDS*, *SITILs*, *SICHL* and *CHRC*. Gene silenced plants under light stress ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress (37°C ; 5 days) showed increased oxidative damage (O_2^- and H_2O_2) and decreased level activities of *SISODs*. These result indicate that O_2^- and H_2O_2 could up-regulate the antioxidant defense system that ultimately lead to improved thermotolerance in over-expressed *SITIL1*, *SITIL2* and *SICHL* plants, and the other side decreased thermotolerance in gene silenced plants. These results suggest that lipocalins play an important role in abiotic oxidative stress tolerance (eliminating ROS) in tomato plants.

CHAPTER I

INTRODUCTION

Tomato is an economically important food worldwide and highly accumulated lycopene and β -carotene in ripe stage of tomato fruit. 'Micro-Tom' is a cultivar that was originally bred for home gardening. 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 (Scott and Harbaugh 1989). Mutant's library derived from an ethyl methanesulfonate (EMS) mutagenesis has been produced for genetic studies in 'Micro-Tom' (Watanabe et al. 2013).

1.1. Lipocalin proteins

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 macro molecules (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 homeostasis,

signal transduction, and responses to stress (Bishop 2000; Flower et al. 2000; Grzyb et al. 2006; Malnoe 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) existed. Phylogenetic analyses showed that plant lipocalins exhibited homology with the bacterial outer membrane lipoprotein (Ble), the insect protein Lazarillo and the mammalian apolipoprotein D (ApoD), 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 (Chi et al. 2009; Levesque-Tremblay et al. 2009; Boca et al. 2014). In *Arabidopsis*, AtTIL is constitutively expressed in all tissues except for dry seeds and development stages, 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 per oxidation, which is a key mechanism that lipocalins protect plants from the abiotic stresses (Boca et al. 2014).

1.2. Plastid differentiation

Tomato is climacteric fruit and largely used for studying the physiological and molecular basis of the ripening processes and development mechanisms (Kahlau et al. 2008; Matas et al. 2010; Karlova et al. 2011). Plastids are a family of cellular organelles that comprise a variety of different types found in a plant cell, differentiation in specific cell types in relation to fruit ripening (Gupta et al. 2011). The plastids were divided into five groups according to their function in the cell. Chloroplasts are present in green tissues and are involved in photosynthesis. Chromoplasts non-photosynthetic plastid that accumulate carotenoids and occur in plant organs such as fruit, flower, root and tuber. Leucoplasts are colorless plastids mainly existing in root tissues and are the site of several reactions. Amyloplasts are non-pigmented organelles found in some plant cells that are related to gravitropism and responsible for the synthesis and storage of starch granules. Etioplasts are chloroplasts that have not been exposed to light, they are usually found in flowering plants grown in the dark (Enami et al. 2011).

To better understand the mechanism of plastid differentiation from chloroplast to chromoplast, In my laboratory by Suzuki et al. (2015) analyzed and compared plastid proteome and plastid morphologies with ‘Micro-Tom’ and two other varieties, ‘Black’ and ‘White Beauty’. The result of study showed that compared plastid proteome of ‘Micro-Tom’ with ‘Black’ and ‘White Beauty’ using the two-dimensional gel electrophoresis, the differences of spot number and isoelectric points of TIL (temperature-induced lipocalin) (Fig. 1) and decreasing CHRC (plastid-lipid-associated protein) and HrBP1 (hairpin binding protein-1) in ‘Black’ and ‘White Beauty’ were detected (Suzuki et al. 2015).

Chromoplast-specific carotenoid associated protein (CHRC) is a nuclear-encoded plastid protein related to carotenoids that exists uniquely in plastid, involved in carotenoid accumulation and stabilization (Vishnevetsky et al. 1996, 1999; Kilambi et al. 2013). CHRC is up regulated

during chromoplast differentiation in certain fruits (Deruere et al. 1994) and flowers (Vishnevetsky et al. 1996). CHRC also termed fibrillin/CDSP34 protein, are known to accumulate in fibrillary-type chromoplast such as those of ripening pepper fruits, and in leaf chloroplast from *Solanaceae* plants under abiotic stress conditions (Langenkamper et al. 2001) and have a role in chromoplastogenesis during flower development and fruit ripening (Leitner-dagan et al. 2006).

Harpin binding protein-1 (HrBP1) was reported to be localized in chromoplast and played a role in resistance against viruses, bacteria, fungi and pests in plants, and improve plant growth and development via those signaling pathways (Chen et al. 2012). The relation of *CHRC* and lipocalins with fruit maturation and plastid differentiation is still unknown. According to previous study, I believed these proteins (*TIL1*, *TIL2*, *CHL*, *CHRC*, and *HrBP1*,) might play a major role in chromoplast differentiation, carotenoid accumulation or other plant growth and development.

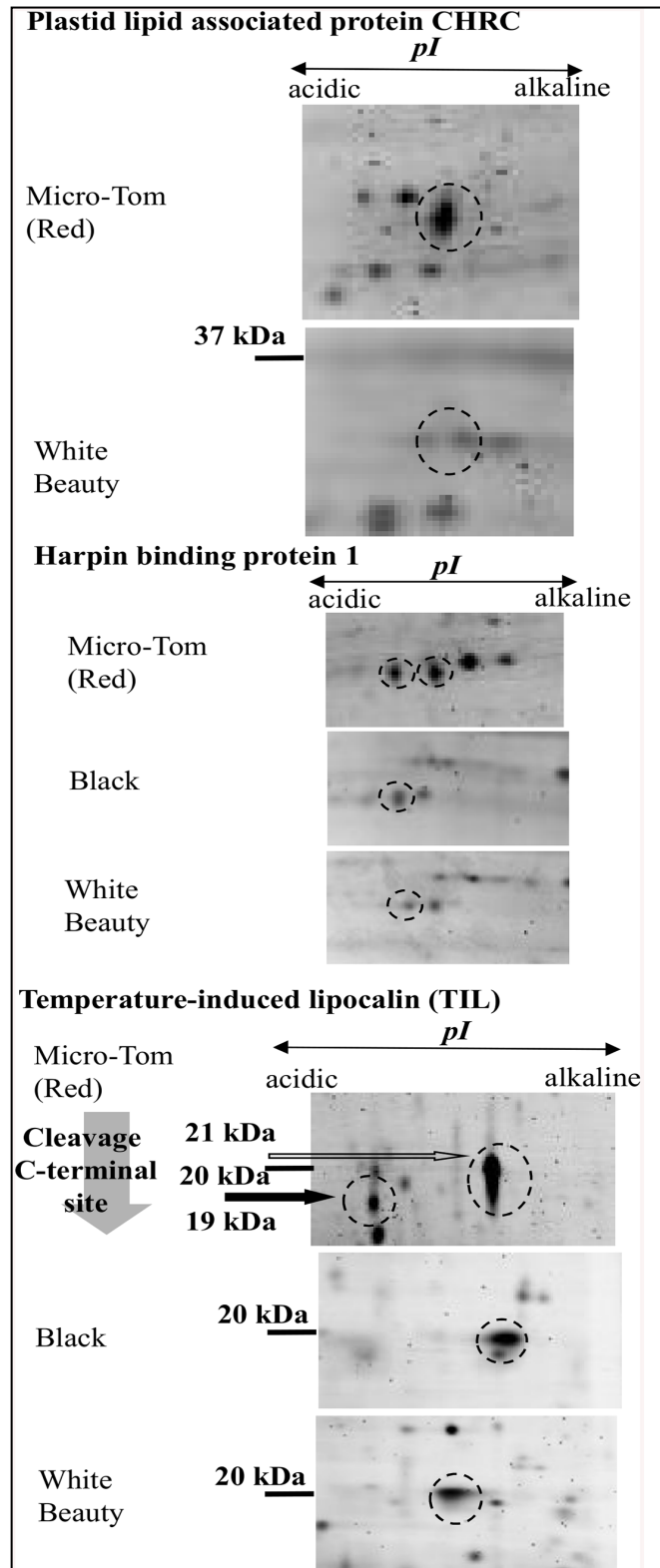


Fig. 1 Proteins of varying levels of accumulation in ‘Micro-Tom’ (Red), ‘Black’ and ‘White Beauty’ (Suzuki et al., 2015).

1.3. Virus-induced gene silencing (VIGS)

RNAi (RNA interference) is a biological process that can silence gene expression triggered by dsRNA (double-stranded RNA) (Agrawal et al. 2003). RNAi or RNA silencing was first discovered in plants as a mechanism for post-transcriptional gene silencing (Baulcombe 2000). VIGS (virus-induced gene silencing) is a powerful tool for gene functional analyses in several plants such as tomato (Liu et al. 2002, 2005; Fu et al. 2005, 2006; Orzaez et al. 2006, 2009; Li et al. 2013), barley (Holzberg et al. 2002; Bruun-Rasmussen et al. 2007; Oikawa et al. 2007), wheat (Holzberg et al. 2002; Liu et al. 2016), cassava (Fofana et al. 2004), tobacco (Liu et al. 2004; Yaegashi et al. 2007; Muruganantham et al. 2009), legume (Constantin et al. 2004; Zang et al. 2009), chili pepper (Del Rosario et al. 2008), *Jatropha* (Ye et al. 2009), *Arabidopsis* (Igarashi et al. 2009), apple (Sasaki et al. 2011), grapevine (Kurth et al. 2012; Park et al. 2016), and *Populus* (Shen et al. 2015).

VIGS is an interesting and rapid alternative for knocking down expressions of genes without the plant genetic transformation (Ratcliff et al. 2001; Liu et al. 2002; Lu et al. 2003; Brigneti et al. 2004; Burch-Smith et al. 2004; Robertson 2004; Fu et al. 2005; Orzaez et al. 2009). Barley stripe mosaic virus (BSMV) is an established VIGS vector for barley and wheat; however, silencing using this vector is generally transient, with efficient silencing often being confined to the first two or three systemically infected leaves. When barley plants were infected by BSMV vector including barley *PHYTOENE DESATURASE* (*PDS*) gene as a reporter for silencing, they showed the photobleach phenotype (Bruun-Rasmussen et al. 2007). Cucumber mosaic virus (CMV) is one of the most constraints to the production of tomato and other vegetable crops worldwide. Ntui et al. (2014) generated an RNAi construct containing inverted repeat of 1138 bp fragment of a partial replicase gene of CMV-O and used it to produce transgenic tomato plants expressing CMV-specific dsRNA of the replicase gene. In recent years, several viruses have been used as suitable VIGS vectors in tomato including tobacco rattle virus (TRV) (Deng et al.

2013). In tomato fruits, TRV-based vectors normally produce partial VIGS penetration and patchy tissue distribution as a result from partial and highly variable silencing from fruit to fruit. TRV is a bipartite, positive-sense RNA virus consisting of two types, pTRV1 and pTRV2. pTRV1 is responsible for encoding viral replication and movement factor and facilitates pTRV2 mobility during silencing. pTRV2 typically harbors a coat protein and a fragment from the host gene (Liu et al. 2002).

1.4. The objective of study

The objective of this study is to better understand the functions of lipocalin proteins in tomato plants. In this study, I studied the functions of three lipocalin related genes *SITIL1*, *SITIL2*, and *SICHL* using over-expressed tomato and gene silenced tomato plants. In addition, to elucidate the roles of *SITIL1*, *SITIL2*, and *SICHL* in light and heat response, the changes of phenotypes and gene expression were investigated in over-expressed plants and gene silenced plants under light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and a light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$) and under normal condition (24 °C) and heat condition (37 °C; 5 days).

CHAPTER II

MATERIALS AND METHODS

2.1. Plant materials

‘Micro-Tom’ (*Solanum lycopersicum* cv ‘Micro-Tom’) was used as a model cultivar for tomato research in this study because it has 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 intensity.

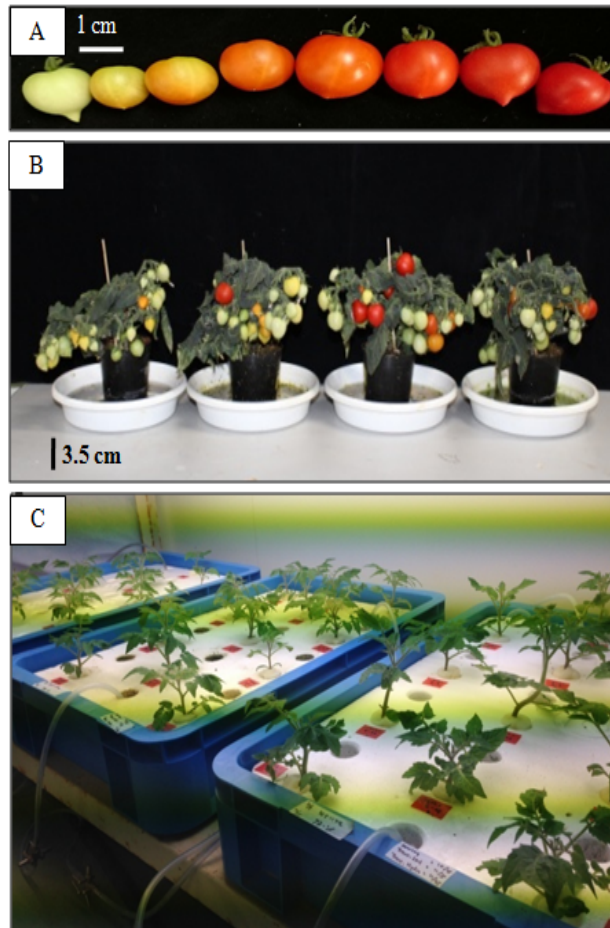


Fig. 2 Tomato (*Solanum lycopersicum*) cv. ‘Micro-Tom’. **(A)** Fruit phenotype of ‘Micro-Tom’ from green stage to red stage. **(B)** The phenotype of ‘Micro-Tom’ plants with pot soil cultivation system. **(C)** The phenotype of ‘Micro-Tom’ with hydroponic cultivation system.

The ripening process of ‘Micro-Tom’ fruit is divided into four stages: mature green fruit stage (30-33 Day Post Anthesis (DPA)); yellow fruit stage (32-33 DPA); orange fruit stage (33-35 DPA); and red fruit stage (41-45 DPA) (Suzuki et al. 2015). *Nicotiana tabacum* (SR1) and *Nicotiana benthamiana* were used for subcellular localization analyzed of SITILs and SICHL. All plants (‘Micro-Tom’, *N. tabacum*, and *N. benthamiana*) growth condition at 24-28 °C with 60±10% relative humidity under a 16 h light/8 h dark cycle. Plant materials were grown at pot soil and hydroponic system using the Enshi formula (808 mg l⁻¹ KNO₃, 492 mg l⁻¹ MgSO₄ 7H₂O, 944 mg l⁻¹ Ca(NO₃) 4H₂O, 152 mg l⁻¹ NH₄H₂PO₄, and 50 mg l⁻¹ Otsuka house 5 (OtsukaAgriTechno. Co., Ltd)) half diluted.

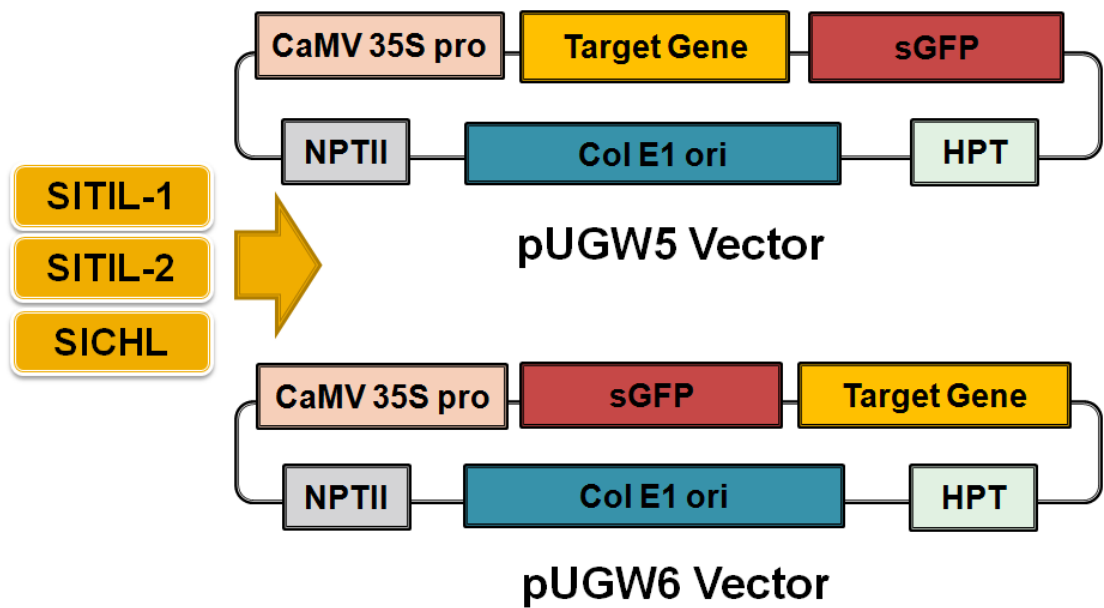


Fig. 3 The construct of subcellular localization analyses using GFP fusion proteins.

2.2. Constructs for the expressing fusion proteins of SITIL1, SITIL2 and SICHL and synthetic green fluorescent protein (sGFP)

I cloned the coding regions of *SITIL1* (LEFL2018O11), *SITIL2* (LEFL1002CA07) and *SICHL* (LEFL2044D22) full-length cDNA clones provided by Prof. Aoki into TOPO vector

(Gateway cloning system, Invitrogen) (Aoki et al. 2010). The coding regions of *SITIL1*, *SITIL2* and *SICHL* were amplified using the primers of SITIL1-F (5'-CACCATGGCTA CAAAAGTAATGG-3') and SITIL1-R (5'-TTTTCCAAGGATTGATTTGAT-3'); SITIL2-F (5'-CACCATGACCACAAAAGAGATGGAAGTAGTG-3') and SITIL2-R (5'-CTATTT TCCCAATATTGATTTGATCCACC-3'); SICHL-F (5'-CACCATGGTTTGCTACAATT TGGTGGCCC-3') and SICHL-R (5'-CACAGACAACCCCCTGGGTTTTGACGG-3'). I transferred each coding region of *SITILs* and *SICHL* into pUGW5 and pUGW6 vectors provided by Prof. Nakagawa using gateway system (Invitrogen). Each coding sequence of *SITIL1*, *SITIL2* and *SICHL* fused in frame to the N-terminus of sGFP in pUGW5-sGFP or the C-terminus of sGFP in pUGW6 vectors (Fig. 3).

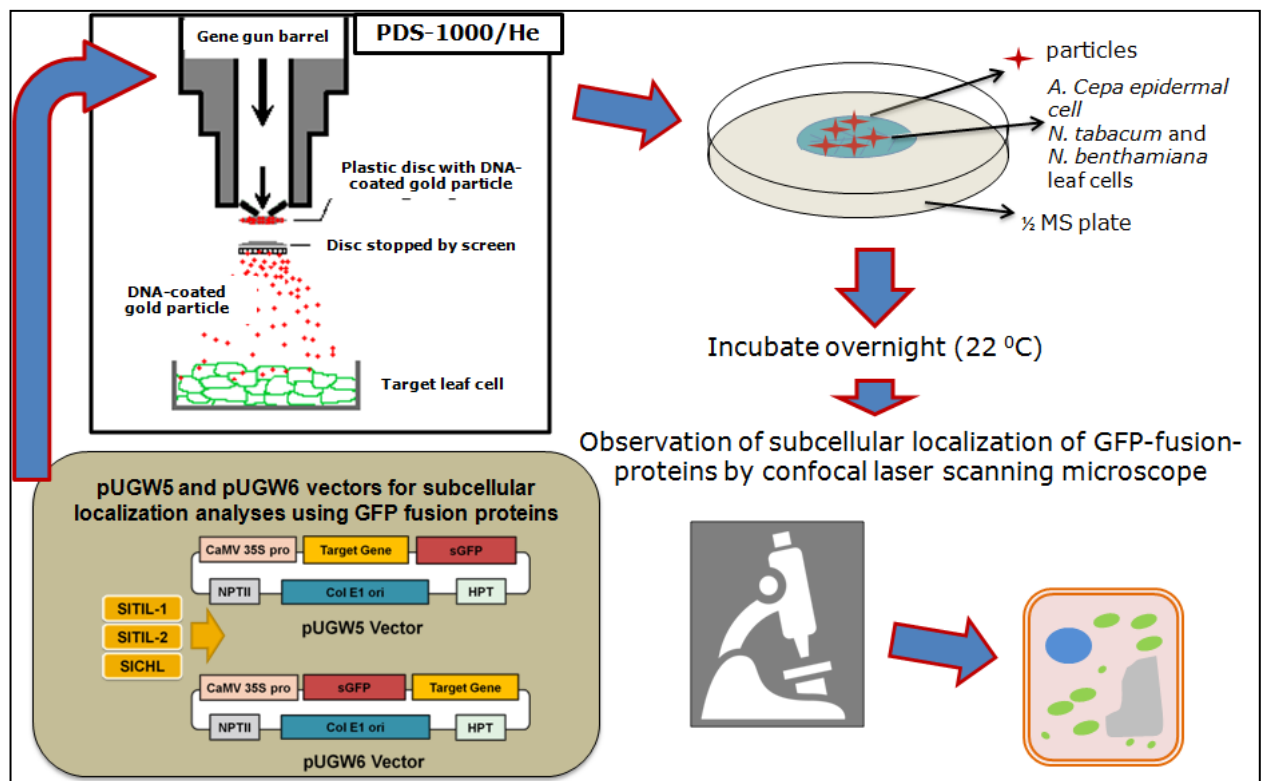


Fig. 4 Method of gene transfer using particle bombardment.

2.3. Observation of subcellular localization of SITIL1, SITIL2 and SIHL

I introduced 35Sp::sGFP-SITIL1, 35Sp::sGFP-SITIL2, 35Sp::sGFP-SIHL, 35Sp::SITIL1-sGFP, 35Sp::SITIL2-sGFP, and 35Sp::SIHL-sGFP constructs (Fig. 3) 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-SITIL1, sGFP-SITIL2, sGFP-SIHL, SITIL1-sGFP, SITIL2-sGFP, and SIHL-sGFP fusion proteins in onion epidermal cells, *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). The methods of particle bombardment and observation of sGFP signals (Fig. 4) were carried out as described by Motohashi et al. (2001).

2.4. RT-PCR analyses in the four fruit developmental stages of ‘Micro-Tom’

Total RNA was isolated from fruits (mature green, yellow, orange, and red stages) using RNeasy mini kit (Qiagen, Germany) following the manufacturer’s instructions and treated extensively with RNase-free DNase I. First strand cDNA was synthesized from about 1 µg total RNA from fruits using PrimeScript™ first-strand cDNA synthesis kit (Takara, Japan). PCR was carried out standard conditions included 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 1 min at 68 °C, using the primers SITIL1-F and SITIL1-R; SITIL2-F and SITIL2-R; SIHL-F and SIHL-R; CHRC-F (5′-TCTAGACAAAACCAAATTACAGCTCA-3′) and CHRC-R (5′-CTCGAGTGTGATGAGTTCAACGATTTCC-3′). *Actin* cDNA (425 bp) as an internal standard of gene expression was amplified using Actin-F (5′-GTTGGTGATGAAGCACAA-3′) and Actin-R (5′-CAAGACTTCTGGGCATCT-3′). RT-PCR analyses were performed in three replicates.

2.5. Promoter sequence analyses

Promoter sequences are usually the sequence immediately upstream the transcription starts site (TSS) or first exon. *cis*-elements in promoters (1000 bp upstream from the start codons) of *SITIL1*, *SITIL2*, *SICHL*, *CHRC*, and *HrBPI* genes (Figs. 16,17,18,19,20) were predicted using the PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). *CHRC* gene was used as typical gene related to chromoplast development.

2.6. Isolation of *SITIL1*, *SITIL2* and *SICHL*

Schematic structures of genomic DNA of *SITIL1*, *SITIL2* and *SICHL* genes shown in Fig. 5. I cloned the coding regions of *SITIL1* (LEFL2018011), *SITIL2* (LEFL1002CA07) and *SICHL* (LEFL2044D22) full-length cDNA clones provided by Prof. Aoki into TOPO vector (Gateway cloning system, Invitrogen, USA) (Aoki et al. 2010). The coding regions of *SITIL1*, *SITIL2* and *SICHL* were amplified using the primers of SITIL1-F and SITIL1-R; SITIL2-F and SITIL2-R; SICHL-F and SICHL-R. I transferred each coding region of *SITIL1*, *SITIL2* and *SICHL* into pGWB8 binary vector provided by Prof. Nakagawa using gateway system (Invitrogen, USA).

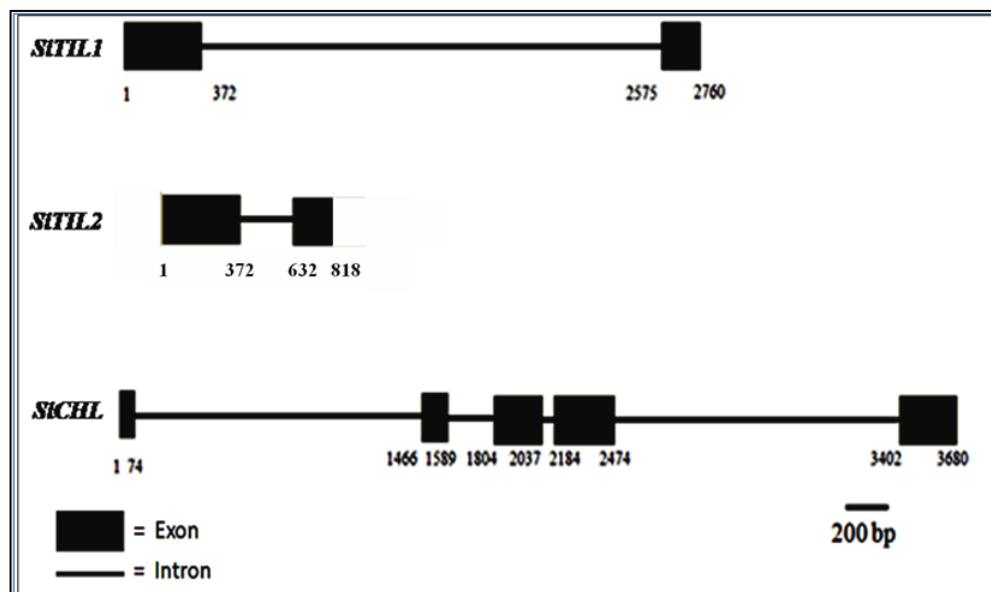


Fig. 5 Schematic structures of genomic DNA of *SITIL1*, *SITIL2* and *SICHL* genes.

2.7. Generation of over-expression of *SITILs* and *SICHL* tomato plants

The *SITIL1*, *SITIL2* and *SICHL* coding sequences were cloned into pGWB8 binary vector, under control of the CaMV 35S promoter, to construct an expression plasmid pGWB8-*SITIL1*, pGWB8-*SITIL2* and pGWB8-*SICHL*. Over-expressed plants (35Sp::*SITIL1*, 35Sp::*SITIL2* and 35Sp::*SICHL*) were generated using *Agrobacterium tumefaciens* strain GV 3101 harboring pGWB8-35Sp::*SITIL1*, pGWB8-35Sp::*SITIL2*, and pGWB8-35Sp::*SICHL*. The construct and transformation protocol of over-expressed *SITIL1*, *SITIL2* and *SICHL* were described previously (Sun et al. 2006) (Fig. 6). My study used macro elements, micro elements, tomato vitamin, CaCl₂, and FeSO₄·EDTA described (Table 1) to make Murashige and Skoog (MS) media, co-cultivation media, and callus induction media (selection I) described (Table 2), shoot induction media (selection II) and root induction media (Table 3) for generated over-expressed tomato plants.

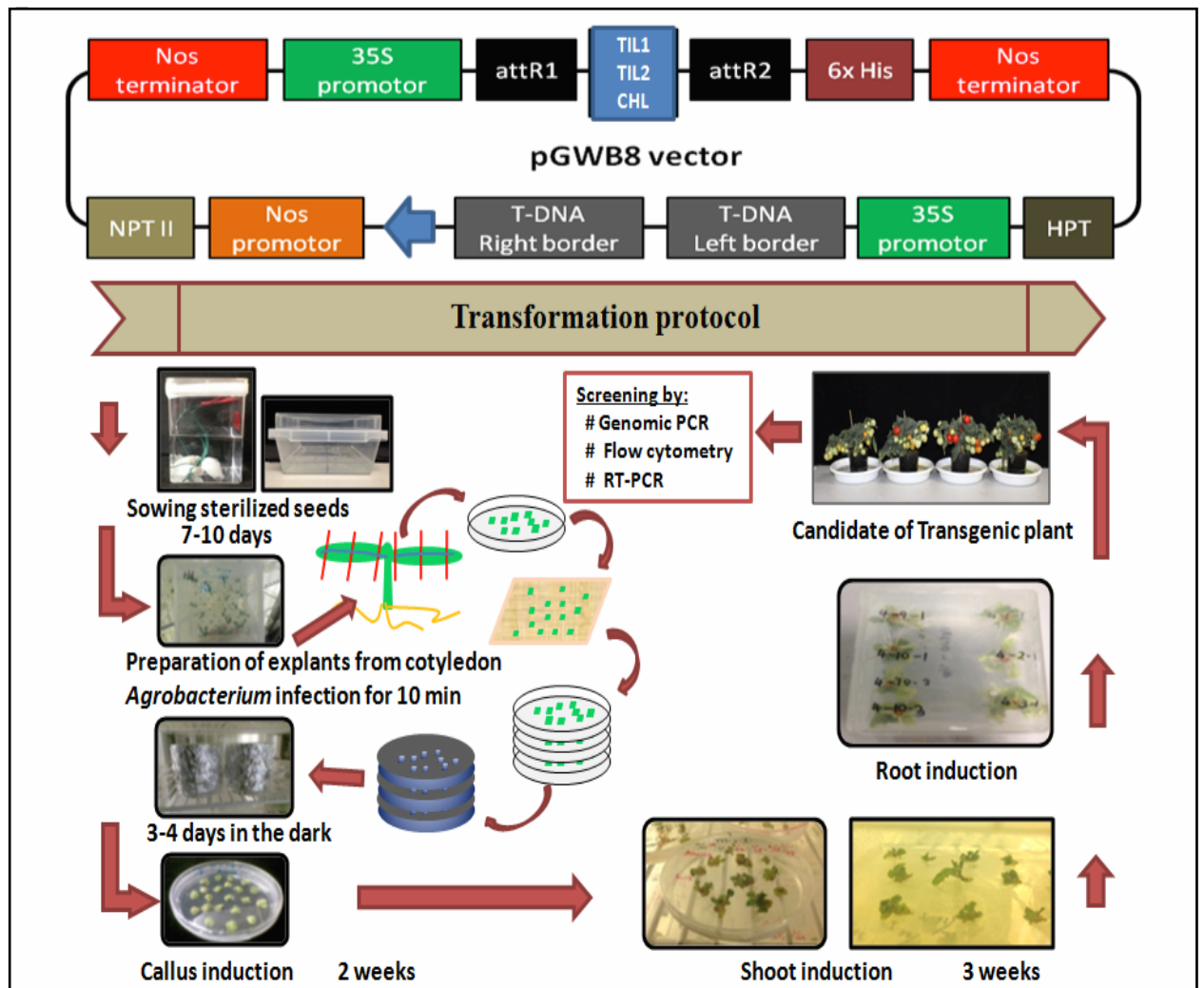


Fig. 6 The construct and transformation protocol of over-expressed *SITILs* and *SICHL*.

Table 1. Composition of Macro elements, microelements, tomato vitamin, CaCl_2 , and $\text{FeSO}_4 \cdot \text{EDTA}$.

Macro elements	
KNO_3	1900 mg l^{-1}
NH_4NO_3	1650 mg l^{-1}
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370 mg l^{-1}
KH_2PO_4	170 mg l^{-1}
Micro elements	
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	23.8 mg l^{-1}
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6 mg l^{-1}
H_3BO_3	6.2 mg l^{-1}
KI	0.83 mg l^{-1}
$\text{Na}_2\text{M}_0\text{O}_4 \cdot 2\text{H}_2\text{O}$	0.25 mg l^{-1}
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025 mg l^{-1}
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025 mg l^{-1}
Tomato vitamin	
Myo-inositol	100 mg l^{-1}
Glycine	2 mg l^{-1}
Thiamin	1 mg l^{-1}
Nicotine	0.5 mg l^{-1}
Pyridoxine	0.5 mg l^{-1}
CaCl_2	
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440 mg l^{-1}
$\text{FeSO}_4 \cdot \text{EDTA}$	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8 mg l^{-1}
EDTA . Na_2	37.3 mg l^{-1}

Table 2. Composition of Murashige and Skoog (MS) media, co-cultivation media, and callus induction media (selection I).

MS media (pH=5.8)	
Macro elements	10 ml l ⁻¹
Micro elements	1 ml l ⁻¹
FeSO ₄ .EDTA	2 ml l ⁻¹
Sucrose	30 g
Tomato vitamin	1 ml
CaCl ₂	10 ml l ⁻¹
Gerlite	3 g
Co-cultivation (pH=5.8)	
MS	Each for mg l ⁻¹
Sucrose	30 g
Gerlite	3 g
Zeatin	1.5 mg (pH=5.8)
Tomato vitamin	1 ml
1 N NaOH	330 µl
DW	1 L
Selection I (pH=5.8)	
MS	Add each for mg l ⁻¹
Sucrose	30 g
Gerlite	3 g
Zeatin	1.5 mg (pH=5.8)
Tomato vitamin	1.0 ml
1 N NaOH	330 µl
Augmentin	375 mg (1 tablet)
Kanamycin	100 mg
DW	1 L

Table 3. Composition of shoots induction media (selection II) and root induction media.

Selection II (pH=5.8)	
MS	Add each for mg l ⁻¹
Sucrose	30 g
Gerlite	3 g
Zeatin	1.0 mg
Tomato vitamin	1.0 ml
1 N NaOH	330 µl
Augmentin	375 mg (1 tablet)
Kanamycin	100 mg
DW	1 L
Root induction media (pH=5.8)	
1/2 MS	Each for mg 500 ml ⁻¹
Sucrose	15 g
Gerlite	3 g
Tomato vitamin	500 µl
1 N NaOH	115 µl
Augmentin	375 mg (1 tablet)
Kanamycin	50 mg
DW	1 L

2.8. Molecular analyses of over-expression plants

The putative over-expressed tomato plants were analyzed by a PCR assay in order to detect the presence of *Neomycin phosphotransferase II (NPT II)* and *Hygromycin phosphotransferase (HPT)* genes. RT-PCR analyses were used to confirm over-expressed plants. Total RNA was isolated from leaves using RNeasy mini kit (Qiagen, Germany) following the manufacturer's instructions and treated extensively with RNase-free DNase I. First strand cDNA was synthesized from about 1 µg total RNA from fruits using PrimeScript™ first-strand cDNA synthesis kit (Takara, Japan). PCR was carried out standard conditions included 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 1 min at 68 °C, using the primers *NPT II*-F

(5'-GAAGGGACTGGCTGCTATTG-3') and *NPT* II-R (5'-ATGTCCTGATAGCGGTCCGC-3'); *HPT*-F (5'-GTGTCACGT TGCAAGACCTG-3') and *HPT*-R (5'-GATGTTGGCGACCTCGTATT-3'); *SITIL1*-F and *SITIL1*-R; *SITIL2*-F and *SITIL2*-R; *SICHL*-F and *SICHL*-R; 35S-F (5'-GAGGAGCATGGTGGAAAAAG-3') and 35S-R (5'-CCCTGTCCTCTCCAAATGAA-3'); *SITIL1*-F (5'-ATCACAGGCATAAGGGGAAG-3') and 6x-HIS-R (5'-ATGATGAT GATGATG-3'); *SITIL2*-F (5'-ATGACCACAAAAGAGATGGAAGTA-3') and 6x-HIS-R (5'-ATGATGATGATGATG-3'); *SICHL*-F (5'-ATCACA GGCATAAGGGGAAG-3') and 6x-HIS-R (5'-ATGATGATGATGATG-3'). *Actin* cDNA (425 bp) as an internal standard of gene expression was amplified using *Actin*-F (5'-AGATGGTGTCTAGCCACACAG -3') and *Actin*-R (5'-ACCACCACTGAGGACGATGT -3'). RT-PCR analyses were performed in three replicates.

2.9. Ploidy analyses

The ploidy level of regenerated plants was determined by a flow cytometry (Beckman Coulter EPICS XL-MCL). Their leaves from over-expressed plants (35Sp::*SITIL1*, 35Sp::*SITIL2*, 35Sp::*SICHL*) were used for nuclei isolation. Pieces of tissue (1 cm²) were chopped individually on a 50 mm glass plate with a platinum coated blade (Feather, Japan) in 2 ml of nucleus isolation buffer/chopping buffer. The chopping buffer (1000 ml) contained 6.302 g Na₂SO₄, 6.057 g Tris, 10 ml 2-Mercaptoethanol, 10 ml 1% Triton X-100 (w/v), 50 g Polyvinyl pyrrolidone (PVP), and 0.025 g Propidium iodide (PI). After chopping, there-suspended sample was passed through a CellTrics® 30 µm (Partec). Samples were kept under cool conditions (ice box) during the analyses process. I added 100 µl PI (0.5 mg/µl) for each sample and then analyzed the ploidy by a flow cytometry. Data was plotted on a semi-logarithmic scale, so that the histogram peaks from 2C to 8C were evenly distributed. I used the 2C-peak DNA level of nuclei of leaves from diploid tomato wild type (WT) for the control.

2.10. RNA extraction and RT-PCR analyses

In this study, the expression of *SUPEROXIDEDISMUTASEs* (*SODs*) genes were detected by RT-PCR method. Total RNA was isolated from leaves and fruits using RNeasy mini kit (Qiagen, Germany) following the manufacturer's instructions and treated extensively with RNase-free DNase I. First strand cDNA was synthesized from about 1 µg total RNA using PrimeScript™ first-strand cDNA synthesis kit (Takara, Japan). PCR was carried out at standard conditions included 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 1 min at 68 °C, using the primers SISOD1-F (5'-TCTGGCCTAAAACCTGGACT-3') and SISOD1-R (5'-ACCAGTGAGAGGAATCT GCT-3'); SISOD3-F (5'-CTCCTGGACTTCACGGGTTT-3') and SISOD3-R (5'-CACAAGTG CTCGTCCAA CAA-3'); SISOD6-F (5'-AGGACAGCCATCTGGTGAAC-3') and SISOD6-R (5'-TGGCGAGTAATCCCAAACGA-3'); *Actin* cDNA (425 bp) as an internal standard of gene expression was amplified using Actin-F (5'-AGATGGTGTCTAGCCACACAG -3') and Actin-R (5'-ACCACCACTGAGGACGATGT -3'). RT-PCR analyses were performed in three replicates.

2.11. The observations of the phenotypes and light response in over-expressed *SITIL1*, *SITIL2* and *SICHL*

For seedlings light response, 7-days-old seedlings of over-expressed tomato plants treated under light and dark condition by covering using aluminum foil in the growth chamber with temperature 24 °C with 60±10% relative humidity under a light of 200 µmol photon m⁻² s⁻¹ and 16 h light/8 h dark cycle. I observed the phenotypes at 3, 7, and 12 days after treated and continued RT-PCR analyses (Fig. 7). For study plants light response, one week after transplanted (WAT) over-expressed tomato plants were moved into a growth chamber with temperature 24-28 °C with 60±10% relative humidity under a 16 h light/8 h dark cycle under light condition 1 (405 µmol photon m⁻² s⁻¹) and under light condition 2 (105 µmol photon m⁻² s⁻¹). I observed the

phenotypes and RT-PCR analyses of over-expressed *SITIL1*, *SITIL2*, and *SICHL* every week periodically.

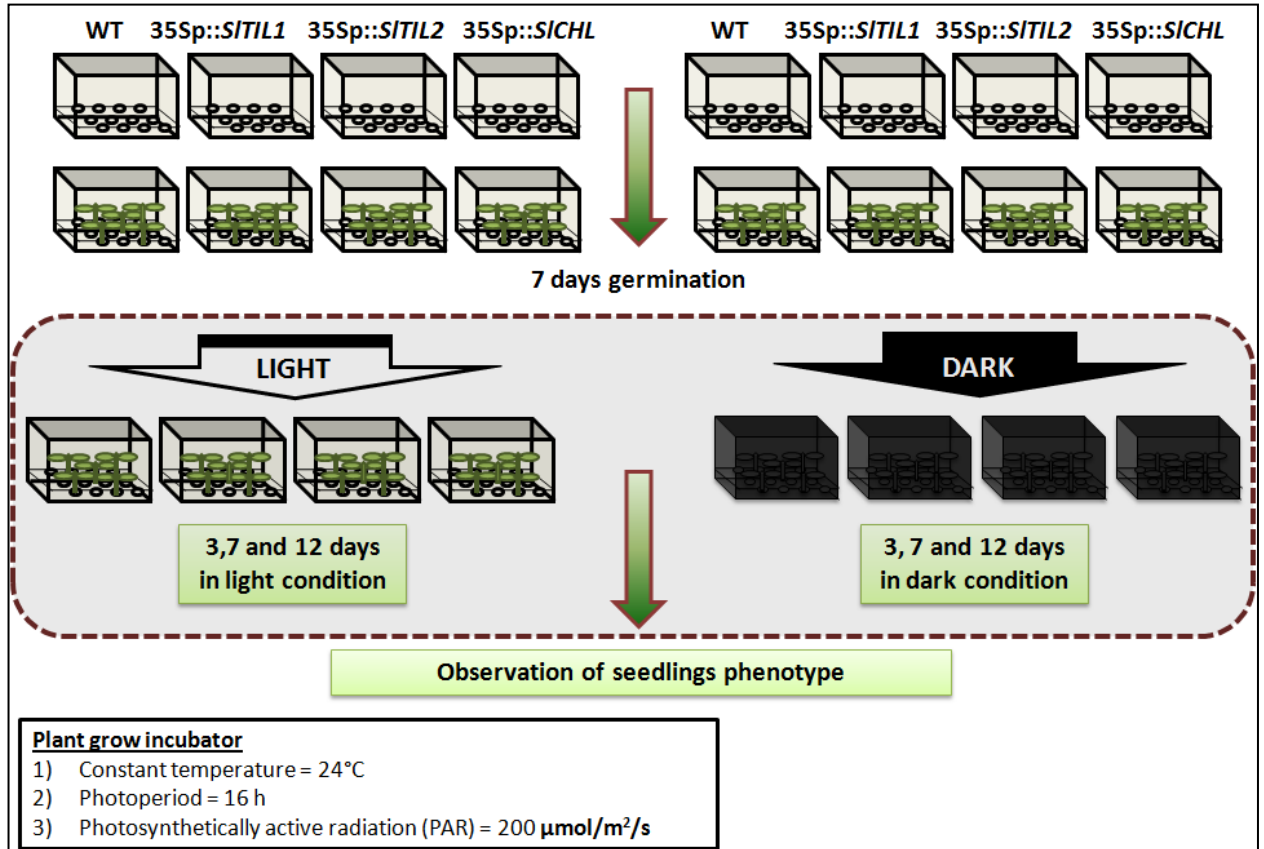


Fig. 7 The design of phenotype observation and RT-PCR analyses of wild-type and over-expressed *SITIL1*, *SITIL2* and *SICHL* in seedling stages.

2.12. Cytological analyses

‘Micro-Tom’ tomato (wild-type) and over-expressed *SITIL1*, *SITIL2* and *SICHL* fruit pericarp samples were cut by hand-sectioning for cytological analyses by a resin-embedding method. These samples were fixed in 1% paraformaldehyde and 3% glutaraldehyde in 0.025 mM phosphate-buffered saline (PBS) and evacuated using a vacuum pump for 15-30 min then stirred using tube rotator for 24 h. Fixed samples were dehydrated through the following series of ethanol concentrations: 30%, 50%, 70%, 80% and 90% for 20 min each and then 100% twice until four times for 30 min. Ethanol in dehydrated samples was exchanged for Technovit 7100

resin (Heraeus Kulzer, Wehrheim, Germany) through the following series of Technovit 7100: ethanol: 1:4, 2:3, 3:2, 4:1 each for 30 min and then 100% Technovit for 30 min and 12 h. Samples were then solidified in Technovit 7100 resin following the manufacturer's protocol. Embedded samples were cut into 5 μ m sections using a microtome with a glass knife. The sections were stained with 1% Toluidine Blue and 2.5% sodium carbonate solution for 10 min, washed with water and then photographed on a Zeiss Axiophot microscope ($\times 4$, $\times 10$ and $\times 20$) with a Spot digital color camera (Diagnostic Instruments) (Fig. 8).

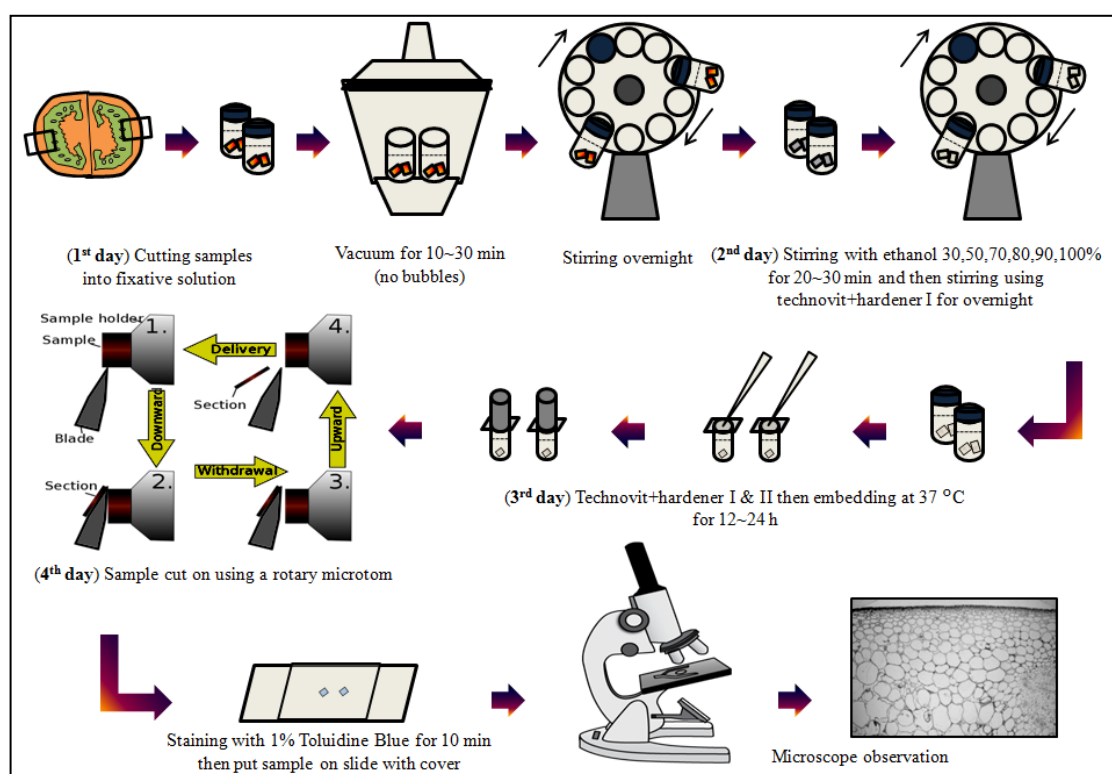


Fig. 8 Cytological analyses in breaker fruit of over-expressed *SITILs* and *SICHL* using Technovit.

2.13. Vector construction for Virus-induced gene silencing (VIGS)

TRV2-VIGS recombinant plasmids were constructed by inserting fragments of *SITIL1*, *SITIL2*, *SICHL*, and *CHRC* cDNA into pTRV2 vector. TRV-based vectors pTRV1, pTRV2 and

pTRV2-PDS were provided by Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org/abrc/catalog/individ_cloned_gene_1.html). Fragments of these cDNA were amplified using the following primers: pTRV2 (95 bp): Coat Protein-F (5'-ACGGGCTAACAGTGCTCTTG-3') and Coat Protein-R (5'-TCCCTTGGTTCGTCGTAAC-3'), *PDS* (286 bp): PDS-F (5'-TAACTGCCAAACCACCACAA-3') and PDS-R (ACCCATTGATTCGCTACCAG-3'), *SITIL1* (505 bp): SITIL1-F (5'-GAATTTCGGAAGTGGTGAAAAATCTTGA-3') and SITIL1-R (5'-GGATCCTTTGGTGTCTTTGGGGCTATC-3'), *SITIL2* (326 bp): SITIL2-F (5'-GAATTCTGAAGGGACTGCCTATAAAGCTG-3') and SITIL2-R (5'-GGATCCCTTAGTGTCTTTGGGGCATC-3'), *SICHL* (360 bp): SICHL-F (5'-GAATTCTTGCGGCATCAATCCTATT-3') and SICHL-R (5'-GGATCCTGCCTGTGATGTAGCCATCAG-3'), *CHRC* (230 bp): CHRC-F (5'-TCTAGACAAAACCAAAATTCA CAGCTCA-3') and CHRC-R (5'-CTCGAGTGTGATGAGTTCAACGATTTCC-3'). pTRV2 vectors were digested with *Eco*RI and *Bam*HI restriction enzymes. Each PCR fragment was inserted into the digested pTRV2-vector resulting in pTRV2-*SITIL1*, pTRV2-*SITIL2* and pTRV2-*SICHL* and pTRV2-*CHRC* vectors. pTRV2-*PDS* 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 I silenced an endogenous *PDS* in tomato using pTRV1 and pTRV2-*PDS*, tomato plants showed photobleached phenotypes.

2.14. Gene silenced treatments

pTRV1, pTRV2-*PDS*, pTRV2-*SITIL1*, pTRV2-*SITIL2*, pTRV2-*SICHL*, and pTRV2-*CHRC* were transferred into *Agrobacterium* strain GV3101. These transferred *Agrobacterium* were grown overnight at 28 °C in 5 ml L-broth medium containing 50 mg l⁻¹ gentamycin and 50 mg l⁻¹ kanamycin for preculture. Precultures were cultured in 50 ml of LB medium containing the same

antibiotics for two days at 28 °C to inoculate. The cells were harvested by centrifugation and resuspended in an infiltration medium (10 mM MgCl₂, 10 mM MES, and 200 mM acetosyringone), adjusted to an optical density at O.D₆₀₀ 2.0 and shaken slowly at room temperature for 3 hours. Resuspensions of pTRV1, pTRV2-*PDS*, pTRV2-*SITIL1*, pTRV2-*SITIL2*, pTRV2-*SICHL*, and pTRV2-*CHRC* were mixed at a ratio of 1:1 and was injected on the bottom of cotyledon and fruit (35 dpa) using a 1 ml syringe without a needle.

2.15. RNA isolation and RT-PCR in gene silenced plants

Total RNA was isolated from control plant, gene silenced plants and over-expressed *SITILs* plants leaf, flower and fruit samples. Fruits are harvested and separated silenced from non-silenced areas by cutting them with a sharp knife. Genomic DNA was removed from extracted total RNA by DNase treatment. RT-PCR condition was denatured at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min used primers: SITIL1-F and SITIL1-R; SITIL2-F and SITIL2-R; SICHL-F and SICHL-R; CHRC-F and CHRC-R. RT-PCR used *SISODs* gene was carried out standard conditions included 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 1 min at 68 °C, used the primers SISOD1-F and SISOD1-R; SISOD3-F and SISOD3-R; SISOD6-F and SISOD6-R. RT-PCR analyses were performed in three replicates.

2.16. The observations of the phenotypes and light response in wild-type plants and gene silenced plants

For study gene silenced plants in light response, 10-days-old after control (*PDS*) were shown bleached phenotypes, gene silenced plants were moved into a growth chamber with temperature 24-28 °C with 60±10% relative humidity under a 16 h light/8 h dark cycle under light condition 1 (405 µmol m⁻² s⁻¹) and under light condition 2 (105 µmol m⁻² s⁻¹). I observed the phenotypes of wild-type plants and gene silenced plants (pTRV2, pTRV2-*PDS*, pTRV2-*SITIL1*,

pTRV2-*SITIL2*, pTRV2-*SlCHL*, and pTRV2-*CHRC*) every week periodically.

2.17. 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) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay. The method for detection O_2^- and H_2O_2 using NBT assay, DAB assay and H_2DCFDA assay were carried out according to Kaur's method (Kaur et al. 2016) (www.bio-protocol.org/e2061). For detection O_2^- and H_2O_2 , leaves of *SITILs* and *SlCHL* over-expressed and silenced plants were cut and then dip 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 dip samples 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 ($100\text{ }^\circ\text{C}$) till the chlorophyll is removed from the samples completely. The samples were dipped for cooling in 20% glycerol then captured the images using a stereo microscope by keeping the samples on a slide.

CHAPTER III

RESULTS

3.1. Characterization of SITIL1, SITIL2 and SICHL

The amino acid sequences of SITILs and SICHL were analyzed using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) (Figs. 10,11). The result showed that SITIL1 and SITIL2 have high similarity with StTIL (*Solanum tuberosum* TIL), AtTIL (*Arabidopsis thaliana* TIL), NtTIL (*Nicotiana tabacum* TIL), PbTIL (*Populus balsamifera* TIL), PeTIL (*Populus euphratica* TIL), TcTIL (*Theobroma cacao* TIL), PaTIL (*Prunus armeniaca* TIL), PpTIL (*Prunus persica* TIL), and MtTIL (*Medicago truncatula* TIL) (Fig. 10).

The phylogenetic tree of TILs was made using Phylogeny.fr (http://www.phylogeny.fr/simple_phylogeny.cgi) (Fig. 9). SITIL1 from ‘Micro-Tom’ showed highest similarity with StTIL in potato and AtTIL in *Arabidopsis* comparing with other TILs (Fig. 9). SITIL2 from ‘Micro-Tom’ showed highest similarity with NtTIL. The amino acid sequences of TILs were conserved in *Solanaceae*. The amino acid sequence of SICHL showed high similarity with SpCHL (*Solanum pennelii* CHL) and StCHL (*S. tuberosum* CHL) (Figs. 9 and 11).

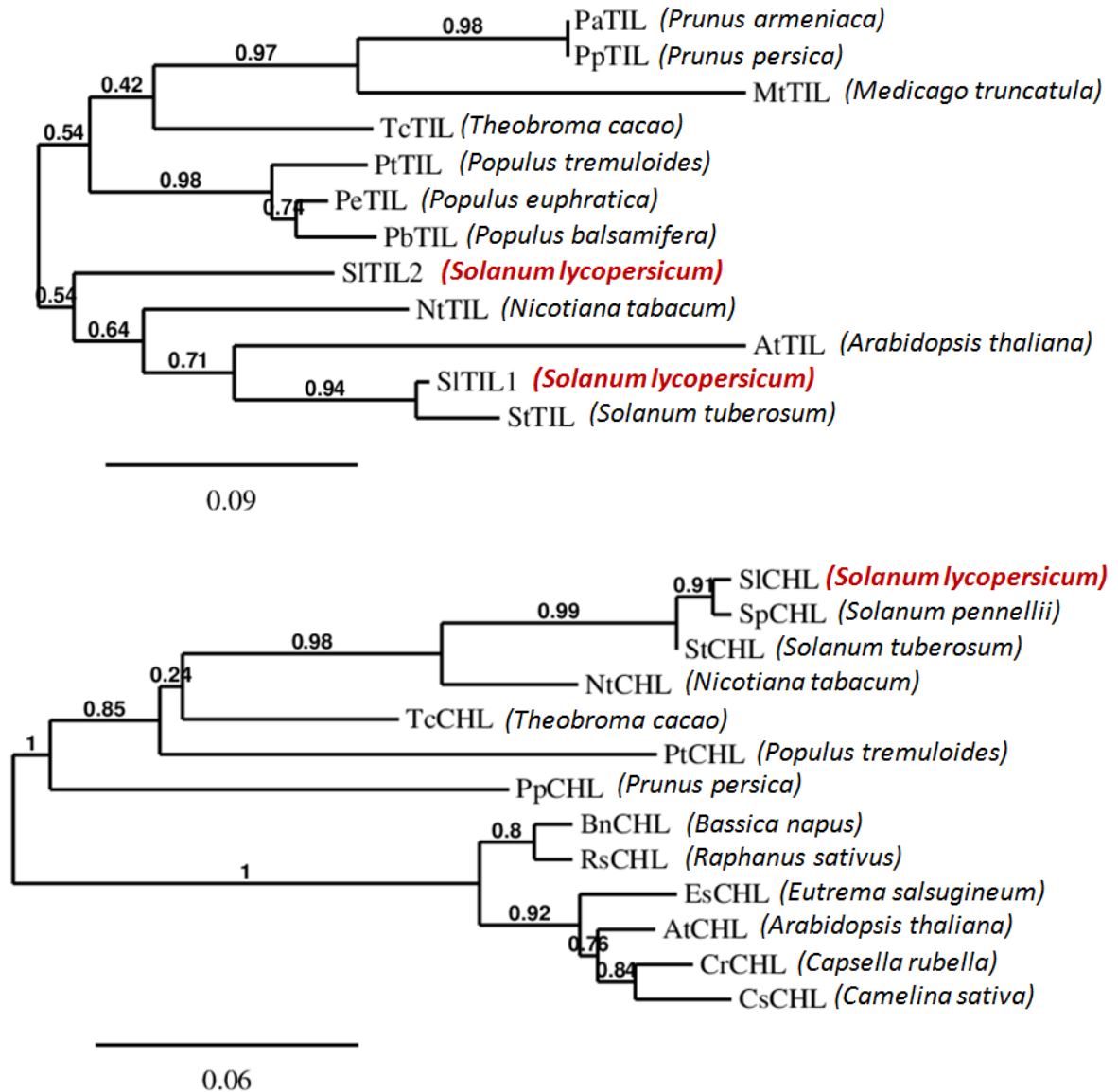


Fig. 9 Phylogenetic trees of TIL proteins and CHL proteins in plants. The values near branch represent bootstrap value, and the scale bar indicates 0.09 (TILs) and 0.06 (CHL) amino acid substitutions per site. A phylogenetic tree of TIL proteins in plants. The GenBank accession numbers of the published *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). 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 *SITIL1* (LEFL2018O11), *SITIL2* (LEFL1002CA07),

SiCHL (LEFL2044D22) were obtained from 'Micro-Tom' Database
(<http://www.pgb.kazusa.or.jp/mibase/index.html>).

MtTIL	---MGEPEVVKGVDLERYMGRWYEIASFSPFFQPTNGENTRATYILNSNGTVDVNETWN	61
NtTIL	-MATKVM EVVKLDVEKYMGRWYEIASFSPSRFQPKNGVNTRATYTLNPDGTVHVLNETWC	61
<u>SITIL1</u>	-MATKVM EVVKNLDLKRYMGRWYEIASFSPSRFQPKDGVNTRATYTLNSDGTVHVLNETWC	61
<u>SITIL2</u>	-MTTKEME VVKNLDEKYMGRWYEIASFSPSRNQPKDGVNTRATYTLNQDGTVHVLNETWS	61
StTIL	-MATKVM EVVKNLDLKRYMGRWYEIASFSPSRFQPKDGVNTRATYTLNSDGTVHVLNETWC	61
PaTIL	-MAKKTMDVVKGLDLQRYMGRWYEIASFSPSRFQPKNGENTRATYTLRDDGTVNVNETWS	61
PpTIL	-MAKKTMDVVKGLDLQRYMGRWYEIASFSPSRFQPKNGENTRATYTLRDDGTVNVNETWS	61
TcTIL	-MSQKPMEVVKHLDINRYMGRWYEIASFSPSRFQPRGGVNTRATYTLREDGTVHVLNETFT	61
PbTIL	MATKKEME VVKGVDLKRYMGRWYEIASFSPSRFQPKNGVNTRATYTLNEDGTVHVLNETWN	61
PeTIL	MASKKEME VVKGVDLKRYMERWYEIASFSPSRFQPKNGVNTRATYTLNEDGTVHVLNETWN	61
PtTIL	MATKKVME VVKGVDLKRYMGRWYEIASFSPSRFQPKNGVNTRATYTLNEDGTIHVLNETWN	61
	*** :*: :** ***** ** * :***** * :*: *****:	
MtTIL	NGKRESIHGIAYKADPN SDEAKLKVRFLVPPFLPFIIPVGDYWI LYLDQDYQFALIGGPT	121
NtTIL	DGKRGYIEGTAYKADPKSDEAKLKVKFYVPPFLPIIPVTGDYWVLHIDENYQYALIGQPS	121
<u>SITIL1</u>	NGKRGFIEGTAYKADPN SDEAKLKVRFYVPPFLPIIPVTGDYWVLYIDEDYQYALIGQPS	121
<u>SITIL2</u>	-GKRGSIEGTAYKADPKSDEAKLKVKFYIPPFLPIIPVIGDYWVLYIDDDYQYALIGQPS	121
StTIL	NGKRGFIEGTAYKADPN SDEAKLKVRFYVPPFLPIIPVTGDYWVLYIDEDYQYALIGQPS	121
PaTIL	DGKRSSIIEGTAYKADPSSEEAKLKVKFYVPPFLPIIPVVG DYWVLFIDEDYQYALIGQPS	121
PpTIL	DGKRSSIIEGTAYKADPSSEEAKLKVKFYVPPFLPIIPVVG DYWVLFIDEDYQYALIGQPS	121
TcTIL	DGKRGYIEGTAYKADPKSDEAKLKVKFYVPPFLPIIPVVG DYWVLFIDDDYQYALIGQPS	121
PbTIL	DGKRGYIEGSAYKADPN SDEAKLKVKFYVPPFLPIIPVVG DYWVLSLDEDYQYALIGQAS	121
PeTIL	DGKRGYIEGSAYKADPN SDEAKLKVKFYVPPFLPIIPVVG DYWVLSLDDYQYALIGQPS	121
PtTIL	DGKRGSIEGSAYKADPN SDEAKLKVKFYVPPFLPIIPVVG DYWILYLD DDYQYALIGQPS	121
	*** * * ***** :*:*****: *****:*** . ***** * :*: :*:***** :	
MtTIL	ELFLWILSRQTYLDDESYNKL VQAKDDGYDVTKLHKTPQSNPPPQ-----	181
NtTIL	RNYLWVLCRQNSLDDEIYNQLVEKAKEEGYDVSKLHKTPHTNPPPPENDDAPKDTKG IWWI	181
<u>SITIL1</u>	RRYLWILSRQTRL DDEIYNQLVEKAKEEGYDVSKLHKTPQSDSPDSEDS PKDTKG IWWI	181
<u>SITIL2</u>	-KYLWILCRQPHLDEEIYNQLVEKAKEEGYDVSKLHKTPQADPPPDGEDAPKDTKGFWWI	181
StTIL	RRYLWILSRRTCLDDEIYNQLVEKAKEEGYDVSKLHKTPQSDSPPESEDS PEDTKG IWWI	181
PaTIL	RNYLWILSRQPRLDDEIYNQLVQRAKDEEYDVSKLHKTPQSETPPEEGE GPKDTKG IWWF	181
PpTIL	RNYLWILSRQPRLDDEIYNQLVQRAKDEEYDVSKLHKTPQSETPPEEGE GPKDTKG IWWF	181
TcTIL	RKYLWILCRQPHMDDEIYNQLVQAKADEGYDVSTLHKTPQSDPPPEGEETPKDTKG IWWI	181
PbTIL	RKYLWILCRKTHMEDDIYNQLVEKAKEEGYDVEKLHKTPQTDPPPE-EEGPKDTKG IWWF	181
PeTIL	RKYLWILCRKTHMEDEIYNQLVEKAKEEGYDVEKLHRTPTQTDPPPE-EEGPKDTKG IWWI	181
PtTIL	RSYLWILCRKTHMEDEIYNQLVEKAKEEGYDVGKLHKTPQTDPPPE-EEGPKDTKG IWWI	181
	. ***: * : :*: *****:***** :*** . *****: **:	
MtTIL	----- 187	
NtTIL	KSILGK 187	
<u>SITIL1</u>	KSILGK 187	
<u>SITIL2</u>	KSILGK 187	
StTIL	KSILGK 187	
PaTIL	KSLLGK 187	
PpTIL	KSLLGK 187	
TcTIL	KSIFGK 187	
PbTIL	QSILGK 187	
PeTIL	KSILGK 187	
PtTIL	KSILGK 187	

Fig. 10 Alignment of the deduced amino acid sequences of SITIL1 and SITIL2 in *Solanum lycopersicum* and other TILs (Mt, *Medicago truncatula*; Nt, *Nicotiana tabacum*; St, *Solanum tuberosum*; Pa, *Prunus armeniaca*; Pp, *Prunus persica*; Tc, *Theobroma cacao*; Pb, *Populus*

balsamifera; Pe, *Populus euphratica*; Pt, *Populus tremuloides*). Identical residues are in gray.

<u>SlCHL</u>	MVCYNLVAQQSPPI LVQSQYPSKPRGLSVKVVSSCCTECPIFGKVEVQHVISGLAASILF	61
SpCHL	MVCYNLMAQQSPPI LVQSQYPSKPRGLSVKVVSSCCTECPIFGKVEVQHVISGLAASILF	61
StCHL	MFCYNLVAQQSPPI LVQSQYPSKPRGLPVKVVSACTECPIFRKVEVKHVISGLATSILC	61
*, ****:***** ***** *****:***** ****:*****:***		
<u>SlCHL</u>	LSQSNMAFAADLPHYNSVFQLANVADSMP TLPLEK GNDGGKLM MRGMTAKDFDPI RYSG	121
SpCHL	LSQSNMGFAADLPHYNSVFQLANVADSMP TLPLEK GNDGGKLM MRGMTAKDFDPI RYSG	121
StCHL	LSPSNMAFAADLPHYNSVFQLANVADSMP TLPLEK ENDGGKLM MRGMTAKDFDPI RYSG	121
** ***, ***** ***** *****		
<u>SlCHL</u>	RWFEVASLKRGFAGGGQEDCHCTQGIYTVD MNAPAIQVDTFCVHGGPDGYITGIRGRVQC	181
SpCHL	RWFEVASLKRGFAGGGQEDCHCTQGIYTVD MNAPAIQVDTFCVHGGPDGYITGIRGRVQC	181
StCHL	RWFEVASLKRGFAGGGQEDCHCTQGIYTVD MNAPAIQVDTFCVHGGPDGYITGIRGRVQC	181

<u>SlCHL</u>	LNEEDKEKDETDLERQEMIREKCYLRFP TLPFIPKEPYDVIATDYDNFALVSGAKDKSFV	241
SpCHL	LNEEDKEKDETDLERQEMIREKCYLRFP TLPFIPKEPYDVIATDYDNFALVSGAKDKSFV	241
StCHL	LNEEDKEKDETDLERQEMIREKCYLRFP TLPFIPKEPYDVIATDYDNFALVSGAKDKSFV	241

<u>SlCHL</u>	QIYSRTPNPGPEFIDRYKNYLASF GYDPSKIKDTPQDCEVKTTSQLSAMMSMSGMQQALN	301
SpCHL	QIYSRRPNPGPEFIDRYKNYLASF GYDPSKIKDTPQDCEVKTTSQLSAMMSMSGMQQALN	301
StCHL	QIYSRTPNPGPEFIEKYKNYLASF GYDPSKIKDTPQDCEVKTTSQLSAMMSMSGMQQALN	301
***** *****:*****		
<u>SlCHL</u>	NQFPDLELR RPVQFNPF TSVFETLKKLAELYFK	334
SpCHL	NQFPDLELR RPVQFNPF TSVFETLKKLAELYFK	334
StCHL	NQFPDLELR RPVQFNPF TSVFETLKKLAELYFK	334

Fig. 11 Alignment of the deduced amino acid sequences of SlCHL in *Solanum lycopersicum* and other CHLs (Sp, *Solanum pennellii* and St, *Solanum tuberosum*). Identical residues are in gray.

3.2. Observation of subcellular localization of SITIL1, SITIL2 and SlCHL

To know intracellular localization of lipocalin proteins in tomatoes, I analyzed the subcellular localization of SITILs and SlCHL using a particle bombardment. The fusion proteins of SITIL1-sGFP, sGFP-SITIL1, SITIL2-sGFP, and sGFP-SITIL2 were accumulated in the plasma membrane and around membranes of the onion (*A. cepa*) epidermal cells (Figs. 13B,C,D,E). In case of *N. tabacum* leaf cells, I sometimes detected the fluorescence around plastids and nuclear and in reticulate structures (Figs. 12B,C,D,E). In the control cells expressing sGFP (pUGW5-sGFP and pUGW6-sGFP), the fluorescence were found in the cytosol and the

nucleus (Figs. 12A and 13A). The fusion proteins of SICHL-sGFP were localized in the chloroplasts of *N. tabacum* and *N. benthamiana* leaf cells (Figs. 12F,G).

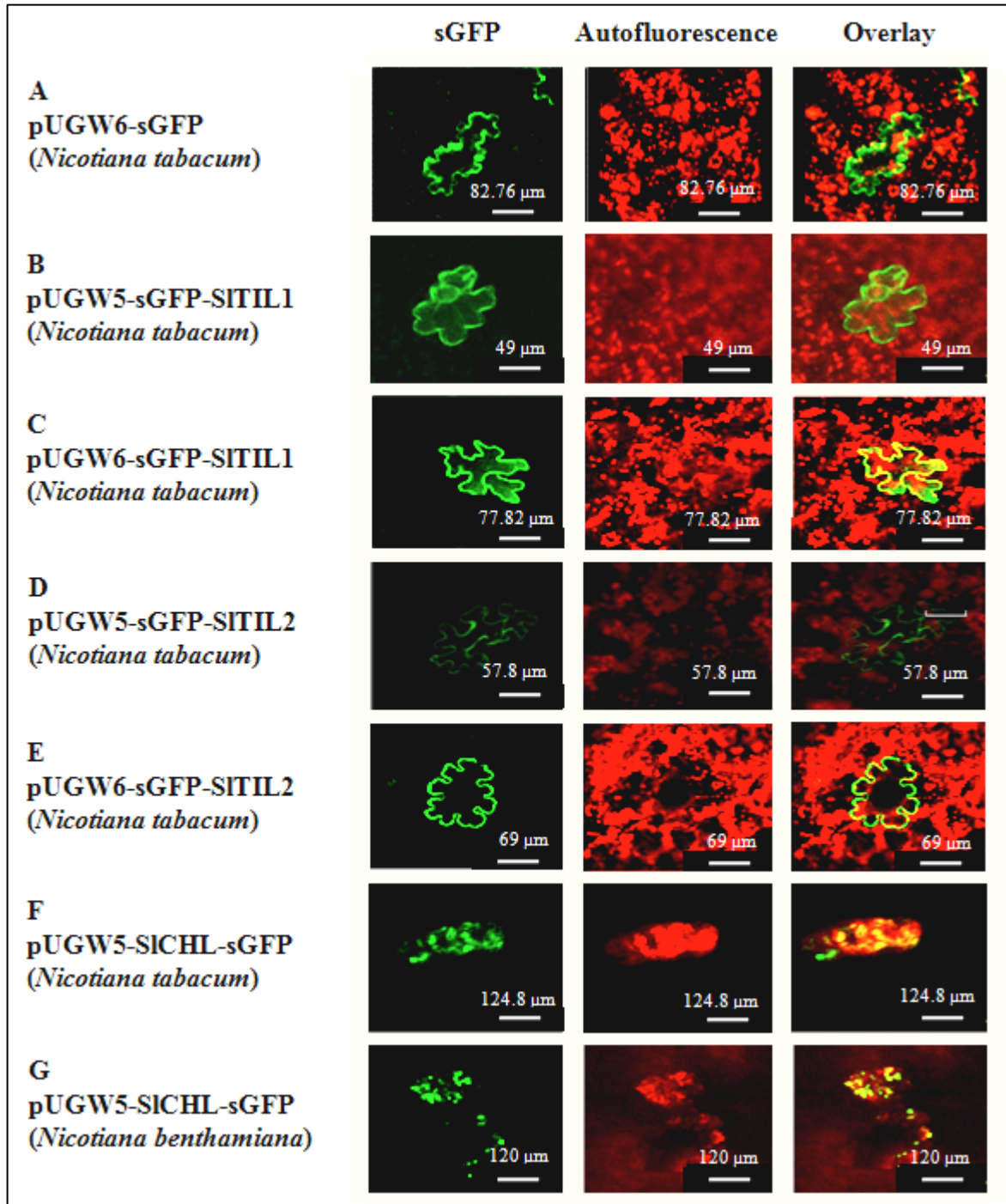


Fig. 12 Subcellular localization of SITIL1, SITIL2, SICHL in *Nicotiana tabacum* and *N. benthamiana* leaf cells. (A) Fluorescent microscopic images of pUGW6-sGFP protein in *N. tabacum* leaf cells. (B) pUGW5-sGFP-SITIL1 fusion protein in *N. tabacum* leaf cells. (C) pUGW6-sGFP-SITIL1 fusion protein in *N. tabacum* leaf cells. (D) pUGW5-sGFP-SITIL2 fusion protein in *N. tabacum* leaf cells. (E) pUGW6-sGFP-SITIL2 fusion protein in *N. tabacum* leaf

cells. (F) pUGW5-SICHL-sGFP fusion protein in *N. tabacum*. (G) pUGW5-SICHL-sGFP fusion protein in *N. benthamiana* leaf cells.

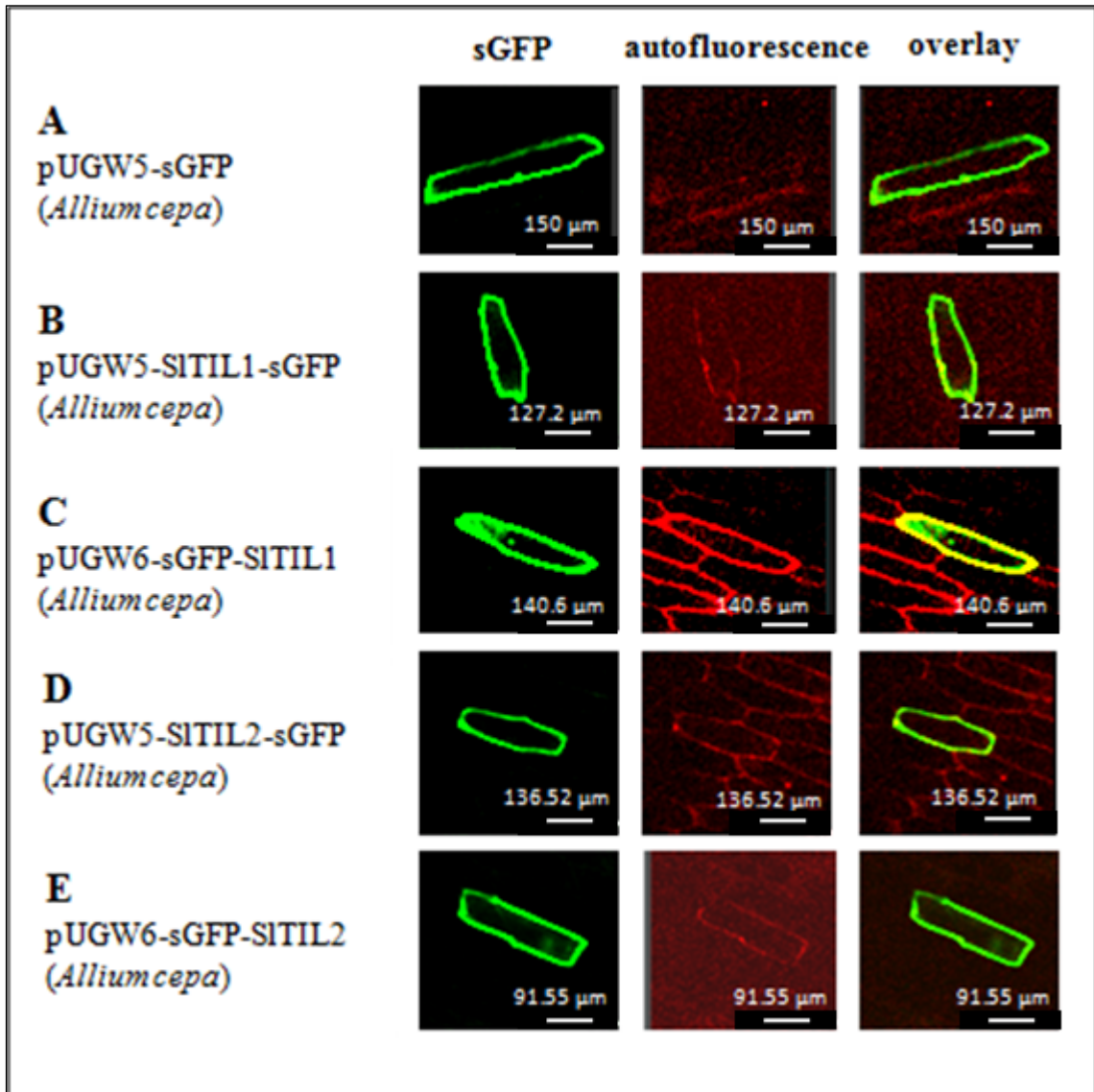


Fig. 13 Subcellular localization of SITIL1, SITIL2, SICHL in onion epidermal cells. (A) Fluorescent microscopic images of sGFP in onion epidermal cells (pUGW5-sGFP). (B) Fluorescent microscopic images of pUGW5-SITIL1-sGFP fusion protein in onion epidermal cells. (C) Fluorescent microscopic images of pUGW6-sGFP-SITIL1 fusion protein in onion epidermal cells. (D) Fluorescent microscopic images of pUGW5-SITIL2-sGFP fusion protein in onion epidermal cells. (E) Fluorescent microscopic images of pUGW6-sGFP-SITIL2 fusion protein in onion epidermal cells.

3.3. RNA analyses in the four fruit developmental stages of ‘Micro-Tom’

Expression analyses of *SITIL1*, *SITIL2*, *SICHL*, and *CHRC* genes were carried out using

RT-PCR (Fig. 14A). I analyzed the expression of lipocalin genes at the four fruit development stages of ‘Micro-Tom’ (mature green, yellow, orange and red stages) (Fig. 14B). The data showed that *SITIL1* transcripts were slightly increased from the mature green stage to the red stage, *SITIL2* transcripts were slightly decreased at the red stage, and *SICHL* transcripts were decreased continuously from the mature green stage to the red stage. *CHRC* transcripts were decreased from yellow or orange stages and then kept same levels of transcripts (Fig. 15).

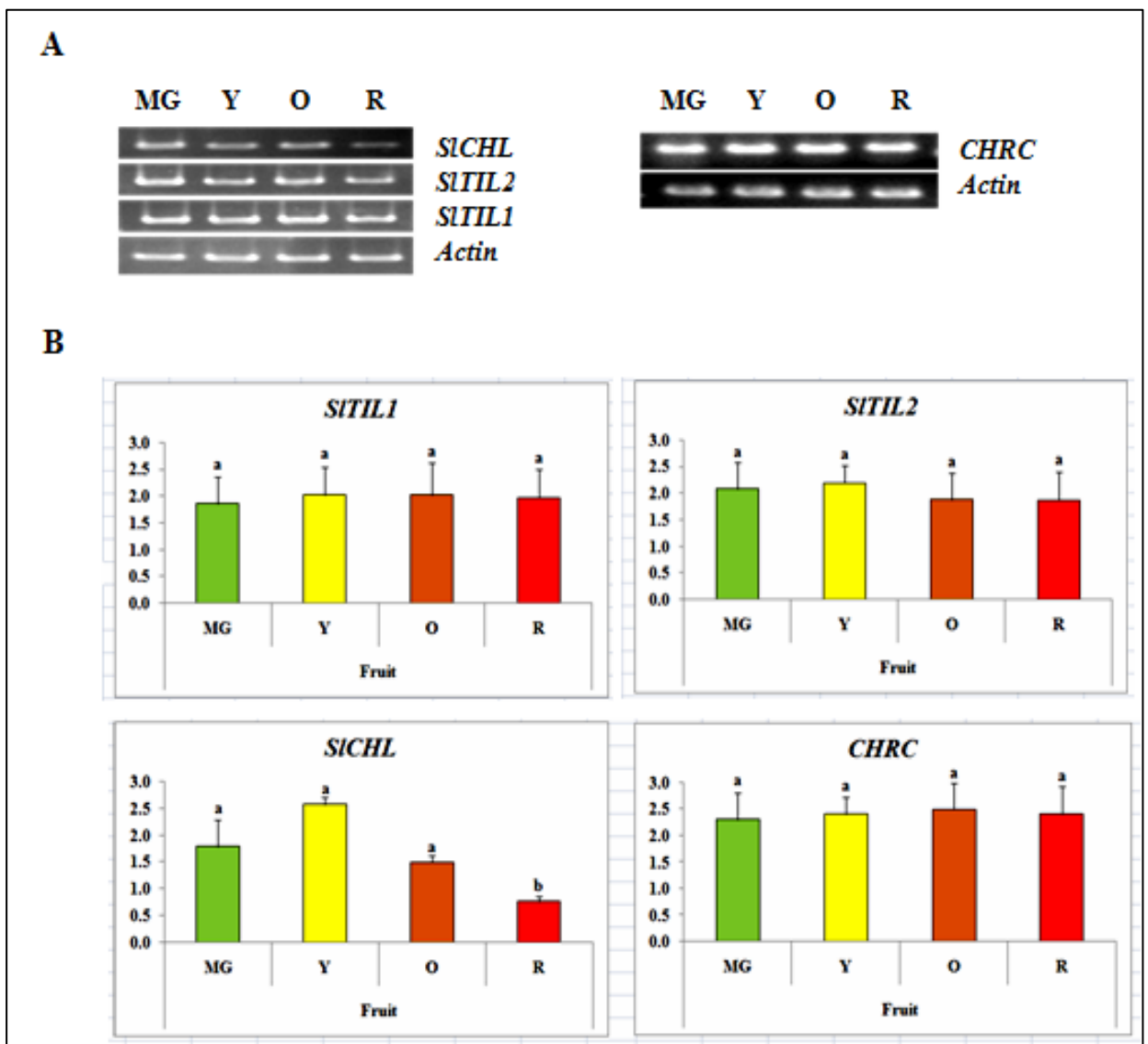


Fig. 14 RNA analyses of *SITIL1*, *SITIL2*, *SICHL*, and *CHRC*. (A) RNA analyses of these genes at each developmental stage of ‘Micro-Tom’ tomatoes. MG: mature green, Y: yellow color stage, O: orange color stage, R: red color stage. (B) Expression analyses of these genes at each

developmental stage of ‘Micro-Tom’ tomatoes. Data are mean values of three separate experiments $\pm$ SD. Columns with different letter are significantly different at $p < 0.05$ by t -test.

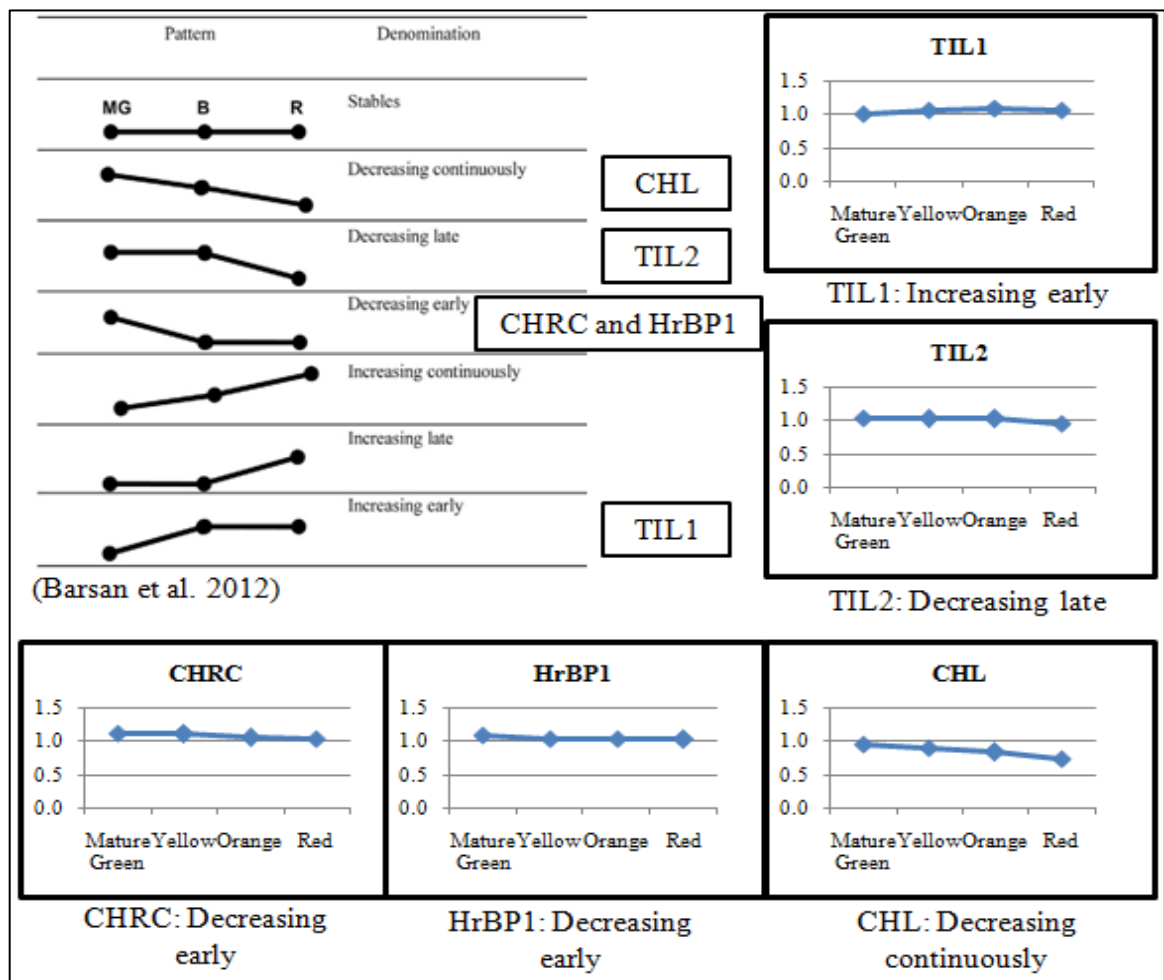


Fig. 15 Abundance patterns of *SITIL1*, *SITIL2*, *SICHL*, *CHRC* and *HrBP1* at each developmental stage of ‘Micro-Tom’ fruits (mature green, yellow, orange and red color).

3.4. Analyses of *cis*-elements in putative *SITIL1*, *SITIL2*, *SICHL* and *CHRC* promoters

To better understand the regulation of *SITIL1*, *SITIL2*, *SICHL*, and *CHRC* transcripts, *cis*-elements in their promoter regions that 1000 bp upstream from the start codon (Figs. 16,17,18,19,20) were predicted by PlantCARE. The results showed that three major classes of *cis*-elements were identified, including light-responsive elements, stress-responsive elements and hormone-responsive elements. A relatively large number of light-responsive *cis*-elements in each promoter were identified (Table 4). Only ACE was a motif found in *SITIL1*, *SITIL2*, *SICHL*, and *CHRC* promoter. Stress-responsive *cis*-elements were identified which reflected plant responses

to drought (MBS in *SITIL2*, and *CHRC* promoters), heat (HSE in *SITIL1*, *SICHL*, and *CHRC* promoters), anaerobic induction (ARE in *SITIL1*, and *SITIL2* promoters). Seven kinds of hormone-responsive *cis*-elements were identified in each promoter (Table 4). The coding sequences of *SITIL1* and *SITIL2* exhibited high similarity (84 %), but each promoter had specific *cis*-elements related to heat stress responsiveness (*SITIL1*) and ethylene responsiveness (*SITIL2*).

5'-AAAATCAATACTTTCACGATAGAAAAAAGAGATATAAAAATTGAAACGGCTTTC
GAAATAAAAGGAACTAAAATGAATAATATCTAATGAATTGGATTTATCTCGCTTTGT
TCAAACGAATTTGAATACGTAACATAAACTCTCTCCGTTCTATATTGATTGATCATTT
TCTTTTTTTATATACGCATATTAATAAAAAATCATAAGTAAAAATATAATTGTATTAATT
CACTCCTTAAAAAACTTCTTACAAATTTTACGAACAAATGTGAAATTTATTAATAAAG
AATTAATTGTAGGGGTAATATAAGGAAAATGTAATTAAAGTTTTTTTGATCTAGTAA
AGTGGATAATTAATATTCCCGTTGTCCACTTTTGACTATCATTTTATGCTTTTCGAAA
GTCAATTGACTAATTTATAAAGTGAAATTAGATTACATTAATTTTGATATTTTAAAC
AAAAAAAATGTAGATATATGAAATAGTATAAATTACATTTTTTTTCATTATATAATCG
TAAAATGTTAGTCAAGCTACTTATCATTTGACTCTAAAAATAATCCTAATAATAAAG
TGGACGGACATAGTATGTAATACTCCTCCATTTAAAAAAGGATGACCTGGTTTGACT
TGGAACGAAGTTTAAGAAAAGAAAGAAGAGGTCAAATATACCAAATGTCACTTAA
TCTTATAGTTTTTAAATATATCACGTAAAAAATTAATAATTAAATATTACCAAAAAAA
AAATCATTCTTTTTTAAACCCTGGCAAAATAAAATGTTTAATTGTAAAGGAAGTT
CCCAAGAAAATTAACCTTTTAAAAATGTAAGGTCCTTAAATATCCACGTCCATCCCAT
AACATGAATTTCTCTTGTATATATATATACTCTCTAAAAACCCAAAATCCTACAA
TCAATGGAAATTCTCCCTATTTAAAGATTCCATTTTATCAAAAAATCTCCCAAATCT
TGAAAACCCCATTTGTTTAAACTTCAAAATA-3'

Fig. 16 Sequence 1000 bp (upstream from the start codons) promoter of *SITIL1* chromosome_ch12_Solanum_lycopersicum.fasta from 3378343 to 3378714.

5'-CTCAACATAGCCCCTCAACTATGGCCATCCCAAATAGTTGGGACTTGAGATCAGC
TATATTTCAATATTCTATATTTTGAGTTCTCTAATGCTAGGTGAAATGCGTGTAGTTC
CAGTTTAATAAAGAAAGAGCTGTATTCTAAAAATGAAATATATCAAGTGTACCATTT
AAGTTGATTTACCAGTATTTACTAAAAATAATAATCTCAGAATGTCTTAAGTTAACA
ATCACCTTCTAGAATAATTTTGTATTCTAGGATGTGTATTATTAAGAAAGAAATT
ACCATTAATAATGAAAAAATAATTTAAACAGCCCACTGTATTATATTGAACAGAATTC

TAGAACCTGCCATTGAACAATCTGCCAAAAAAGGTCAATCATTACACTTGTAAGGTG
 CTTGAATGTCCACGTCTATATCCACAAATATTTTCACAGCACACAATATCTCCTCATCC
 CAATAAACCTTATCAAGAATTCAATCTCTGAAAGAACAAAAAACTTAAAATCCTG
 TATTGTTTGTGTTGTTTGTATTTTAGTTTGAAATAAAATCTAAAAAAGCAAAAAAAGTT
 GTCAATTTTGTGAATTTAAAATATGTAAAATATTATTATCTACTCTCATAATTTTAAA
 AATATTACATGGAAAGTATTATCTTTTAAGTAAATTATTTTTGTTAGGAGTAAGATA
 AGGAAAAAATGGGGAATTAAATTTTAACGAATAAGATAAAAGCTCAAATAATCAAT
 CAACTGACACCAAAAAAAGGATACTTCTATTTTAAAACAAATTAAAAAGAAAAATA
 TTACAAATAAATTTTGAAATGAAGAAATTAATATTACAAAAACCCTTTTCTTGGA
 AGAAGACACTAGTACTTTCTTGAAATTTGACACTCCACCCTATTTACCATTATATAT
 TTGTCTCCCATCACCTTAAACCTTATCAAGAAACCAATTCAAAGAGACCAAAACCC
 TTATACTACACTTTGAAATTTTGCAAAAAAAT-3'

Fig. 17 Sequence 1000 bp (upstream from the start codons) promoter of *SlTIL2* chromosome_ch07_Solanum_lycopersicum.fasta from 203358 to 202984.

5'-TGAGTTCGGAATATAAGAACAATGTCCAGGTTCTAAATCAAATACTTGAATACAT
 ATTCAATAAACTTCTTCATTCAAATACACTGTTCGAAGTAAAGTTATTGAGTTTCATC
 GAATCTAGTTTCTTGCGCAAAAGAACATATTAGAATAGATAAATTCTAATTAACAGA
 CAATCTTAAGAAGTTGATAAACAGCTAAAAAAGTGACAAATATACAAATTATATCT
 TAAATTTATTTGACAAAAAAAACCTCAAAGGTAACATCAAATATTCCAAACTCAA
 TGCAAGGCTATTGACTCAAGAAGCAGAAAAATATATTAGGATTAAAAATGTCATAT
 TTATCCTTATTATTATCCTAACAAGGAACAGATTTAATGAAAAAATTTACCTGAAAA
 TATTTTCGTTGACGGCGGACAACCTTTTCCGTTTTGTTTACTTTTGGGTGCAAGAACAC
 AGAGGAACCTCCCTACCACATTTTAGTAGACCTTTCCATATTAAAAATATATATAAA
 TATTTATAATAAAATAATCTTTTAATTTTACAATCGTAAATTAAATACGTAAATAA
 ATCAAATACTATTATTTATTGTGATTTTAAATATGTTACTCGAAAATTTAAAATGA
 AAGAGTTGTCAAAGATATATGTACCAAATGCTTTTAAATTGTATGCTTCAGTAACT
 TTAGTTTAAATTTATATACCTATTTTAAAGAAGATATCTCTTTCTTTTTTTGCAATT
 TTTTAAATTTTAACTTTTCAAATGACATCAAATAAGACGTAAATTATATCACACTTT
 GGGATGTTTTTAATTTTAAAAAAAATAAATCAAATTAACCTTTTAAATTTTTTAAAAA
 TTAATAAATATTATTAGACAATTATTTTAAATATAGTAAACGGAGAAAAAGGGAGC
 GGTAACATTTTTTTTTTGTGTTACATGCTAACAATTGGCAGAAAAGTGTTTAAATAATTG
 GCTTACAGCTGATGTGAAGTAAGAACACA-3'

Fig. 18 Sequence 1000 bp (upstream from the start codons) promoter of *SlCHL* chromosome_ch12_Solanum_lycopersicum.fasta from 127849 to 127775.

5'-TGAATATTTGATTATTGCAATTTGTTTTATAATTAATTTAGTGCATGCGCATTGTG
TGTGTTGTATCAATATAACATTGCATAAAAAAATTATATATATTTTGCAACTGCAAG
TTACAATTACATAATAATAATAATAATTAATACATTTTGAGAGTTTTCAAAAAAA
GTAAAAAAAATGATATTTTTTGGACAAAACAAATATACTGTTAGATGAATAAATT
GATTTTGAAGTTATAAATGAATTAAGTTAGTAGTTAAAGAATTCTATTATTAGCCA
ATGTAGTTTGATTTTTTGATATCTTAATTTATAATTTTTGCGGTGTCGTGAAGTTTCAT
GACTTTAGTTATAGACATCAATCAACGACTATTCAATTACATATTTTAGTTATACATA
TCAATCAATAATTAATCATATGTATGAATTTAGTTATAAAGTTCAATCAATAATTATT
TAACTACATCATCCCAATGTTATCTTCTTTTTATCATATATTTTAGGATATTTTTTTC
GTGAGAAAAAATCATCATTAAATAATTCTCAAATATACTTTTTACCATATATTTAGA
ATTATTTAATTTTTTAAGATAATCAATATTATAAGAGATTACATAAAAAATCAAGAAA
CATGAAGTAATAGGTATTGATAGGTTTTCTTTTCGTTTATTCACACTCTCAACTAGTA
AATATTTTTTATCCAACATTCAATATTCATTTAACATCTGATATATTTAAATTTACGT
CACATAGAACTATTCAGAAATATTTAGTGAAGGAATAAATAACTCTCCATACTGATA
GTCTCAAATTAATAAATATACTAACTAAATGAAAAGGGTATGTATGTCTTTTTACC
CCAGCTATCCAAGGCAACACGCTCGTTATAAAATGCATCATAACAGTATTGTAATTCT
CATTCATCGAGCTTCCCCTGTTTTCTTCATTCATATTACAAAAAATAGCATTCTG
CTCTTCTCTGTTCCTCTGAAA-3'

Fig.19 Sequence 1000 bp (upstream from the start codons) promoter of *CHRC*.

5'-AAGGTTTTTGAGGTATTTGCTTGAATTGAAAAGATTAGAGATGAAAGATTGAGCG
GGAAAATGATATGTTTTTGAATATTTTTCAAAAAAATTGAGATTCCAAAATTCAA
ATTTGAGTGTTTGGATATAATTTGAAGTTTTGTTTTTTTTTCTTAAACCCAAGAAAA
GAATGTGTAGTCGCGCTTAATAGTCGAGGTAGAAATAAAATTTACGTATATTTTATT
TTTCGAGATCTCAGCTATTGAATTGTTGTTGTTGAAATATGATTTACAAGTTGTGTTT
TGAAATGTAATGTTTAAGTCGGATTTCTAAAATGATTGTCATATGTCATTTTTTTATG
AGCATTTTTCGTGAAATCAAGCTAAAGTTGTTTTTATAACAGTTAGAACAAAGCAAAA
AGAATTTTAATATACAATTCGTACATAAATATGTAATTTAATTTGTTTTTAAAAATGT
AATTACGTAAACATTATTATAATTTATTTATAAAATTTAACAAATAAACACATATAT
CTTATTTAACATTATAAATAATAATTTATGTCCTACGTATATTATAACACGCGTGTG
CACGAAAGAATATGATTTATAAATGTGCCATTGACCATCTACACGTCTCATAGTGTC
CCACATTGCTACCCACTATTACTAATCATAAGGAACCTTTGGTTTTCGATTTACCTAT
CTTAATCAGAAATTTTGATACGAGTTTTTTAATATATAATAGTATTTTATTTTTCATT
AGAGATTTCAATTCGAATATCTTTGTTATAGAGGTTTTATAATTTTTAATATGAAATT

TTTAAATTTAATCGTTTTTAAATATGAATACGAGACACCGAATAAAAGAAAAGGGAA
 AGAAGGAAATGGGTCCCTTAGCTTTTTTATTCAACACAAAAGTAGAAAAATGTAGAT
 TTGTTACTACAACACTACACCACCAATCAAACCTCCACAAAAGATCGATCCTTTTTTCTG
 AAATTCAAGCTCAACCATGGCTTCTC-3'

Fig. 20 Sequence 1000 bp (upstream from the start codons) promoter of *HrBP1*.

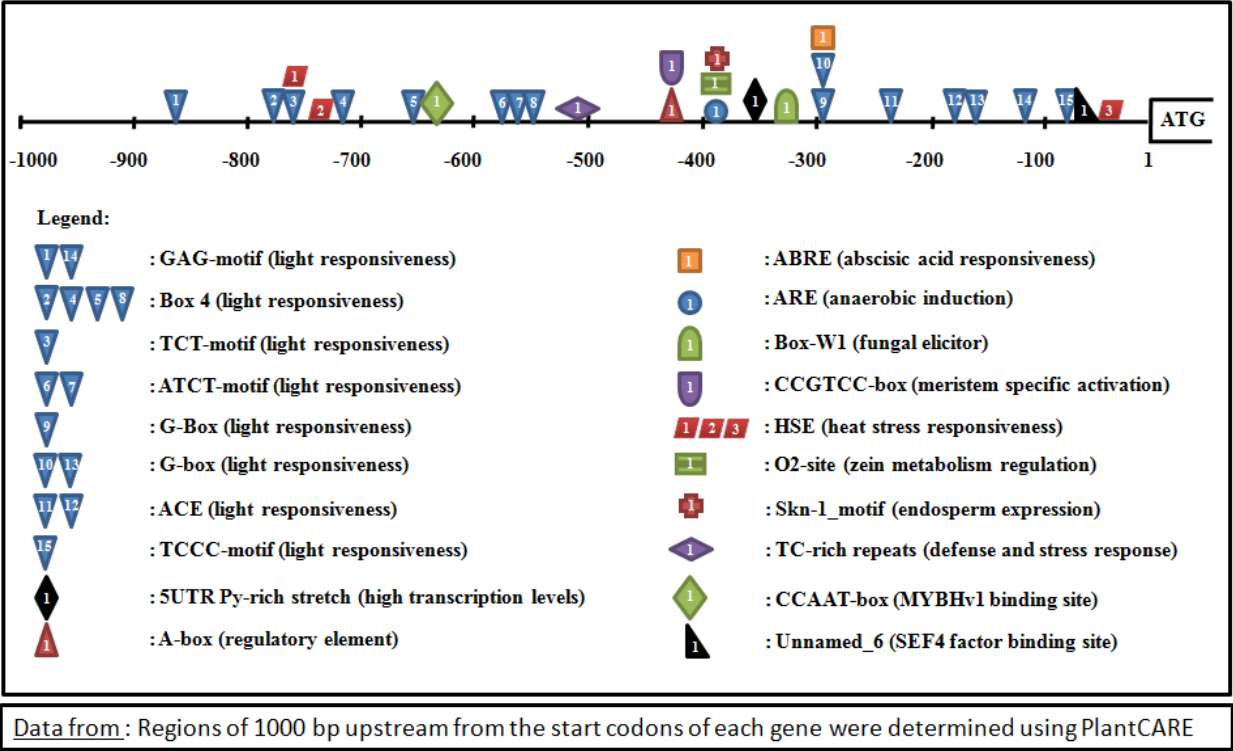


Fig. 21 *cis*-elements in promoter of *SITIL1* gene

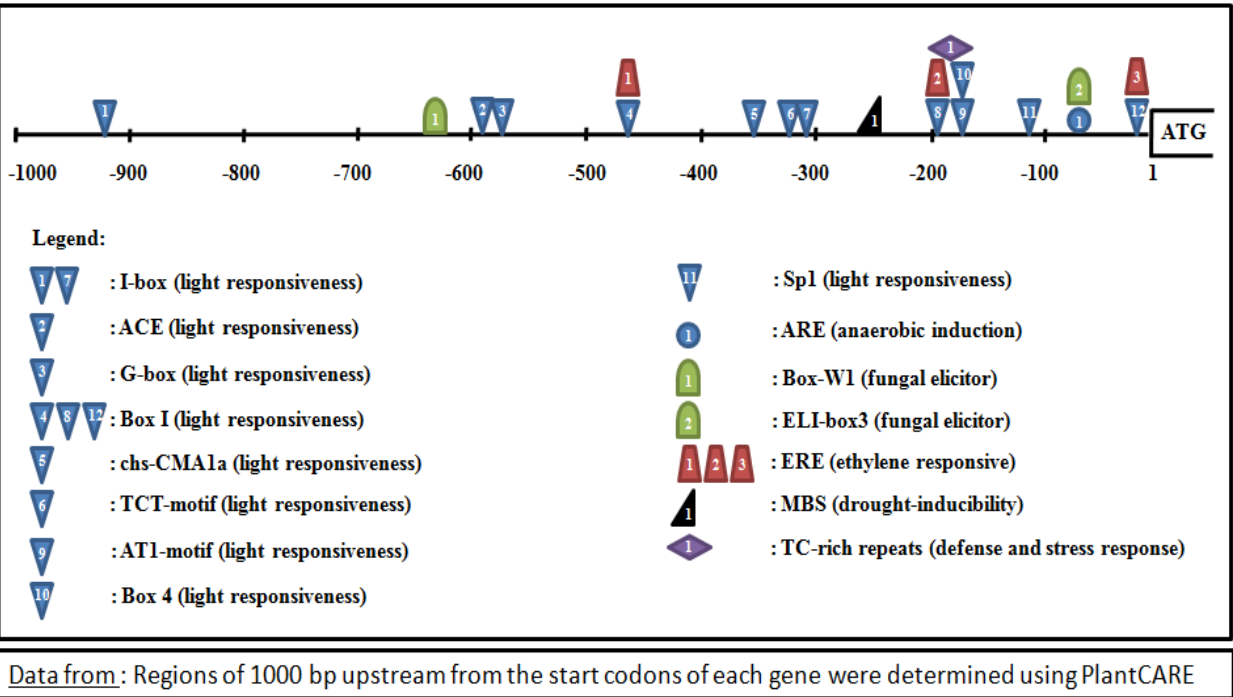


Fig. 22 *cis*-elements in promoter of *SITIL2* gene

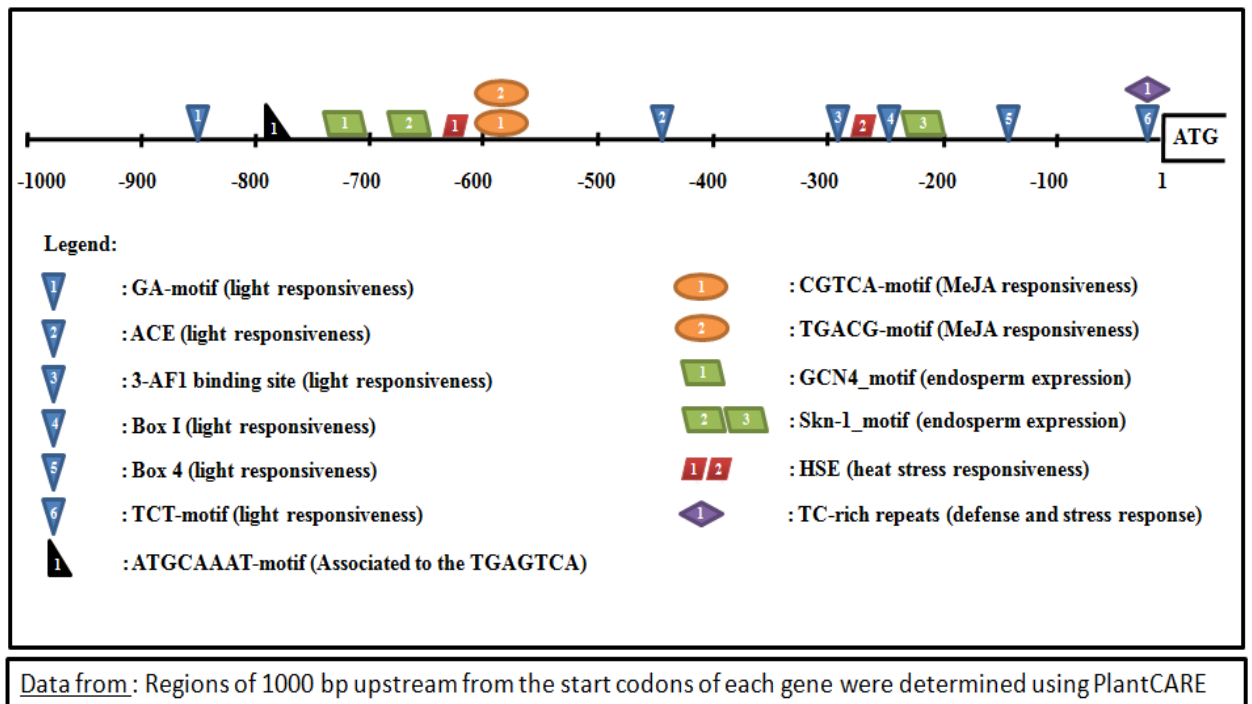


Fig. 23 *cis*-elements in promoter of *SICHL* gene

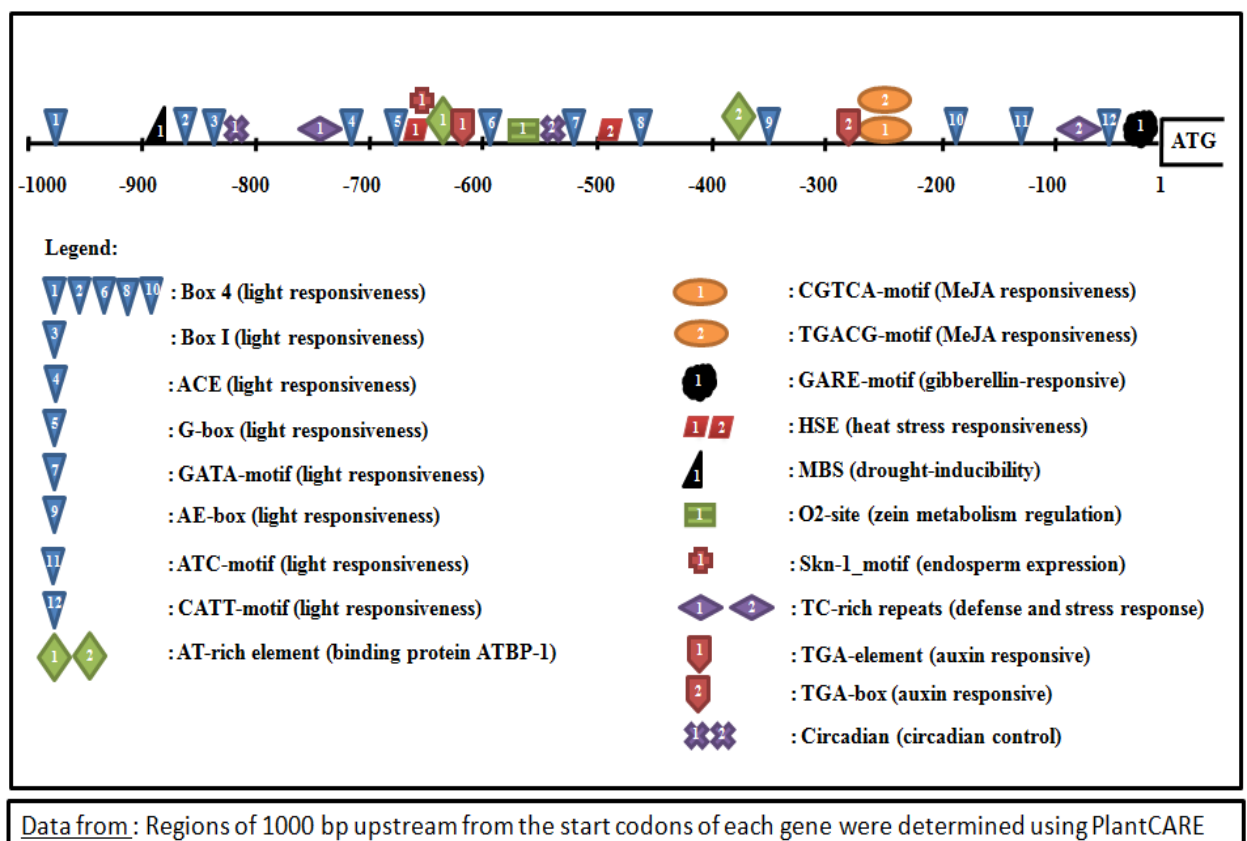


Fig. 24 *cis*-elements in promoter of *CHRC* gene

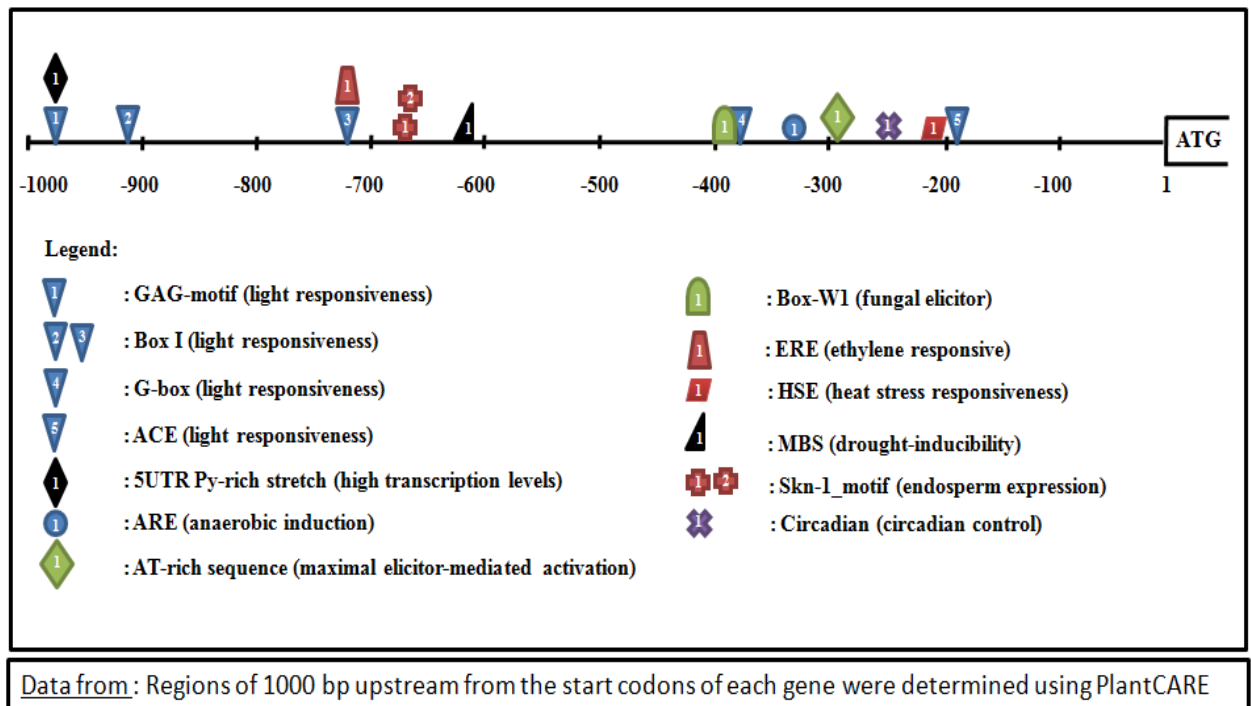


Fig. 25 *cis*-elements in promoter of *HrBPI* gene

3.5. Over-expressed *SITIL1*, *SITIL2*, and *SICHL*

I modified the transformation protocol as described previously (Sun et al. 2006) to get over-expressed tomatoes effectively (Fig. 6). I successfully transformed the constructs of pGWB8-35Sp::*SITIL1*-6xHis, pGWB8-35Sp::*SITIL2*-6xHis and pGWB8-35Sp::*SICHL*-6xHis into ‘Micro-Tom’ tomatoes to investigate their functions. The transformation efficiencies of *SITIL1*, *SITIL2* and *SICHL* constructs were 6.61% (8/121); 8.39% (13/88); and 19.23% (15/78), respectively (Table 5). I observed the ploidy of over-expressed tomatoes using a flow cytometer. The results showed that increased ploidy (4C and 8C nuclei) in these over-expressed plants ranged from 13.95% to 86.05%. The rate of tetraploid over-expressed tomatoes depended on both the genotype and the transformation procedure (Ellul et al. 2003). The percentages of diploid plants over-expressed *SITIL1*, *SITIL2* and *SICHL* were 72.22% (26/36); 68.18% (30/44);

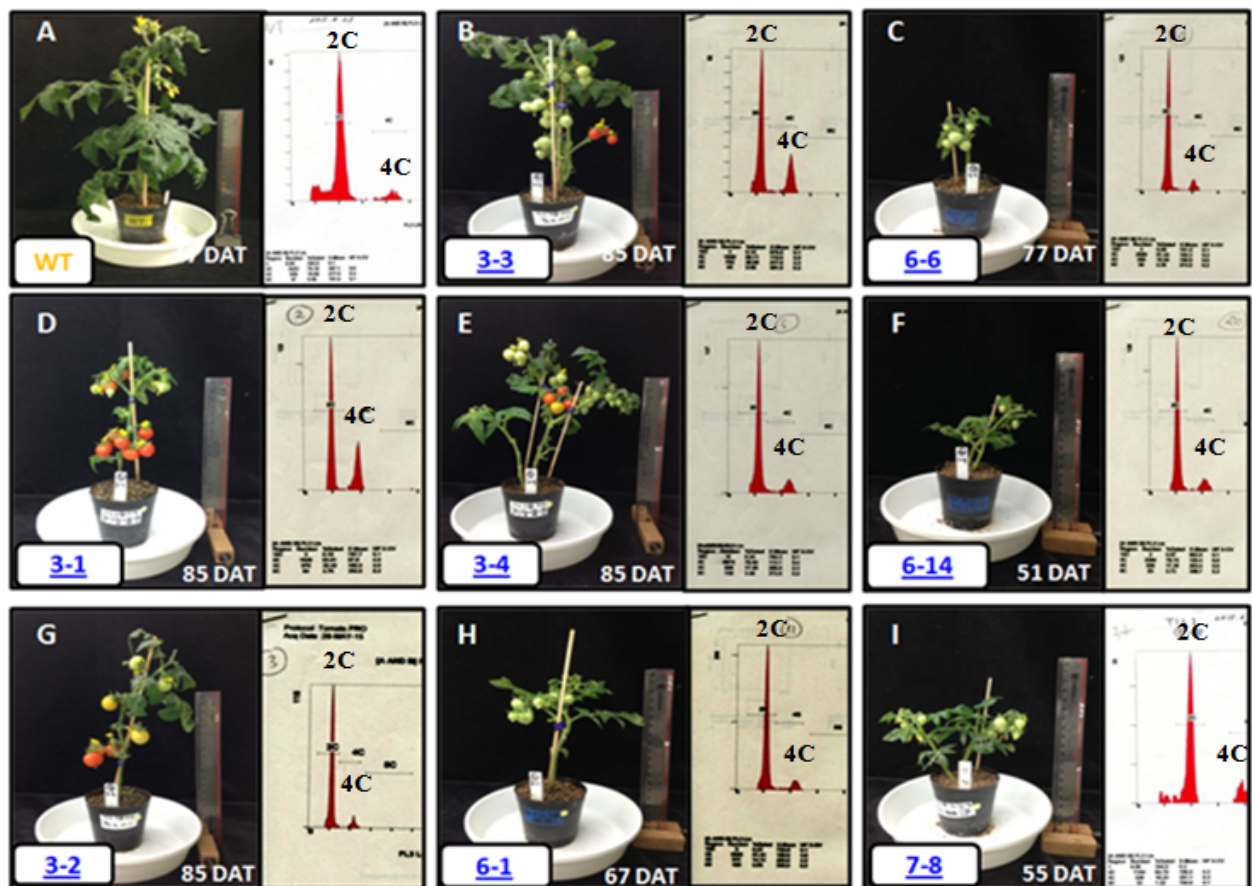
and 86.05% (37/43) respectively (Table 5).

Table 4. The number of *cis*-elements in the promoter of *SITIL1*, *SITIL2*, *SICHL*, *CHRC* and *HrBP1*

Function	<i>SITIL1</i>	<i>SITIL2</i>	<i>SICHL</i>	<i>CHRC</i>	<i>HrBP1</i>
Light responsive	15	12	6	12	5
Fungal elicitor	1	2	-	-	1
Defense and stress responsive	1	1	1	2	-
Heat stress responsive	3	-	2	2	1
Drought-inducibility	-	1	-	1	1
(IAA) Auxin responsive	-	-	-	2	-
(GA) Gibberellin responsive	-	-	-	1	-
MeJA (methyl jasmonate) responsive	-	-	2	2	-
Ethylene responsive	-	3	-	-	1
ABA (abscisic acid) responsive	1	-	-	-	-
Conferring high transcription level	1	-	-	-	1
Regulatory element	1	-	-	-	-
Anaerobic induction	1	1	-	-	1
Related to meristem specific activation	1	-	-	-	-
Zein metabolism regulation	1	-	-	1	-
Endosperm expression	1	-	3	1	2
MYBHv1 binding site	1	-	-	-	-
SEF4 factor binding site	1	-	-	-	-
Associated to the TGAGTCA	-	-	1	-	-
Binding protein (ATBP-1)	-	-	-	2	-
Circadian control	-	-	-	2	1
Maximal elicitor-mediated activation	-	-	-	-	1

Table 5. Screening result of over-expressed *SITIL1*, *SITIL2* and *SICHL*

	35Sp::TIL1	35Sp::TIL2	35Sp::CHL
No.of Explant	701	1056	472
No.of Shoot selected	245	517	384
No.of Root selected	225	346	179
No.of Acclimation plant	139	215	121
No.of Living plant	121	155	78
PCR screening (Hygromycin/HPT & Kanamycin/NPTII)	36	44	43
PCR screening (35S Prom/F; TILs/R)	36	44	43
Ploidy analysis used a flow cytometer :			
No.of diploid (2C)	26 (72.22%)	30 (68.18%)	37 (86.05%)
No.of tetraploid (4C) and octaploid (8C)	10 (27.78%)	14 (31.82%)	6 (13.95%)
PCR screening (TILs/F; 6x-His/R)	15 (12.39%)	23 (14.83%)	25 (32.05%)
RT-PCR screening (TILs/F/R and TILs/F; 6x-His/R)	8 (6.61%)	13 (8.39%)	15 (19.23%)



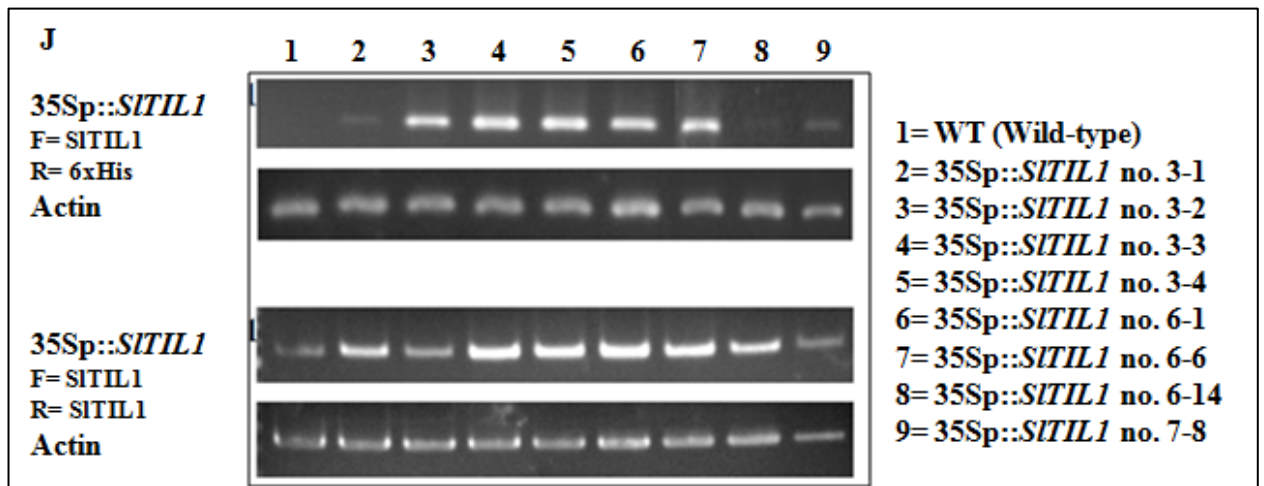


Fig. 26 The phenotypes of over-expressed *SITIL1*, ploidy analyses and RT-PCR analyses. (A) The phenotype and 2C-histogram peak DNA level of nuclei of leaves from diploid tomato wild-type (WT) for the control. (B-I) The phenotypes and 2C-histogram peak DNA level of nuclei of leaves from diploid over-expressed *SITIL1* (T1 generation). (J) RT-PCR analyses of over-expressed *SITIL1*.

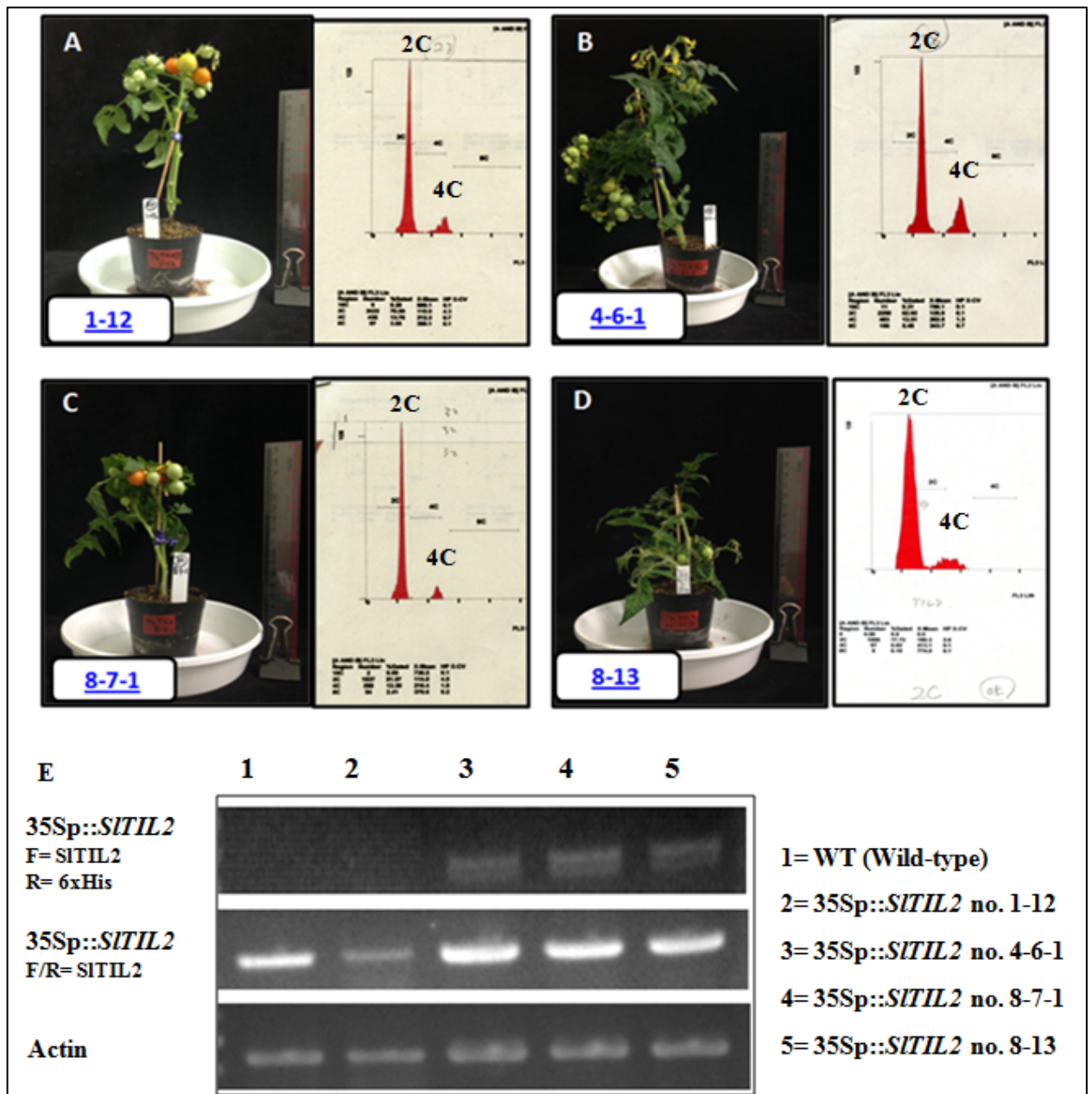


Fig. 27 The phenotypes of over-expressed *SITIL2* and ploidy analyses. (A,B,C,D) The phenotypes and 2C-histogram peak DNA level of nuclei of leaves from diploid over-expressed *SITIL2* (T1 generation). (E) RT-PCR analyses of over-expressed *SITIL2*.

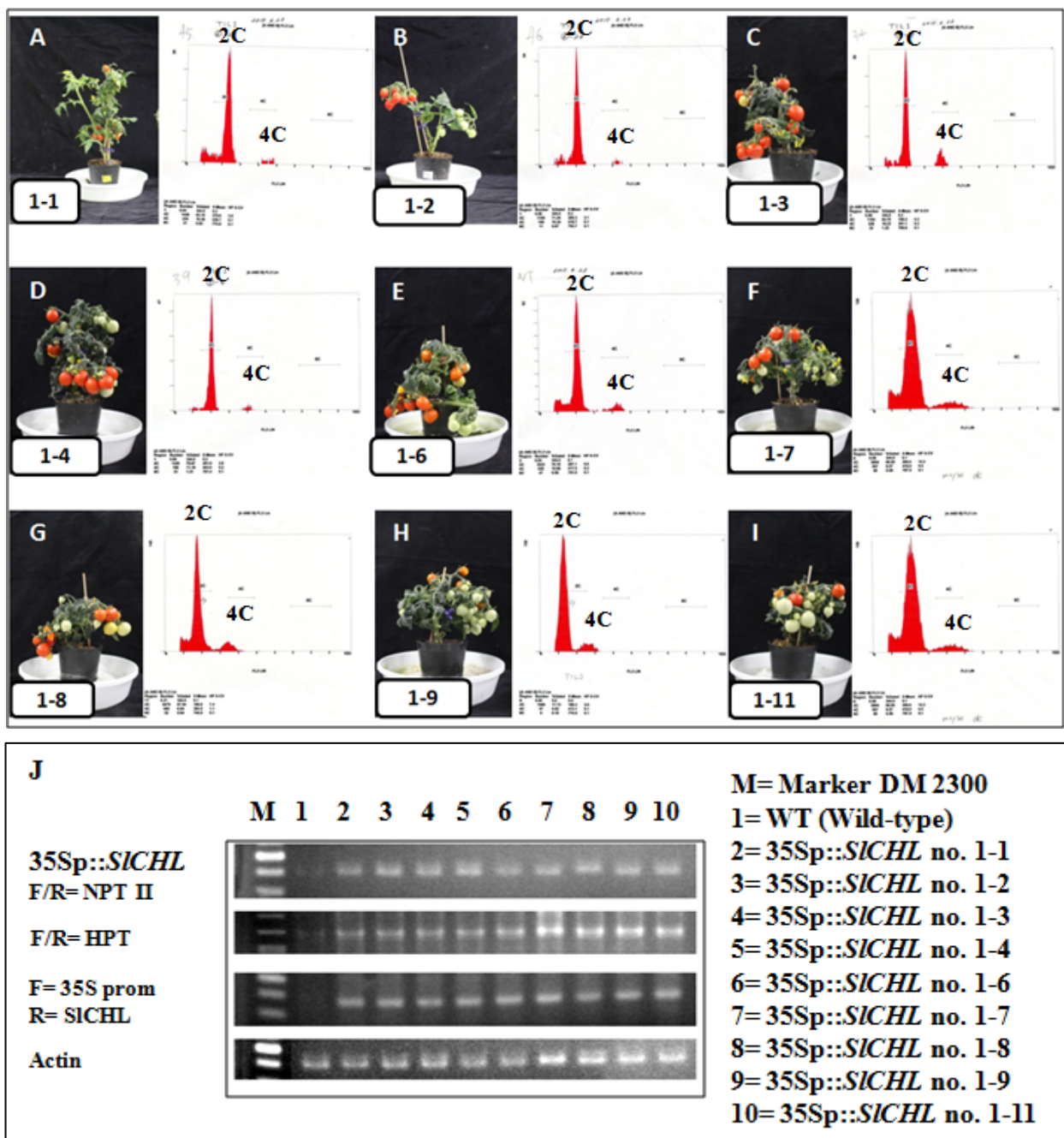


Fig.28 The phenotypes of over-expressed *SICHL*, ploidy analyses and RT-PCR analyses. (A-I) The phenotypes and 2C-histogram peak DNA level of nuclei of leaves from diploid over-expressed *SICHL* (T1 generation). (J) RT-PCR analyses of over-expressed *SICHL*.

3.6. Light responses of tomato seedlings (germination stages) over-expressed *SITIL1*, *SITIL2* and *SICHL* under light and dark condition

To investigate the functions of TILs and CHL, I successfully transformed the constructs of pGWB8-35Sp::*SITIL1*-6xHis, pGWB8-35Sp::*SITIL2*-6xHis and pGWB8-35Sp::*SICHL*-6xHis into ‘Micro-Tom’ tomatoes. The changes of phenotype of over-expressed *SITIL1*, *SITIL2*, *SICHL* and wild-type plants on seedling stages were observed at the 3rd, 7th and 12th days in light and dark conditions. Under light condition, the seedling phenotypes of over-expressed *SITILs* and *SICHL* showed bigger and longer of their cotyledon and rapidly grew compared with wild-type plants (Figs. 29,30,31). Over-expressed *SITILs* showed bigger of their hypocotyl and over-expressed *SICHL* showed dark green of their cotyledon and true leaves (Figs. 29,30,31). Under dark condition, etiolation showed in wild-type and over-expressed *SITILs* and *SICHL*, but the phenotype of wild-type plants showed longest hypocotyl and shorter in epicotyle compared over-expressed plants (Figs. 29,30,31). Over-expressed *SITILs* showed more curling in cotyledon and over-expressed *SICHL* showed most green in cotyledon and shorter hypocotyl compared others. Over-expressed *SITIL2* showed most white in epicotyl (Figs. 29,30,31).

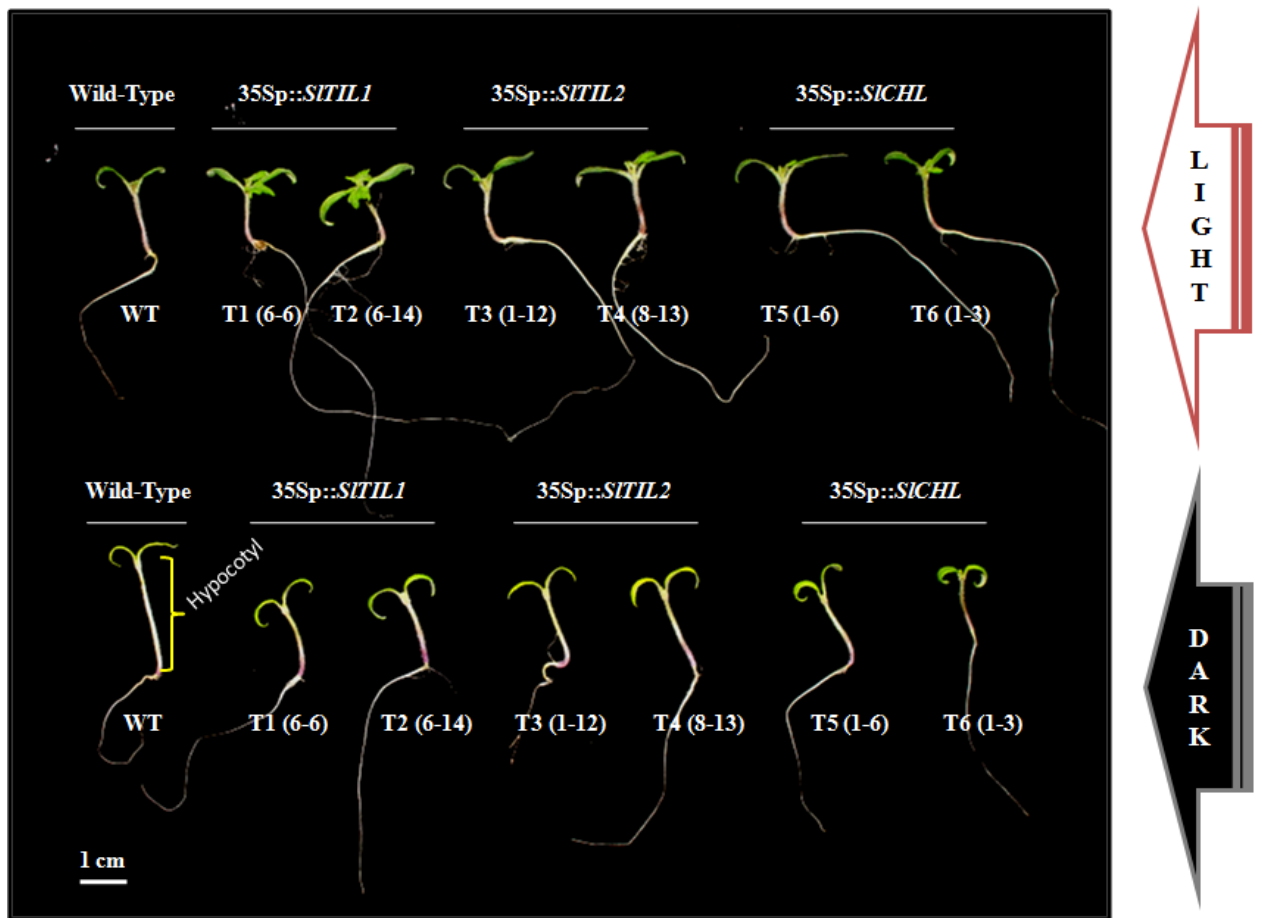


Fig. 29 The phenotypes of seedling stages of over-expressed *SITIL1*, *SITIL2* and *SICHL* at 3 days in light and dark condition.



Fig. 30 The phenotypes of seedling stages of over-expressed *SITIL1*, *SITIL2* and *SICHL* at 7 days in light and dark condition.

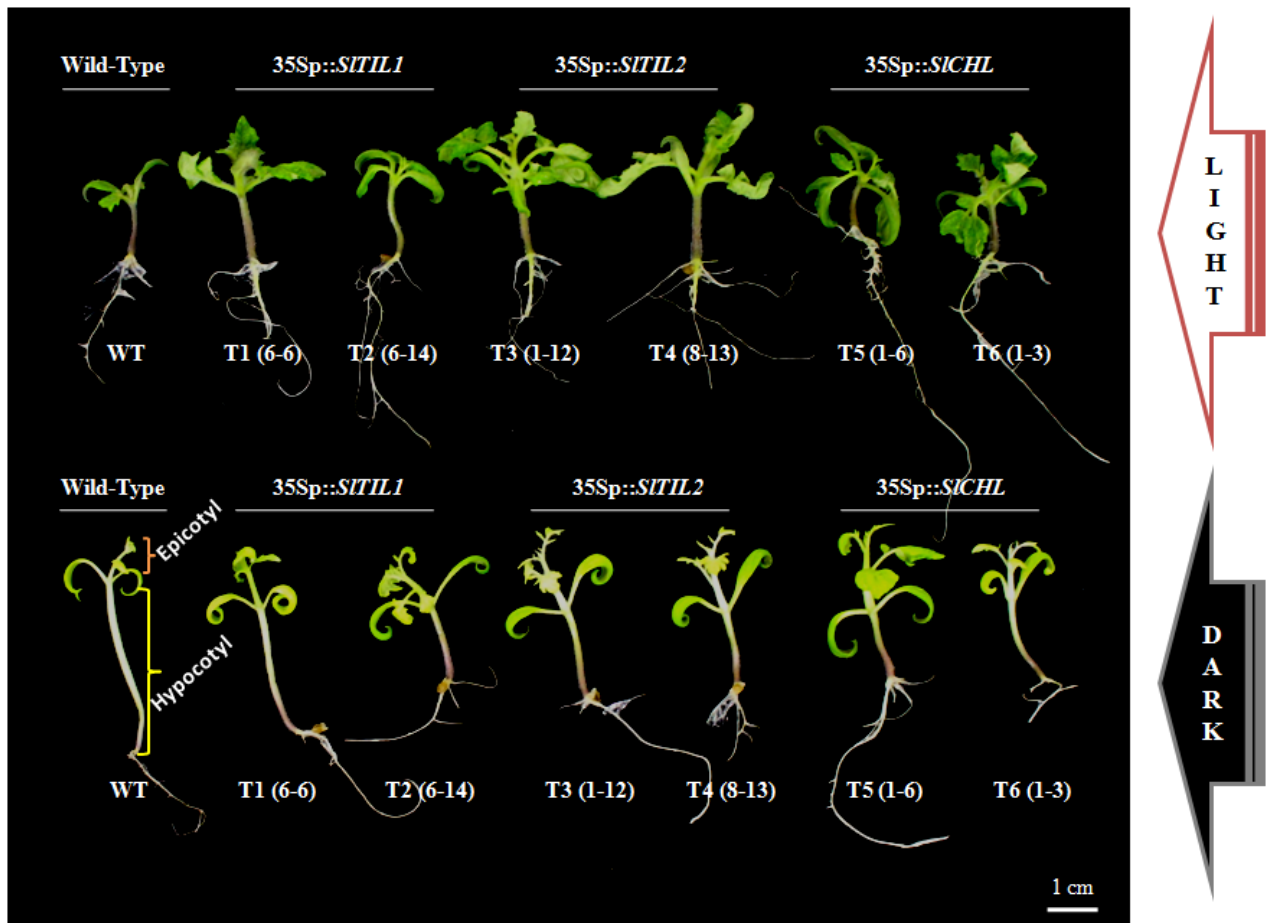


Fig. 31 The phenotypes of seedling stages of over-expressed *SITIL1*, *SITIL2* and *SICHL* at 12 days in light and dark condition

3.7. Light responses of over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced plants under light condition1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$)

When I compared with the phenotype of over-expressed *SITIL1*, *SITIL2*, *SICHL* and wild-type plants, over-expressed *SITILs* and *SICHL* plants showed to increase the number of leaves with curling (Figs. 32A,B,E,F), longer terminal leaflets (Figs. 32B,F), bullwhip phenotype in leaves (Figs. 32B,F), early flowering (data not shown), increasing number of flowers (data not shown), increasing number of inflorescences (data not shown), and bigger peduncle and fruits (Figs. 32C,G, D, H and Fig. 34). The over-expressed *SITIL1* showed longer leaves with curling, over-expressed *SITIL2* showed earlier flowering, and over-expressed *SICHL* showed dark greening in seedling and mesocarp of mature green fruit and earlier ripening compared others

(Figs. 35A,B). The plant growth of over-expressed *SITILs* and *SICHL* showed rapid growth compared wild-type plants at light condition 1 and light condition 2 (Figs. 33A,B). The plant growth of gene silenced plants showed growth much slower compared wild-type plants at light condition 1 and light condition 2 (Figs. 37A,B).

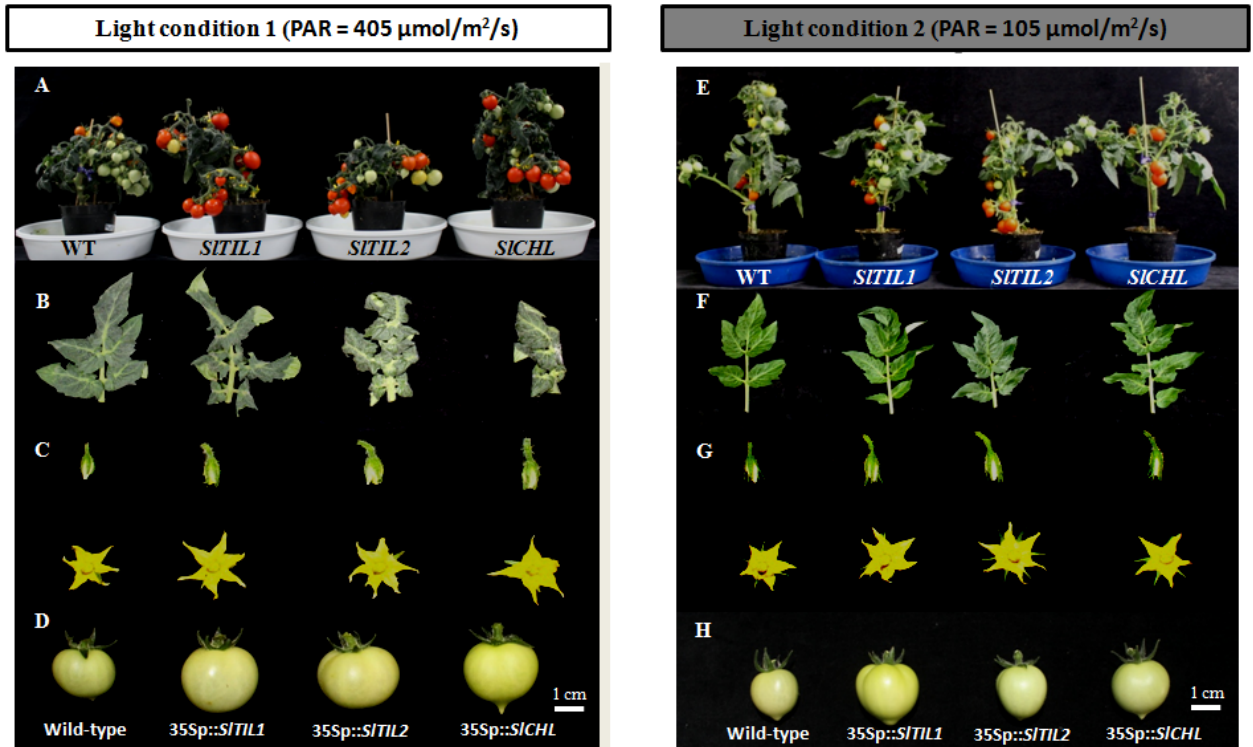


Fig. 32 Phenotypes of over-expressed *SITIL1*, *SITIL2* and *SICHL* under light condition 1 (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and light condition 2 (105 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (A,B,C,D) Phenotypes of over-expressed tomatoes, leaves, flowers and fruits at light condition 1 (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (E,F,G,H) Phenotypes of over-expressed tomatoes, leaves, flowers and fruits at light condition 2 (105 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

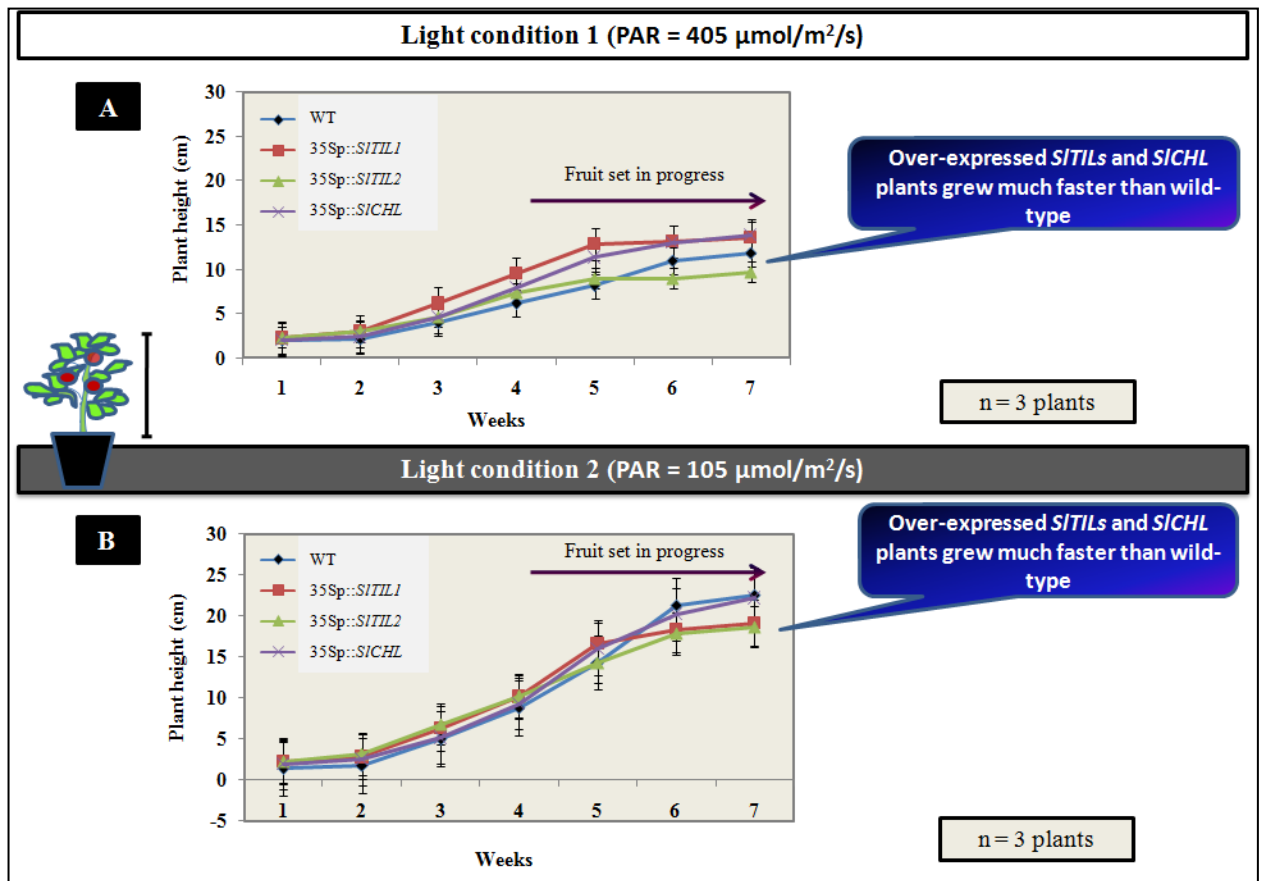


Fig. 33 Their growth of over-expressed *SITIL1*, *SITIL2* and *SICHL* tomato plants. (A) The height of over-expressed *SITIL1*, *SITIL2* and *SICHL* over-expressed plants compared wild-type plants under light condition 1 (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (B) The height of over-expressed *SITIL1*, *SITIL2* and *SICHL* tomato plants compared wild-type plants under light condition 2 (105 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

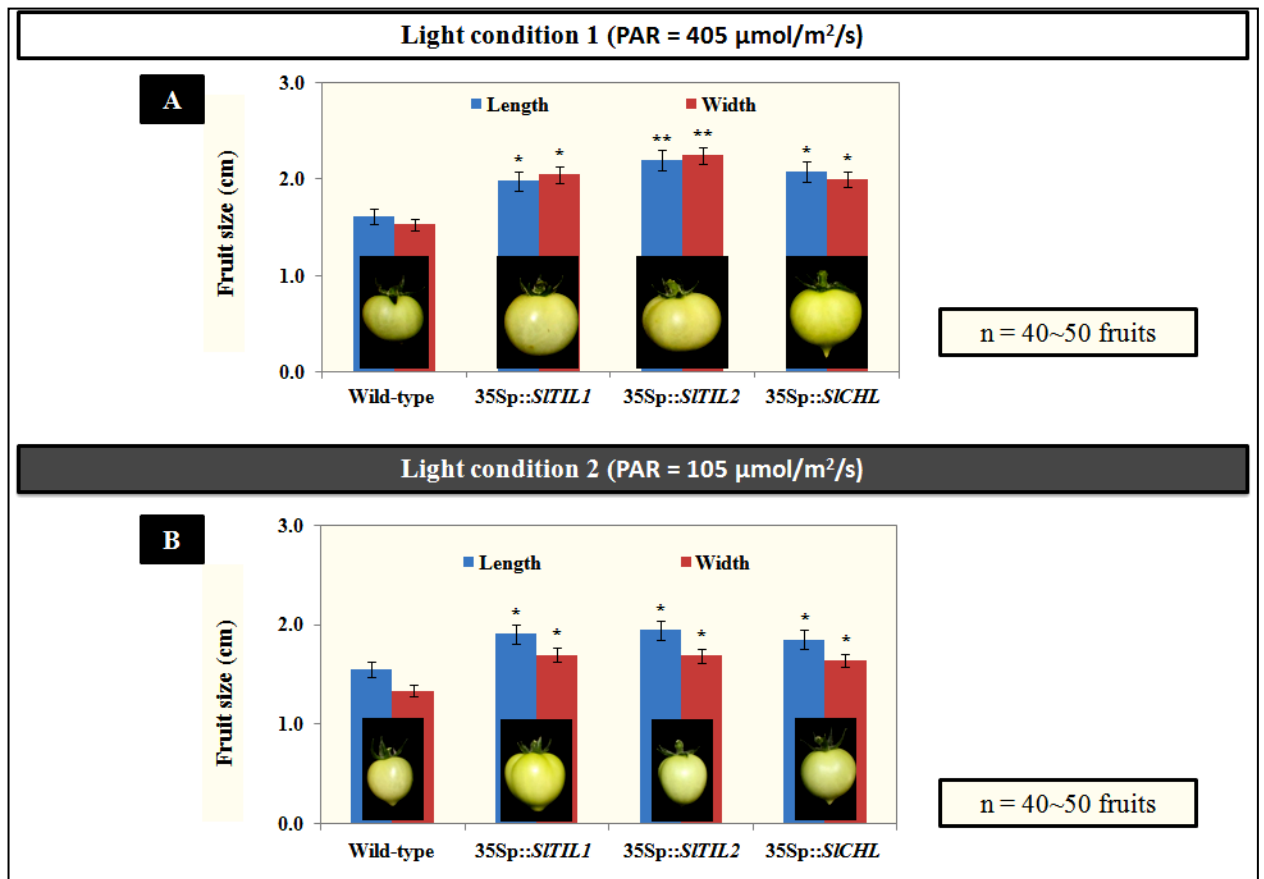


Fig. 34 Their fruit size of over-expressed *SITIL1*, *SITIL2* and *SICHL* tomato plants. (A) Fruit size of over-expressed *SITIL1*, *SITIL2* and *SICHL* tomato plants compared wild-type plants under light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) Fruit size of over-expressed *SITIL1*, *SITIL2* and *SICHL* tomato plants compared wild-type plants under light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$).

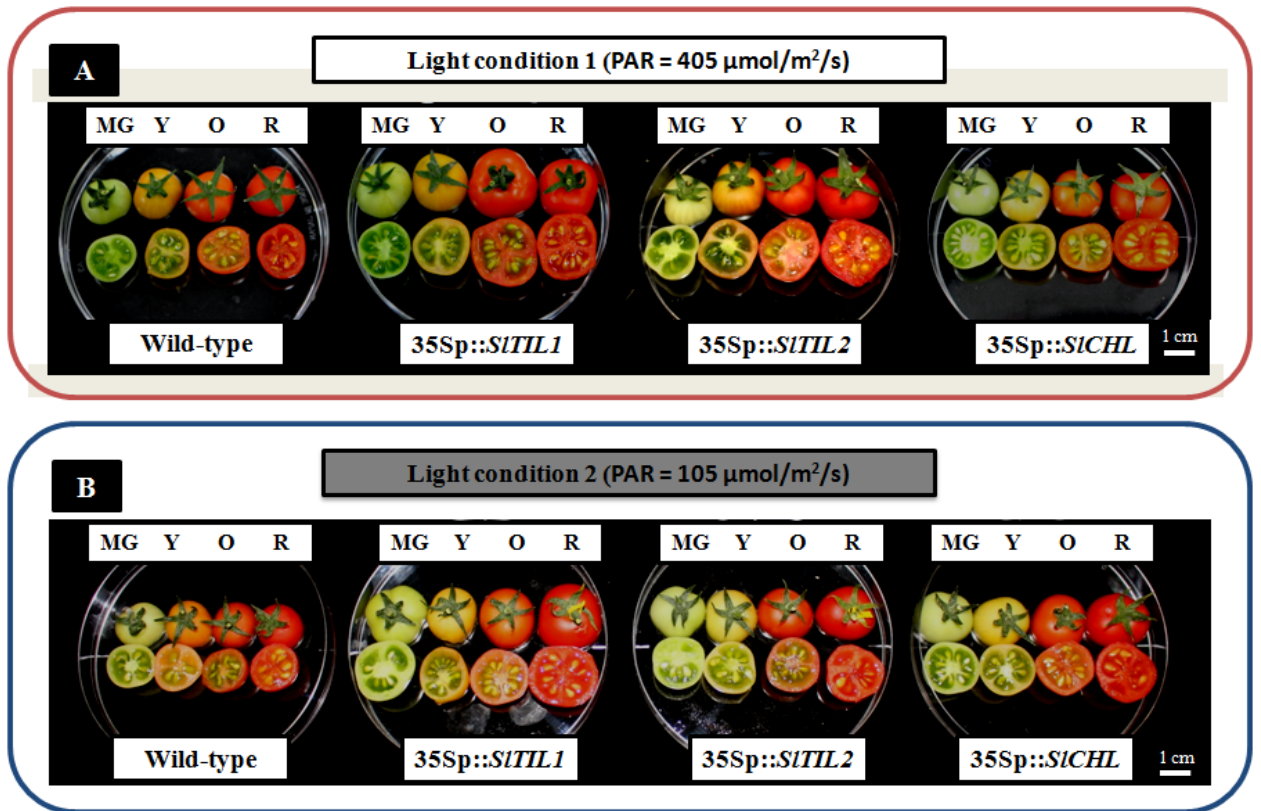


Fig. 35 Fruit phenotypes of over-expressed *SITIL1*, *SITIL2* and *SICHL* tomatoes at the four developmental stages. MG: mature green, Y: yellow color stage, O: orange color stage, R: red color stage. (A) Under light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) Under light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$).

I suppressed *PDS*, *SITIL1*, *SITIL2*, *SICHL* and *CHRC* expression in tomatoes at light condition 1 and light condition 2 using VIGS-system to observe their phenotypes. Their gene silenced plants showed the different phenotypes compared with pTRV2-plants as a control in light condition 1 and light condition 2 (Fig. 36). *PDS* silencing inhibited carotenoid biosynthesis causing the photo-bleached phenotype in leaves, flowers and fruits in both conditions (Fig. 36). White color region of leaves and petals showed that *PDS* continued to be silenced until flowering stage by VIGS-system. Silencing of *SITIL1*, *SITIL2*, *SICHL* and *CHRC* leaded to delay 10-15 days on flowering and fruit ripening (Fig. 36). The suppressed expression of *SITIL1*, *SITIL2* and *SICHL* showed aberrant shape of leaves, such as curling (Fig. 36B,F), and yellowing in pericarp of fruits (Fig. 36D,H). *SITIL1*, *SITIL2*, *SICHL* and *PDS* silencing plants showed leaves with

curling, and shorter leaves compared wild-type plants at light condition 1 (Fig. 36B). But the suppressed expression of *CHRC* showed not curling (Fig. 36B,F), little yellowing in pericarp of fruits (Fig. 36D,H). Their fruits of gene silenced plants at the maturation stage showed the different colour compared with pTRV2-plants as a control (Fig. 36D,H). Their fruit of gene silenced plants were smaller than control fruits (Fig. 38 A, B). Gene silenced plant growths were slower compared wild-type plants (Fig. 37). But their growth recovered as the days passed in *CHRC* and *SICHL* silenced plants under light condition 2 (Fig. 37 B).

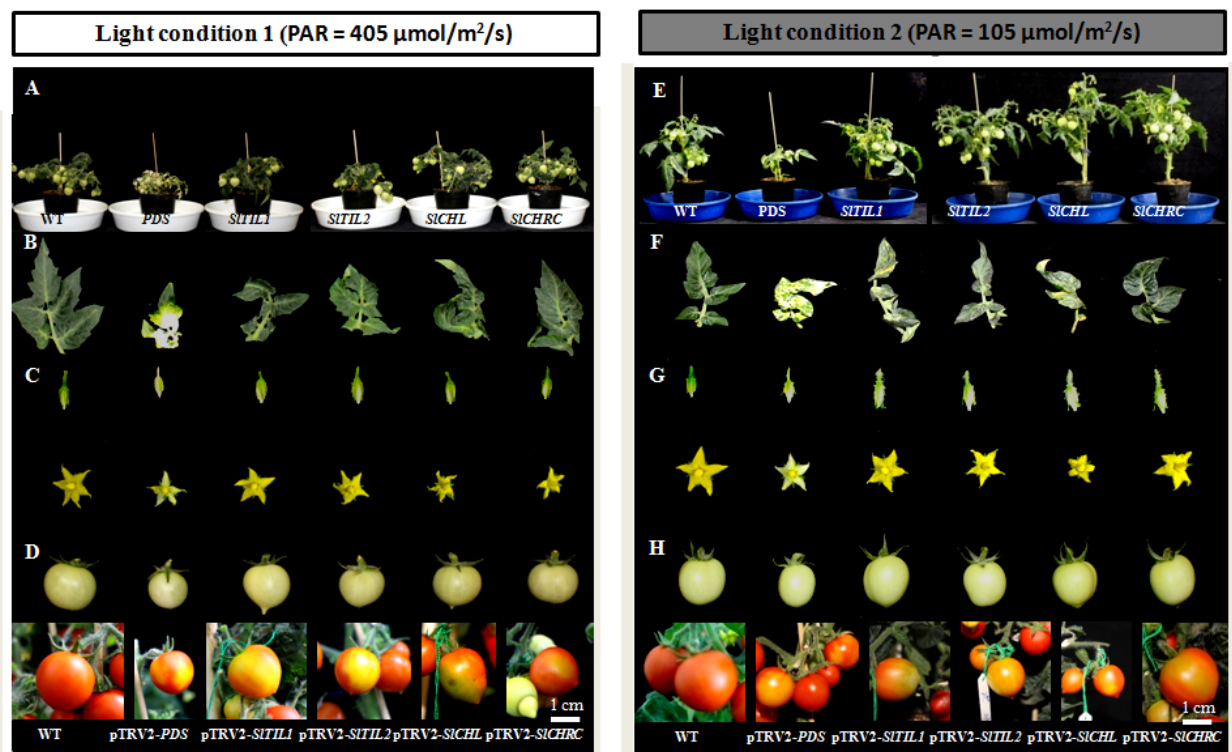


Fig.36 Phenotypes of gene silenced plants under light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$). (A,B,C,D) Phenotypes of gene silenced on plants, leaves, flowers and fruits at light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$). (E,F,G,H) Phenotypes of gene silenced on plants, leaves, flowers and fruits at light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$).

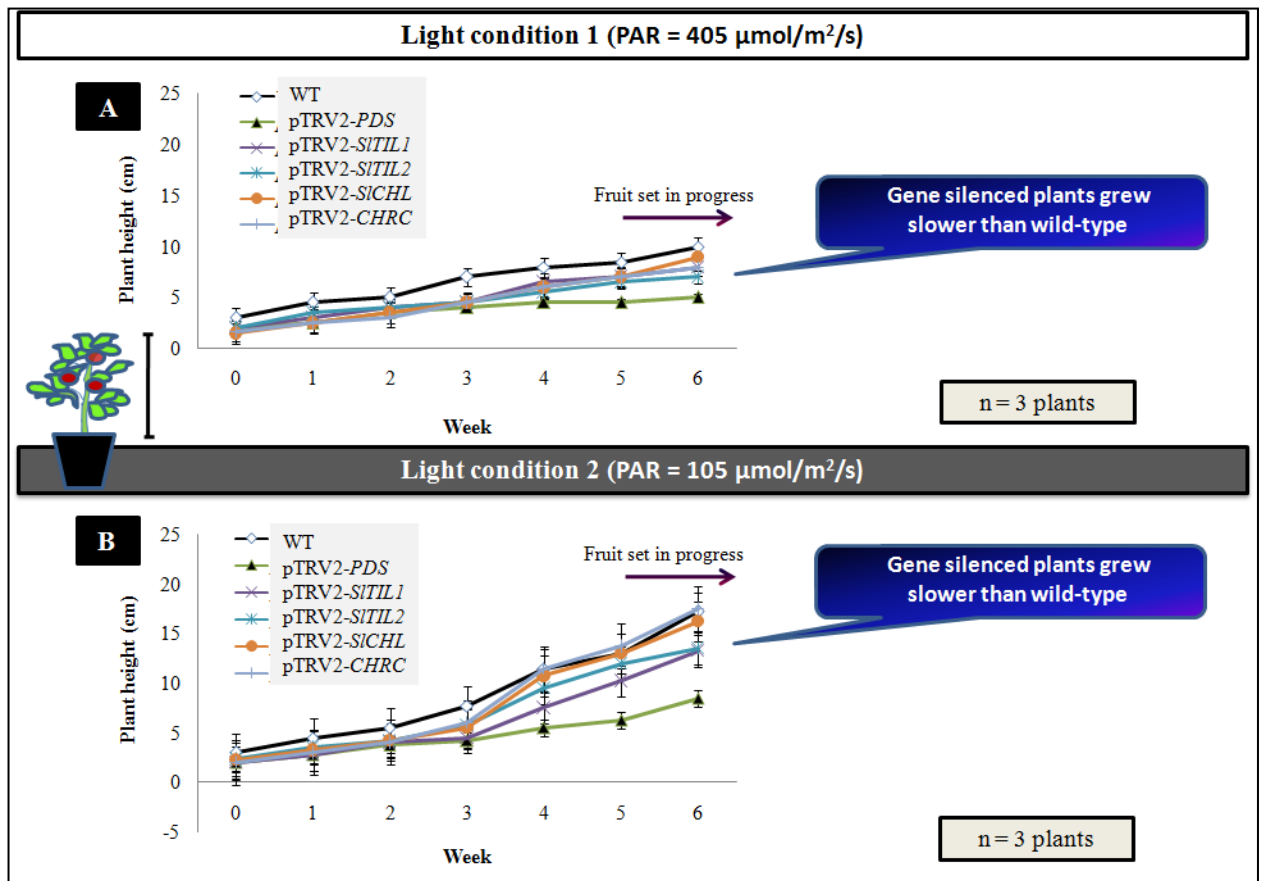


Fig. 37 Their growth of gene silenced plants. (A) The height of gene silenced plants compared wild-type plants under light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) The height of gene silenced plants compared wild-type plants under light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$).

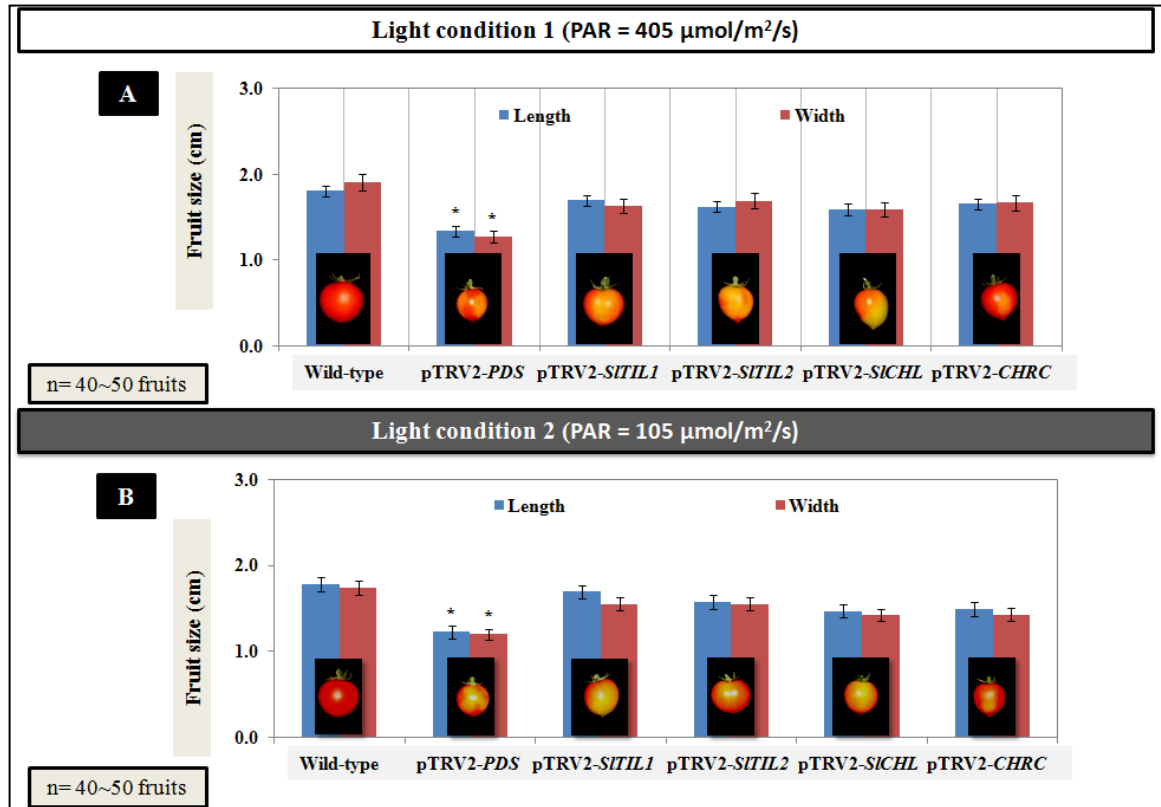


Fig. 38 Their fruit size of gene silenced plants. (A) Fruit size of gene silenced plants compared wild-type plants under light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) Fruit size of gene silenced plants compared wild-type plants under light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$).

3.8. Cytological analyses

The structure and pericarp cells in their fruits at breaker stages of wild-type plants and over-expressed *SITILs* and *SICHL* are shown in Figure 39 and 40. Most pericarp cells expanded a lot in over-expressed *SITILs* and *SICHL* at light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$) compared wild-type plants as a control (Fig. 39 and 40). In pericarp of wild-type plants between exocarp and mesocarp have intercellular space (Fig. 39A,B,C and 40A,B,C), but intercellular space was not found in over-expressed *SITILs* (Fig. 39D-I and 40D-I) and *SICHL* pericarp cells (Fig. 39J,K,L and 40J,K,L). Cell structure of vascular bundles in over-expressed *SITILs* and *SICHL* showed more tenuous compared wild-type vascular bundles cells that mean cells in over-expressed *SITILs* and *SICHL* tomatoes are rapidly developed (Fig. 39 blue circles).

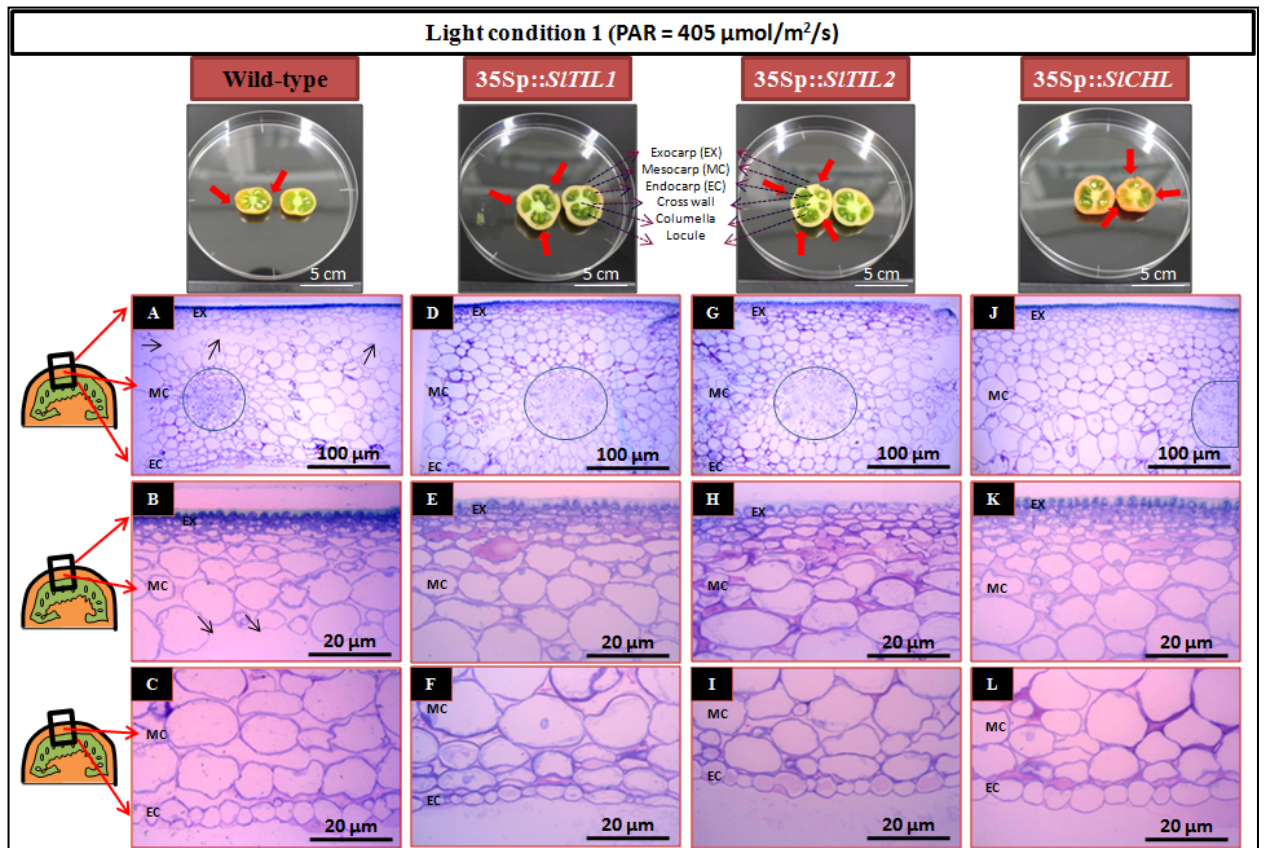


Fig. 39 The pericarp transversal structure (breaker stages) of over-expressed *SITIL1*, *SITIL2* and *SICHL* under light condition 1 (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (A,B,C) The pericarp transversal structure of wild-type plants. (D,E,F) The pericarp transversal structure of over-expressed *SITIL1*. (G,H,I) The pericarp transversal structure of over-expressed *SITIL2*. (J,K,L) The pericarp transversal structure of over-expressed *SICHL*. EX: Exocarp, MC: Mesocarp, EC: Endocarp. Blue circles: Surrounding vascular bundles.

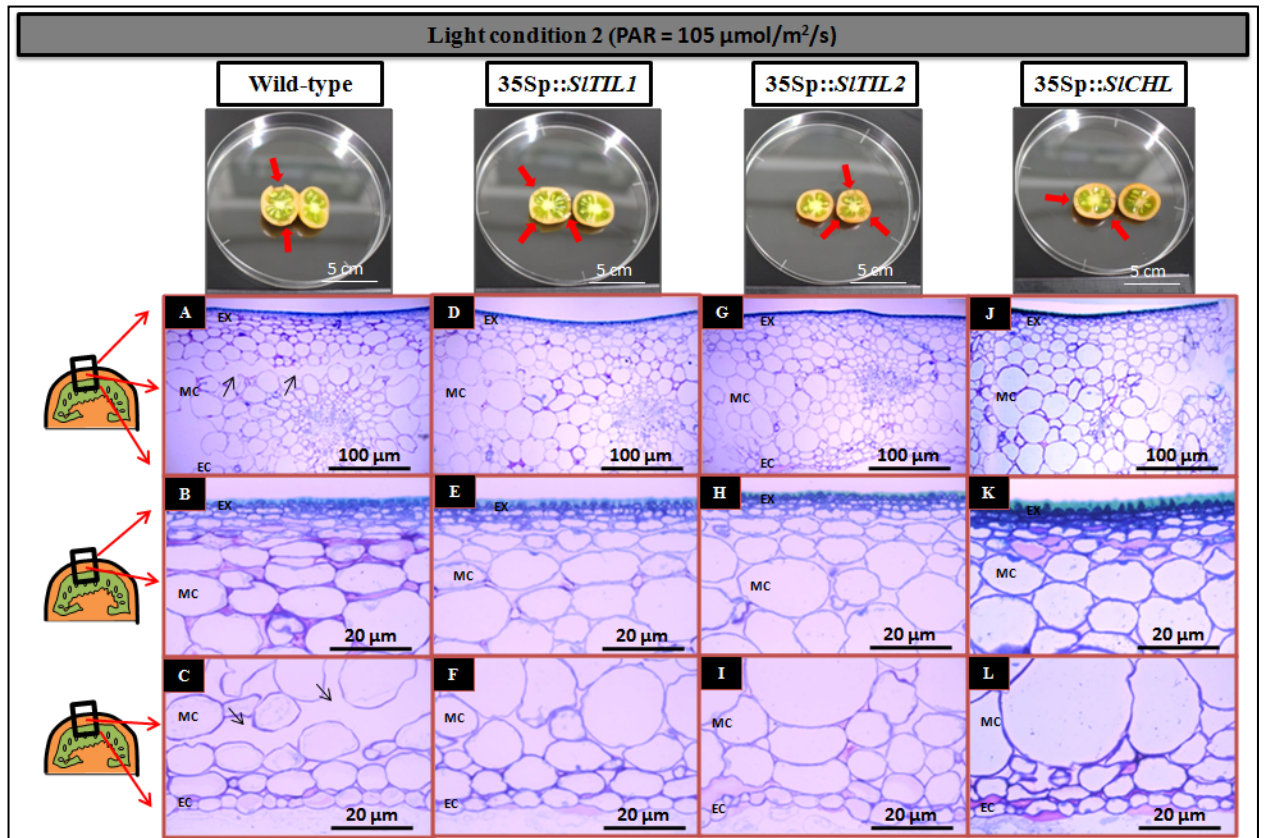


Fig. 40 The pericarp transversal structure (breaker stages) of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$). (A,B,C) The pericarp transversal structure of wild-type plant. (D,E,F) The pericarp transversal structure of over-expressed *SITIL1*. (G,H,I) The pericarp transversal structure of over-expressed *SITIL2*. (J,K,L) The pericarp transversal structure of over-expressed *SICHL*. EX: Exocarp, MC: Mesocarp, EC: Endocarp. Blue circles: Surrounding vascular bundles.

3.9. The mRNA levels of *SISODs* in over-expressed and gene silenced *SITIL1*, *SITIL2* and *SICHL* plants

In this study, the expression of *SISODs* in over-expressed and silenced *SITIL1*, *SITIL2* and *SICHL* plants were determined by RT-PCR. In *SITIL1*, *SITIL2* and *SICHL* over-expressed plants, the expression of *SISOD1*, *SISOD3* and *SISOD6* showed highly expressed in seedlings, leaves, flowers and fruits at light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Figs. 41, 42, 43). In contrast, the expression of *SISOD1*, *SISOD3* and *SISOD6* in leaves, flowers, non-silenced fruit, and silenced fruit of *SITIL1*, *SITIL2* and *SICHL* was very low (Fig.44).

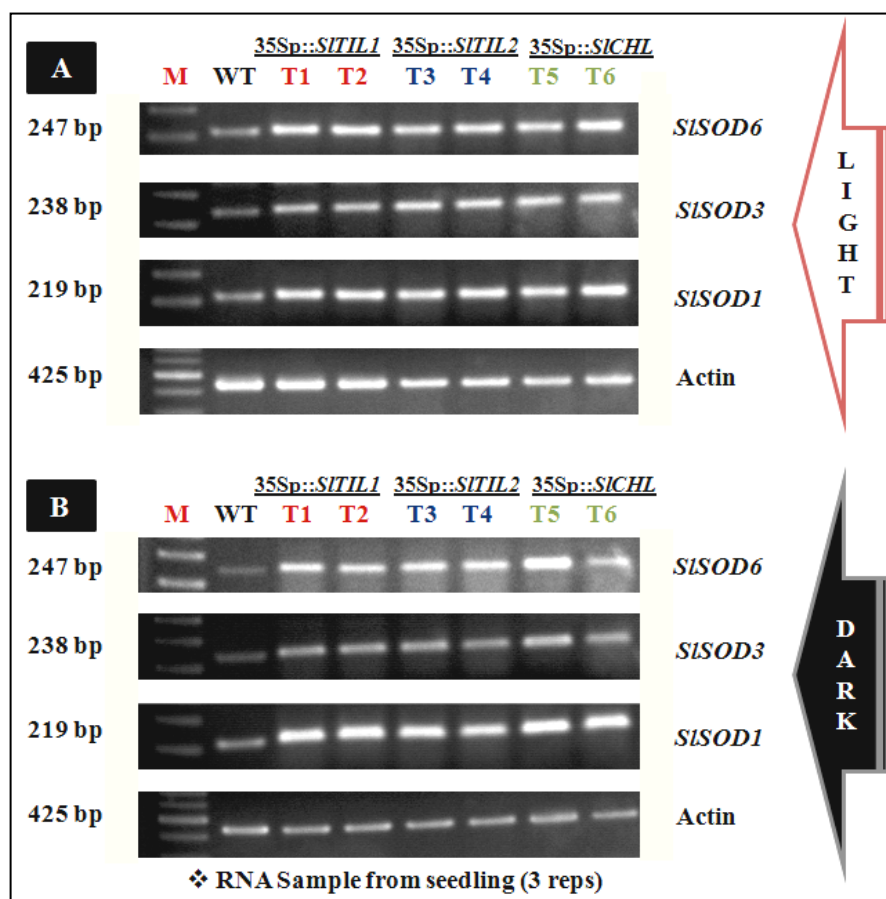


Fig. 41 The expression of *SISODs* (*SISOD1*, *SISOD3* and *SISOD6*) in over-expressed *SITIL1*, *SITIL2* and *SICHL* on seedlings. (A) The expression of *SISODs* in over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition. (B) The expression of *SISODs* in over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under dark condition.

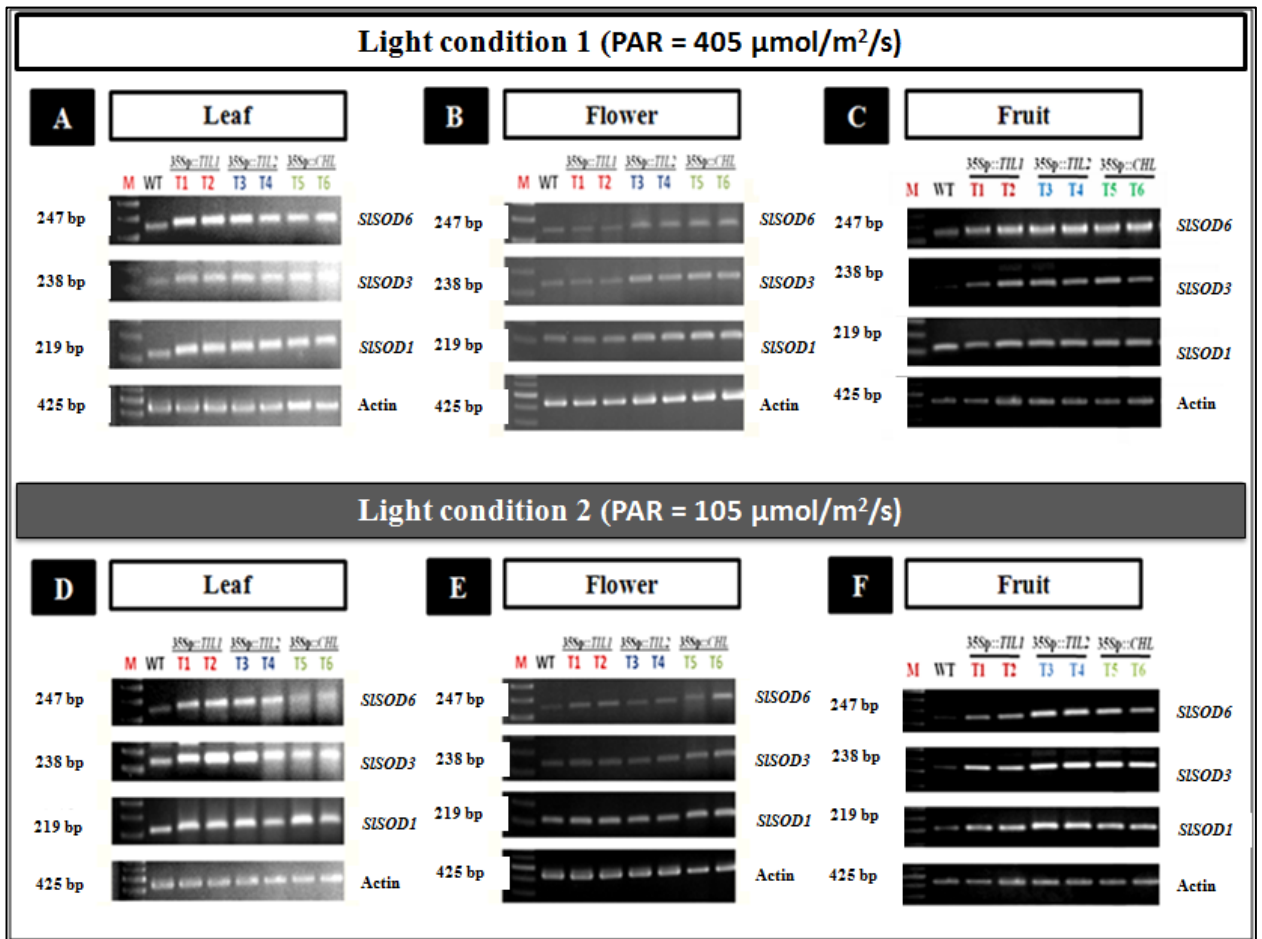


Fig. 42 The expression of *SISODs* (*SISOD1*, *SISOD3* and *SISOD6*) in over-expressed *SITIL1*, *SITIL2* and *SICHL*. (A) The expression of *SISODs* in leaves of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition 1 (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (B) The expression of *SISODs* in flowers of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition 1 (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (C) The expression of *SISODs* in fruits of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition 1 (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (D) The expression of *SISODs* in leaves of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition 2 (105 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (E) The expression of *SISODs* in flowers of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition 2 (105 $\mu\text{mol m}^{-2} \text{s}^{-1}$). (F) The expression of *SISODs* in fruits of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants under light condition 2 (105 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

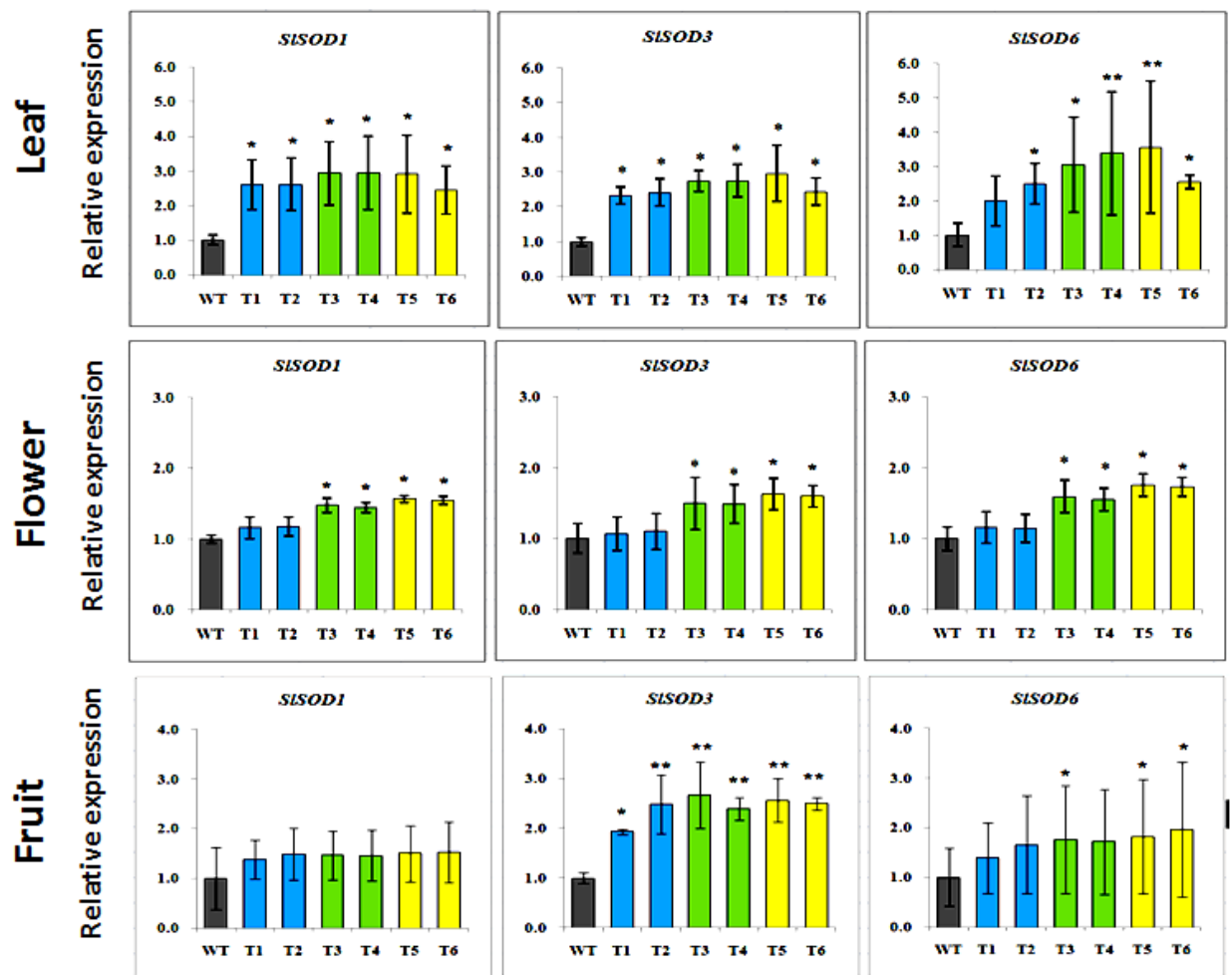


Fig. 43 The relative expression of *SISODs* (*SISOD1*, *SISOD3* and *SISOD6*) in over-expressed *SITIL1*, *SITIL2* and *SICHL* on leaf, flower and fruit. WT: Wild-type, T1-T2: 35Sp::*SITIL1*, T3-T4: 35Sp::*SITIL2*, T5-T6: 35Sp::*SICHL*.

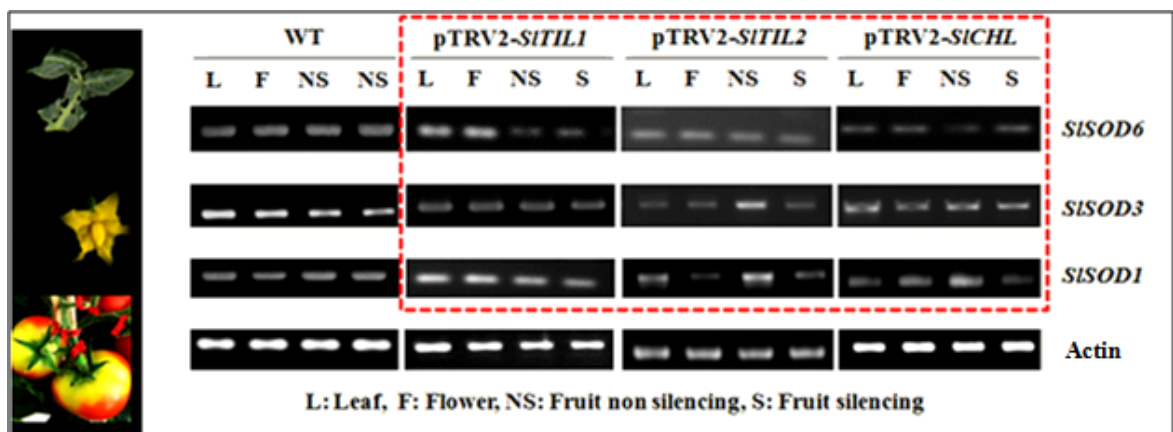


Fig. 44 The expression of *SISODs* (*SISOD1*, *SISOD3* and *SISOD6*) in various tissues of *SITILs* and *SICHL* silenced plants. L: leaves, F: flowers, NS: non-silenced fruits, S: silenced fruits.

3.10. Detection of ROS in over-expressed and gene silenced *SITIL1*, *SITIL2* and *SICHL* plants

Superoxide ions (O_2^-) and hydrogen peroxide (H_2O_2) are the reactive oxygen species (ROS) that play a significant role in regulation of many plant processes. The level of O_2^- ions is determined qualitatively using nitrobluetetrazolium (NBT) assay while the H_2O_2 is qualitatively estimated using 3,3-diaminobenzidine (DAB) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay. To further investigate the effects of lipocalins on ROS, the results of NBT and DAB staining and H_2DCFDA assay showed that the level of O_2^- ions (Fig. 45) and H_2O_2 (Figs. 46 and 47) are lower than control (WT) under light condition ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat condition (37°C ; 5 days) in over-expressed *SITILs* and *SICHL*. These results demonstrated that *SITILs* and *SICHL* over-expressed plants have tolerance of ROS (O_2^- ions and H_2O_2) under light condition ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress condition (37°C ; 5 days). Contrary, NBT and DAB staining and H_2DCFDA assay in *SITILs* and *SICHL* gene silenced plants showed that their levels of O_2^- ions (Fig. 48) and H_2O_2 (Figs. 49 and 50) were significantly increased under light condition ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress condition (37°C ; 5 days). These results demonstrated that the *SITILs* and *SICHL* gene silenced plants of oxidative damage were not partly diminished.

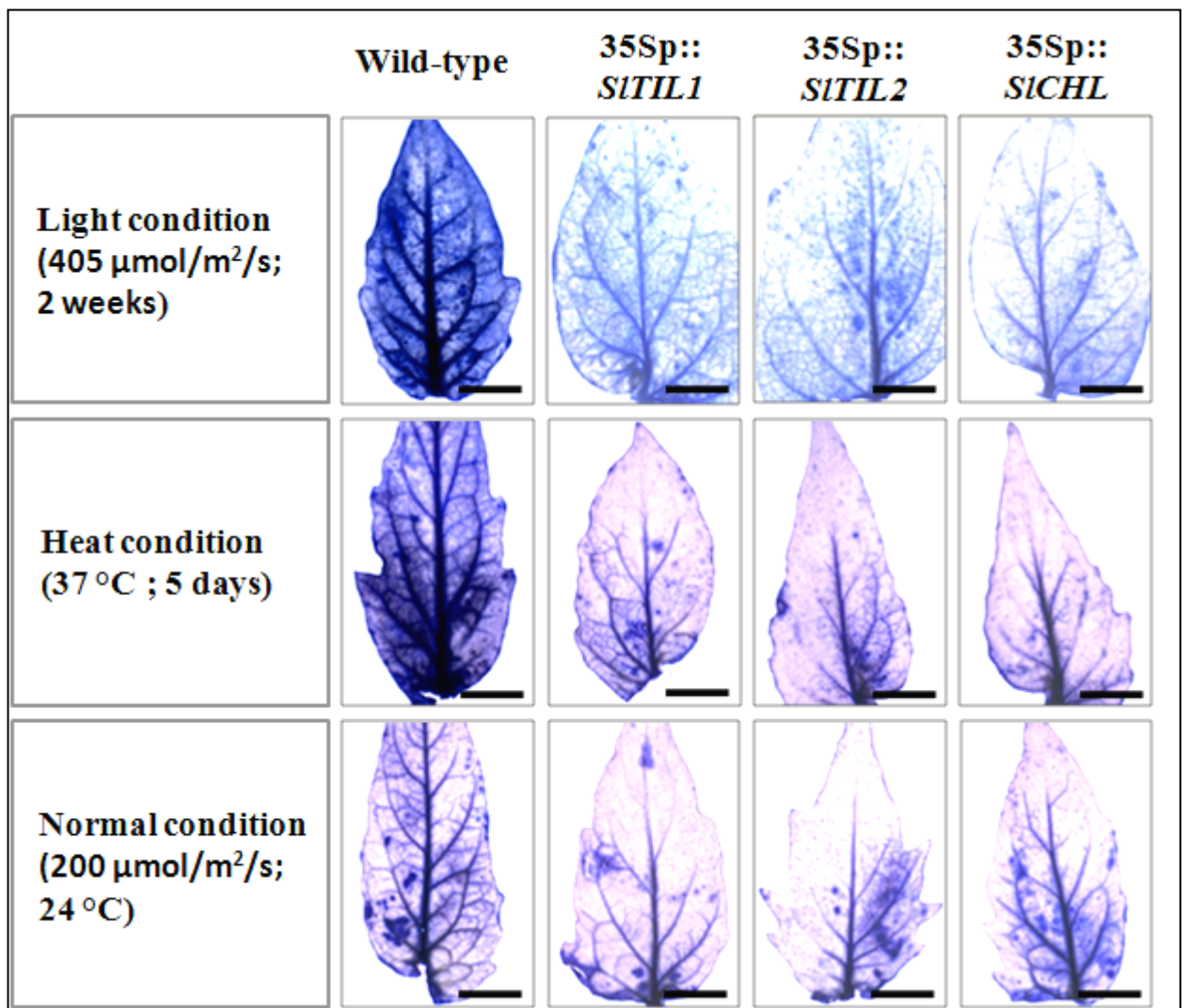


Fig. 45 Stereomicroscope image of NBT assay of over-expressed *SITIL1*, *SITIL2* and *SICHL* leaves under light condition (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress condition (37 °C; 5 days) showing the presence of superoxide ions ($\text{O}_2^{\cdot -}$) indicated by presence of blue coloured formazan. Bars show 5 mm.

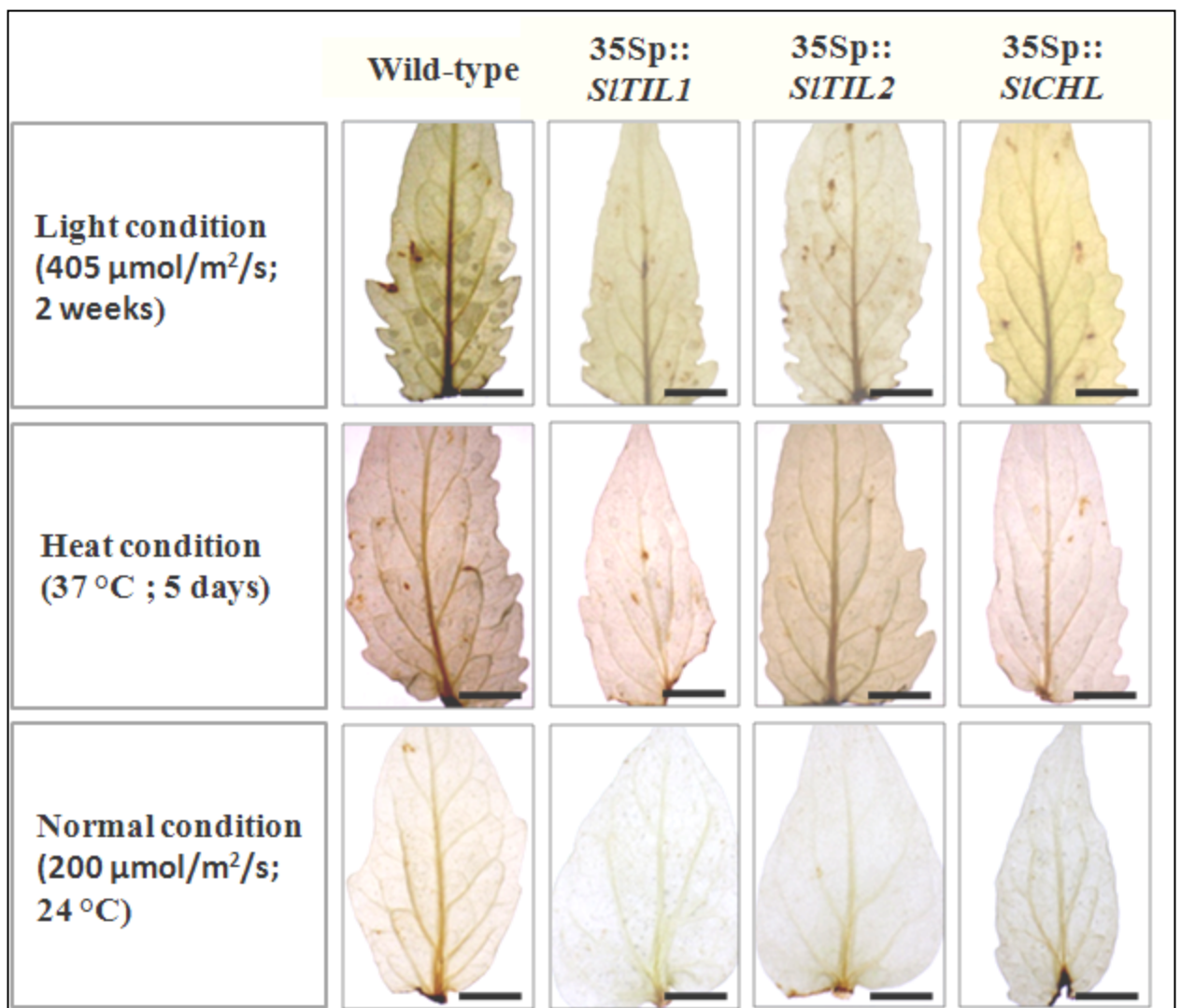


Fig. 46 Stereomicroscope image of DAB assay of over-expressed *SITIL1*, *SITIL2* and *SICHL* leaves under light condition (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress condition (37 °C; 5 days) showing the presence of hydrogen peroxide (H_2O_2) indicated by presence of brown coloured product. Bars show 5 mm.

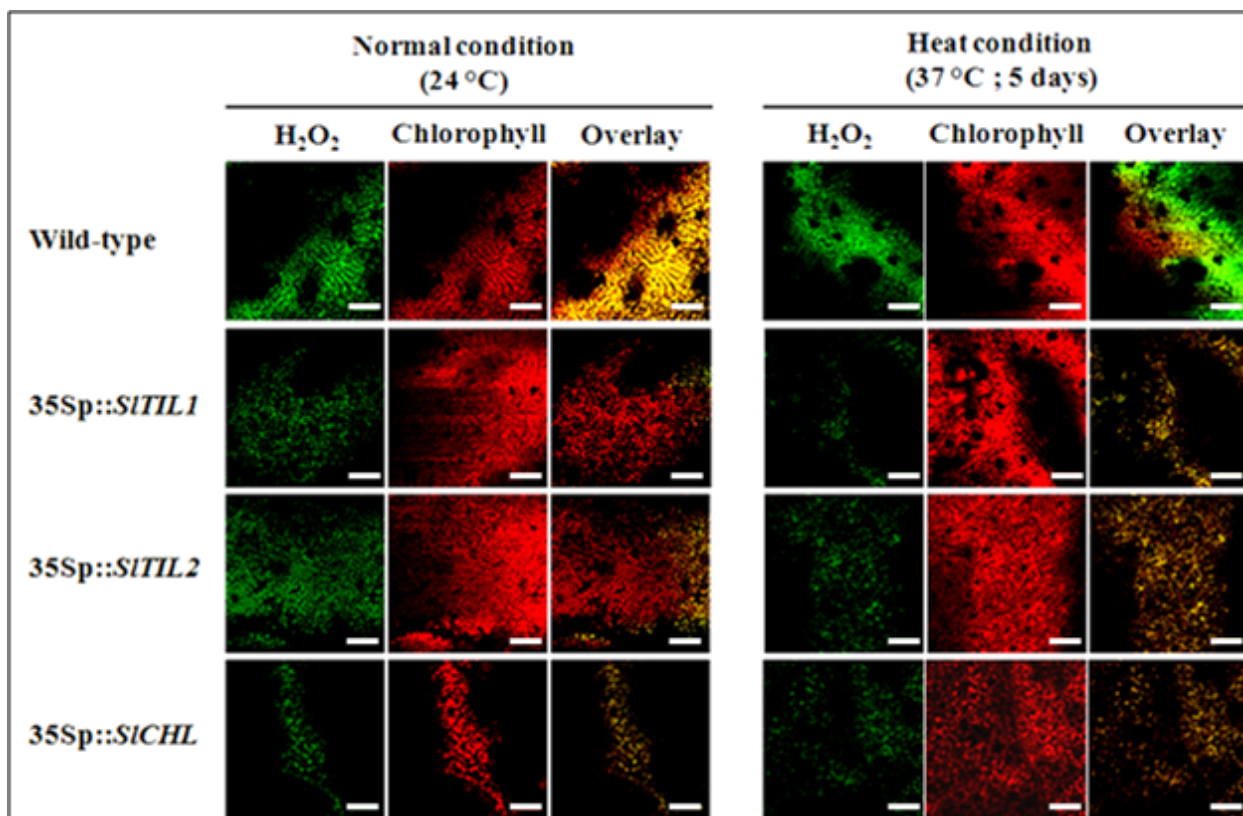


Fig. 47 Confocal H₂DCFDA staining images of over-expressed *SITIL1*, *SITIL2* and *SiCHL* leaves under heat stress condition (37 °C; 5 days). Green color indicates ROS predominantly hydrogen peroxide; Red color indicates chlorophyll; Overlay of ROS and chlorophyll. Bars show 50 μm.

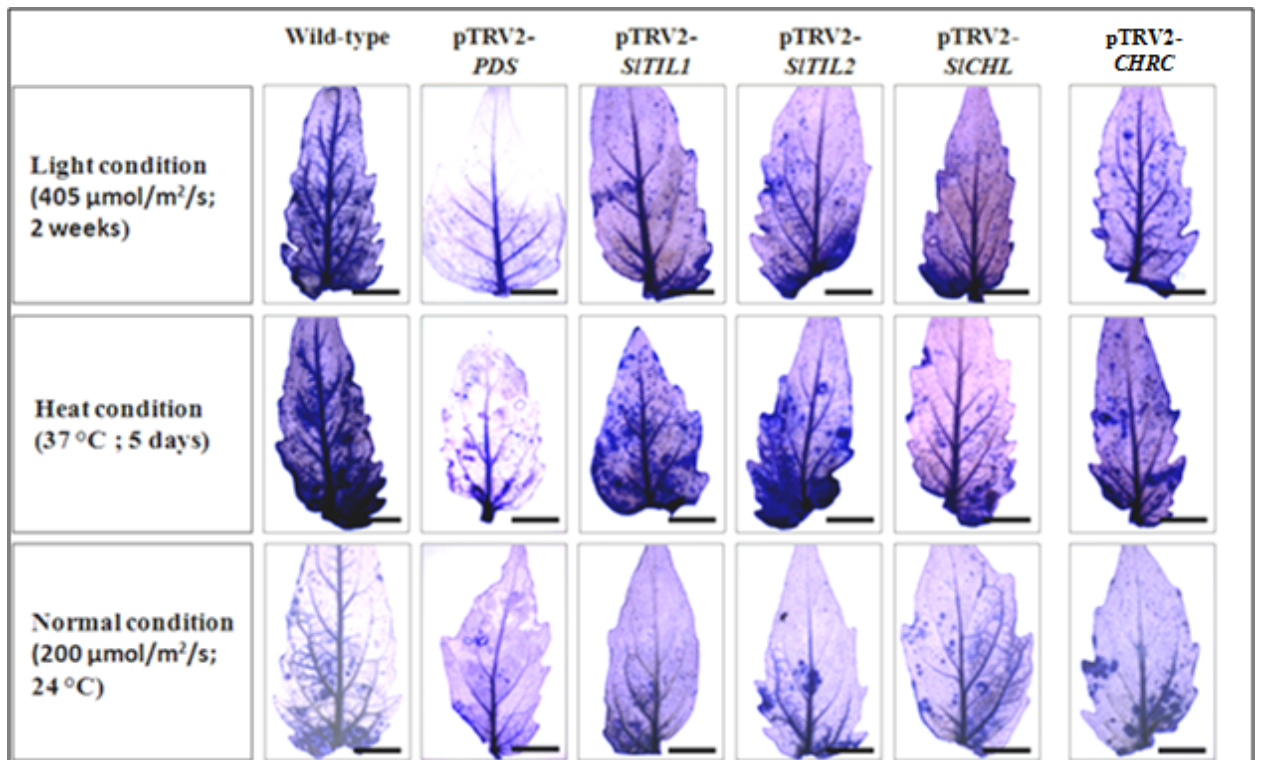


Fig. 48 Stereomicroscope image of NBT assay of gene silenced leaves under light condition (405 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$) and heat stress condition (37 °C; 5 days) showing the presence of superoxide ions ($\text{O}_2^{\cdot -}$) indicated by presence of blue coloured formazan. Bars show 5 mm.

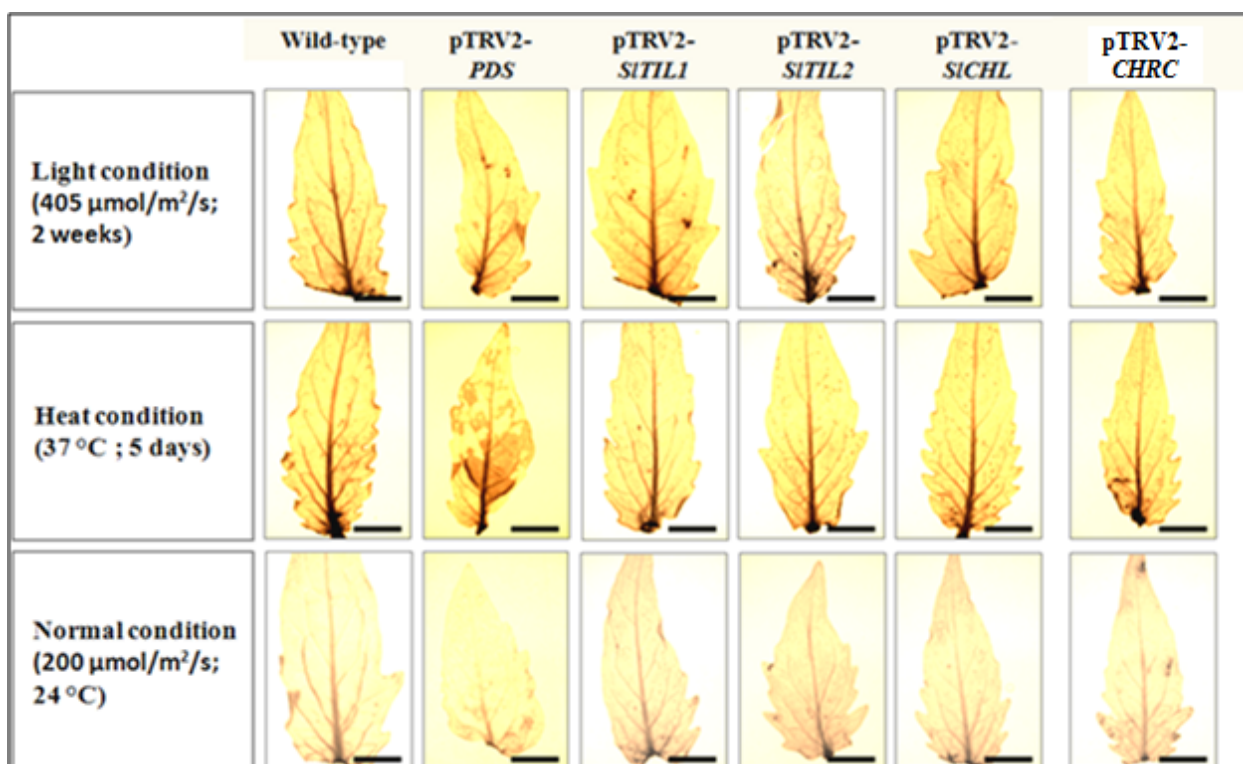


Fig. 49 Stereomicroscope image of DAB assay of gene silenced leaves under light condition (405 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress condition (37 °C; 5 days) showing the presence of hydrogen peroxide (H_2O_2) indicated by presence of brown coloured product. Bars show 5 mm.

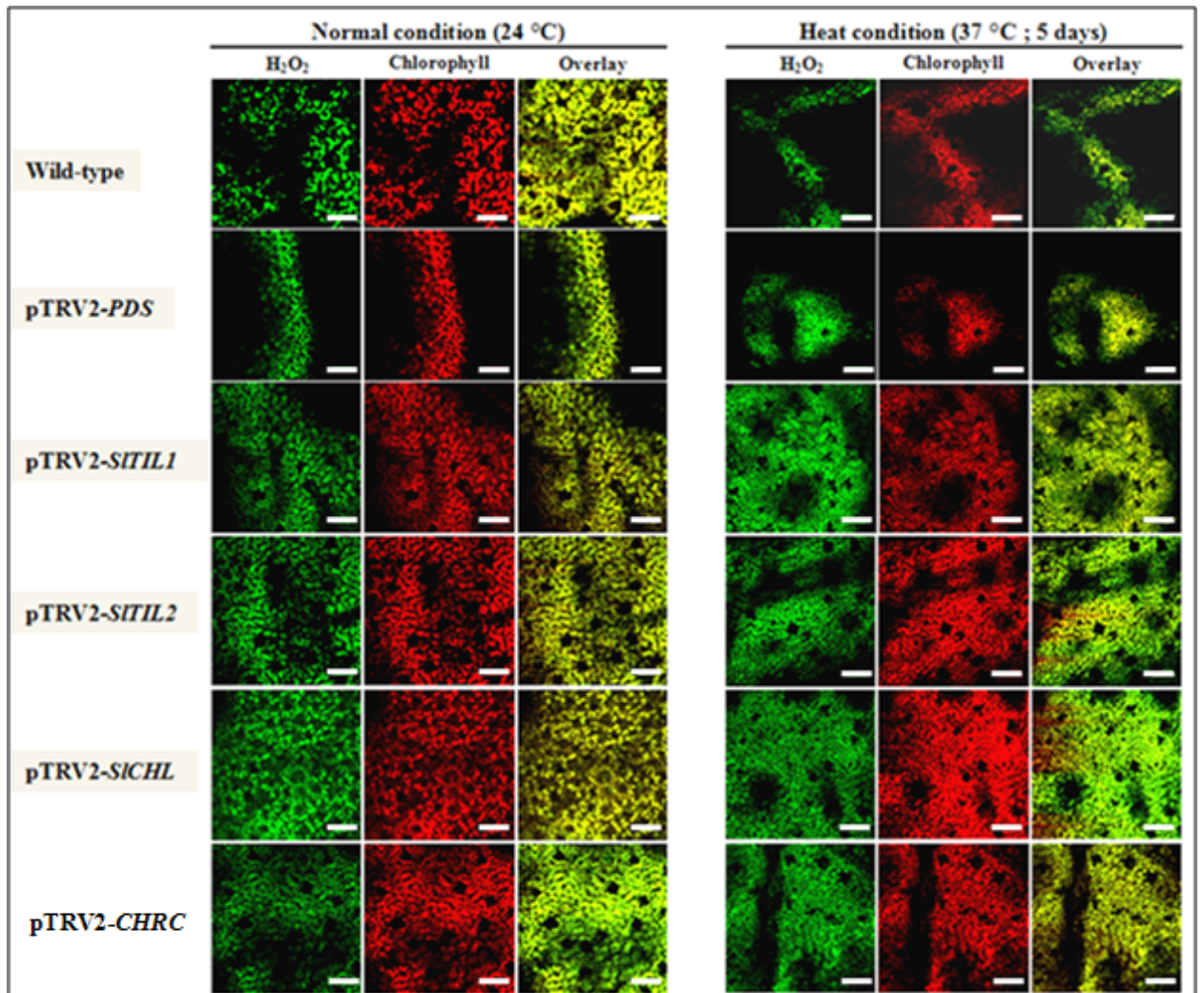


Fig. 50 Confocal H₂DCFDA staining images of gene silenced leaves under heat stress condition (37 °C; 5 days). Green color indicates ROS predominantly hydrogen peroxide; Red color indicates chlorophyll; Overlay of ROS and chlorophyll. Bars show 50 µm.

CHAPTER IV

DISCUSSION

4.1. Subcellular localization and molecular analyses of over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced plants

Phylogenetic tree and the amino acid sequence comparison indicated that SITIL1 and SITIL2 exhibited high identities with that of StTIL, NtTIL, PaTIL, PpTIL, TcTIL, MtTIL, AtTIL, PtTIL, PeTIL, and PbTIL (Fig. 9 and Fig. 10). SITIL1 from ‘Micro-Tom’ showed highest similarity with StTIL in potato and AtTIL in Arabidopsis comparing with other TILs (Fig. 9). SITIL2 from ‘Micro-Tom’ showed highest similarity with NtTIL. The amino acid sequences of TILs were conserved in *Solanaceae*. Sequence comparison of SICHL showed that it was similarity corresponded to SpCHL and StCHL (Fig. 11). Lipocalins Blc, ApoD and Lazarillo were known to be associated with membranes. The similarity between plant TILs and Blc, ApoD, Lazarillo suggested that the plant homologues also were membrane-associated proteins (Charron and Sarhan 2005). TILs are targeted to a variety of cell membranes and organelles in a subcellular localization studies. My studies have found that fusion proteins of SITIL1-sGFP, sGFP-SITIL1, SITIL2-sGFP, and sGFP-SITIL2 accumulated in the plasma membrane and around membranes of the onion epidermal cells and tobacco leaf cells (Fig. 12 and Fig. 13). These results were expected considering previous reports showing that subcellular localization of (*Populus euphratica*) YFP-PeTIL constructs were generated and transiently expressed in *Arabidopsis* protoplast. The protein was clearly localized in the plasma membrane of leaves (Abo-Ogiala et al. 2014). The other results on subcellular localization of AtTIL derivatives containing YFP showed in the plasma membrane reticulate and punctuate structures (Gras and Boronat 2015). In my study, in case of tobacco (*N. tabacum*) leaf cells, subcellular localization of *SITIL1* and *SITIL2* derivatives containing sGFP fused to either the C- or the N-terminal end

(SITIL1-sGFP, SITIL2-sGFP, sGFP-SITIL1 and sGFP-SITIL2) not only shown in the plasma membrane but sometimes also detected the fluorescence around plastids, nuclear and in reticulate structures (Fig. 12B,C).

The subcellular localization of AtTIL indicated that this protein was mainly localized in the plasma membrane in *Arabidopsis* (Charron et al. 2005). However, cell fractionation studies indicated that TIL proteins maybe found in organelle enriched fractions (Chi et al. 2009; Abo-Ogiala et al. 2014; Boca et al. 2014). The intracellular translocation of *Populus euphratica* TIL (PeTIL) from the plasma membrane to the symplast in response to salt stress in *Arabidopsis* protoplast (Abo-Ogiala et al. 2014). Relocation of lipocalins in response to stress has been reported in animal systems (Do Carmo et al. 2007). Proteomic data has shown the presence of AtTIL in mitochondrial membranes (Brugiere et al. 2004), endoplasmic reticulum (Dunkley et al. 2006), golgi (Eubel et al. 2008; Nikolovski et al. 2012; Parsons et al. 2012) and plasmodesmata (Fernandez-Calvino et al. 2011) thereby indicating this protein may be targeted to different cell membranes and organelles. Regardless of its subcellular localization, selective extraction of proteins from membrane fractions indicated that AtTIL behaves as a peripheral membrane protein (Chi et al. 2009). The interaction of AtTIL with cell membranes is intriguing considering that, in contrast with Blc and Lazarillo, this protein does not contain any recognizable signal for membrane targeting. Furthermore, TILs do not contain hydrophobic regions having the features of transmembrane motifs. However, the similarity with ApoD, which has been proposed to interact with the external face of the membrane through a short hydrophobic loop (Bishop 2000; Charron et al. 2005; Giczzyb et al. 2006), it has been suggested that TILs could interact with the plasma membrane by means of a short hydrophobic sequence present in a loop located between β -strands 5 and 6 (Charron and Sarhan 2005).

TargetP and ChloroP predicted that *SICHL* have an N-terminal transit peptide that targets the proteins to the chloroplast. Previous study reported on the immunoblot analyses of AtCHL

was specifically localized in the thylakoid lumen and suggests that import into the lumen probably occurs via interactions with other proteins (Levesque-Tremblay et al. 2009). In this study, I found the subcellular localization of *SICHL* in *N. tabacum* and *N. bentamiana* leaf cells shown localized in the chloroplast (Fig. 12D,E) and I thought that subcellular localization of *SICHL* derivatives containing sGFP fused to the N-terminal end (*SICHL*-sGFP) mainly localized in the chloroplast.

Denominated of abundance pattern of the tomato plastid proteins classified into seven categories: stables, decreasing continuously, decreasing late, decreasing early, increasing continuously, increasing late, and increasing early (Barsan et al. 2012). My data of expression analyses of abundance pattern *SITIL1* showed increasing early, *SITIL2* showed decreasing late, *CHRC* showed decreasing early (Fig. 15). The data of abundance pattern of *SICHL* showed decreasing continuously during fruit ripening or maturation stage (mature green, yellow, orange and red) (Fig. 15). These results indicate that *SICHL* plays a key role in differentiation of chloroplast to chromoplast, related to various color of tomatoes and related to fruit ripening or maturity stage of tomato ('Micro-Tom').

The result of *cis*-elements of *SITIL1*, *SITIL2*, *SICHL*, and *CHRC* in the promoter sequences were predicted by PlantCARE showed that *cis*-elements in the promoters of these genes were related to light, fungal elicitor, defense and stress responsive, heat stress, drought-inducible, auxin, gibberellins, methyl jasmonate, ethylene, abscisic acid, and anaerobic induction, respectively (Table 4). These results showed that *cis*-elements of *SITIL1*, *SITIL2*, *SICHL*, and *CHRC* play an important role in tomato plant stress responses. These genes might contribute to the various roles under abiotic stress condition.

In addition to ripening, ethylene is also known to be involved in other processes such as pathogen and wounding responses, leaf senescence and abiotic and biotic stress responses; so too are other hormones such as abscisic acid (ABA) and sugar signaling (Gazzarini and McCourt

2001). Tomato is a good model system for studying the fruit ripening. In the ripening progresses, fruit color changes from green to red as chloroplast are transformed into chromoplasts, chlorophyll is degraded and carotenoids accumulate (Lucille and Don 2002). As previously stated that lipocalin related proteins such as TIL and HrBP1 could be related to the accumulation of carotenoids, fruit color and the differentiation of chromoplast (Suzuki et al. 2015). In my study, hormone-responsive *cis*-elements of promoter sequences *SITIL2* genes were identified, including ERE which reflected plant responses to ethylene responsive respectively. The coding sequences of *SITIL1* and *SITIL2* were high similarity (84 %), but each promoter had specific *cis*-elements related to heat stress responsiveness (HSE in *SITIL1*) and ethylene responsiveness (ERE in *SITIL2*) (Table 4). These results indicated that *SITIL1* related to stress-responsive (heat stress) and *SITIL2* related to hormone-responsive (ethylene on fruit ripening). Fruit ripening and the role that ethylene plays in it regulation is complex. Identification of additional components involved in ethylene signal transduction, the further characterization of ripening mutants, and additional studies on the biochemistry of ripening are essential for complete understanding of the ripening process (Lucille and Don 2002). Thus, this result will contribute to further understand the various functions of lipocalin related to fruit ripening under complex hormone-responsiveness in tomatoes.

4.2. Light responses of over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced plants

Over-expressed *SITILs* and *SICHL* plants showed early flowering and fruit setting, increasing number of flowers, inflorescences, and fruits, and bigger peduncle, bigger flower and fruit, leaves with curling, longer terminal leaflet, bullwhip phenotype in leaves, bigger petiole and increasing number of leaves compared wild-type plants (Fig. 21A,B, E, F). Over-expressed *SITIL1* showed longest leaves with specific curling (Fig. 21B,F). Over-expressed *SITIL2* showed

earlier flowering, and over-expressed *SICHL* showed earlier ripening (Fig. 21A,E and Fig.24A, B). Fruit growth, the longest phase in fruit development, is initiated by signals related to pollination and to fertilization (McAtee et al. 2013; Sotelo-Silveira et al. 2014). These processes trigger fruit set and rapid fruit growth through cell division and cell expansion, whose importance impacts fruit quality according to genotypes and environmental conditions (Seymour et al. 2013). In my study, the fruit structure and pericarp pattern at breaker stages of wild-type and over-expressed *SITILs* and *SICHL* are shown in Figure 39 and 40. Most pericarp cells expanded a lot in over-expressed *SITILs* and *SICHL* at light condition 1 ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and light condition 2 ($105 \mu\text{mol m}^{-2} \text{s}^{-1}$) compared wild-type as a control (Figs. 39 and 40). In pericarp of wild-type between exocarp and mesocarp have empty space (empty cells) (Figs. 39A,B, C and 40A,B, C), but did not found empty space in over-expressed *SITILs* (Figs. 39D-I and 40D-I) and *SICHL* pericarp cells (Figs. 39J,K, L and 40J,K, L). Cell structure of vascular bundles in over-expressed *SITILs* and *SICHL* showed more tenuous compared wild-type vascular bundles cells that mean cell development in over-expressed *SITILs* and *SICHL* are rapidly developed. These study showed that over-expressed lipocalin genes expanded cell of pericarp transversal structure, indicated that lipocalins contribute to enhancing relative fruit growth after fruit set and during fruit ripening. Overall, these results revealed the key roles of lipocalins in regulating flower and fruit development, and provided a new potential way for improving tomato fruit yield.

Virus-induced gene silencing (VIGS) is an effective tool to down-regulate gene expression that is 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). The tobacco rattle virus (TRV), both of pTRV1 and pTRV2 were used as a VIGS vector in this study. However, I observed no visible virus symptoms in TRV-infected plants when compared to the wild-type, suggesting that the TRV symptoms did not interfere with the gene

silencing phenotype in tomato plants. These results showed that recombinant TRV could efficiently infect tomato plants. This result was already reported by other scientists and I just could reproduce them. TRV vector has been successfully used for gene silencing in a variety of plants, especially in the *Solanaceae* family members such as tomato (Liu et al. 2002). Previous studies were also reported that TRV could be transformed into the different tissues or organs including the leaf (Liu et al. 2002), flowers (Spitzer et al. 2007), fruits (Fu et al. 2005), roots (Kalosian, 2007), and sprouts (Yan et al. 2012) of *Solanaceous* species. The method of TRV infiltration into cotyledons of tomato plant was used and showed the high efficiency of gene silencing. The pTRV2-*PDS* infected cotyledons (young leaves) showed clearly photobleaching indicating that recombinant TRV could efficiently replicate and spread systemically in plants (Fig. 36).

The silencing of *SITIL1*, *SITIL2*, *SICHL* and *CHRC* resulted in patchy coloring of the fruit with areas exhibiting different shades of yellow and red color (Fig. 36D,H). The expression levels of these genes in the yellow colored areas of fruits were reduced respectively compared with the red areas, and also showed that the expression of these genes was up regulated during ripening. These results showed that these genes play a role in suppressing fruit ripening. The lipocalins (*SITIL1*, *SITIL2* and *SICHL*)-silenced plants showed the stress condition such as curling on leave and faster in senescence of leaves and plant growth. This result implies that *SITIL1*, *SITIL2* and *SICHL* genes may have a role in oxidative stress response (Levesque-Tremblay et al. 2009 and Abo-Ogiala et al. 2014). *CHRC* is a nuclear-encoded plastid protein related to carotenoids that exists uniquely in plastid, involved in carotenoid accumulation and stabilization (Vishnevetsky et al. 1996, 1999; Kilambi et al. 2013) and up-regulated during chromoplast differentiation in certain fruits (Deruere et al. 1994) and flowers (Vishnevetsky et al. 1996). In my study, the *CHRC silencing* fruits exhibited yellow areas and did not turn normal red. The results indicated that *CHRC* also could affect the carotenoid accumulation during fruit

development. Gene silenced plants grew more slowly compared than control plants (Fig. 37). In that time, control plant was started to flowering, but after those gene silenced plants increased growth faster compared than control plants. Gene silenced plants delaying flowering 1-2 weeks (Fig. 36A,E). This result showed that the four nuclear-encoded proteins (*SITIL1*, *SITIL2*, *SICHL* and *CHRC*) play roles of fruit ripening in tomato. In the future, this data will provide a platform for further investigation of their biological functions and the evolution of nuclear-encoded chloroplast proteins in tomato.

The lipocalins, the fatty acid-binding proteins (FABPs), and the avidins are three families of ligand binding proteins; together form the calycin protein superfamily (Flower et al. 2000). Functional analyses of *SITIL1*, *SITIL2*, *SICHL* and *CHRC* proteins by classifying them into families, predicting domains and important sites performed using InterPro (<http://www.ebi.ac.uk/interpro/>). The result showed that molecular function of *SITIL1* and *SITIL2* proteins were categorized as transporter activity enables and directed movement of substance (such as macromolecules, small molecules, ions) into, out of or within a cell, or between cells by gene ontology. Protein family members and molecular function of *SICHL* and *CHRC* was none predicted. Here, I describe analyses of the amino acid sequences of *SITIL1*, *SITIL2*, *SICHL* and *CHRC* using the membrane protein prediction SOSUI (<http://harrier.nagahama-i-bio.ac.jp/sosui/>). The result showed that *SITIL1*, *SITIL2*, *SICHL* and *CHRC* were soluble protein. The proportion of membrane proteins are 0.0%, none signal peptide and none hydrophobicity. These results contribute to our understanding of the functional analyses of these proteins in plant and provide clues for further.

Superoxide dismutases (SODs) are critical antioxidant enzymes that protect organism from reactive oxygen species (ROS) caused by adverse condition, and have been widely found in the cytoplasm, chloroplast, and mitochondria of eukaryotic and prokaryotic cells (Feng et al.2016). In recent years, some studies have reported that SODs can protect plants against abiotic and

biotic stresses, such as heat, cold, drought, salinity, abscisic acid and ethylene (Wang et al. 2004; Pilon et al. 2011; Asensio et al. 2012; Feng et al. 2016). In my study, the expression analyses of *SISODs* (*SISOD1*, *SISOD3* and *SISOD6*) showed high expressed in seedling, leaf, flower and fruit of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants (Fig. 41,42,43), but the expression of *SISODs* in gene silenced plants showed very low (Fig. 44). RT-PCR analyses of *SISODs* indicated that *SITIL1*, *SITIL2*, and *SICHL* genes in tomato could play roles in eliminating ROS. These results contribute to our understanding of the functional analyses of lipocalin proteins in plant and provide clues for the study of abiotic stress response such as light and osmotic stress in tomato plants.

4.3. Over-expression of *SITILs* and *SICHL* in tomato ‘Micro-Tom’ confers enhanced light, heat and oxidative stress tolerance

Superoxide ions (O_2^-) and hydrogen peroxide (H_2O_2) are the vital reactive oxygen molecules that play a central role in many processes involved in plant growth and development including abiotic stress tolerance. To get better insights into the ROS mediated regulation of these processes, qualitative and quantitative estimation of different types of ROS is of significant importance. O_2^- is produced by the transfer of electrons from NADPH to oxygen (O_2) mediated by the NADPH oxidase enzyme system. These ions are estimated in rice seedlings using NBT assay which is based upon the principle of reduction of yellow coloured NBT into dark blue coloured insoluble formazan by O_2^- (Kaur et al. 2016).

H_2O_2 is another reactive oxygen molecule that acts as an important signaling molecule regulating different plant processes. The content of H_2O_2 is estimated qualitatively in rice seedlings using DAB and H_2DCFDA assay (Kaur et al. 2016). DAB assay is based upon the principle of formation of deep brown polymerization product on the reaction of DAB with H_2O_2 while H_2DCFDA assay is based upon the principle of fluorescent microscopy. When non-fluorescent H_2DCFDA binds to ROS (predominantly H_2O_2), it gets converted into highly

fluorescent 2',7'-dichlorofluorescein (DCF). DCF gives a green coloured fluorescence when excited with a laser beam of excitation 488 nm using confocal microscope.

ROS generation is regarded as a common cellular response during stressful condition. H_2O_2 is considered a central signaling molecule in plant responses to various environmental stresses (Neill et al. 2002). From another point of view, ROS over-production might causes serious oxidation damage, which could influence the ordinary operation of plant metabolism (Jaspers et al. 2010). Similar to other studies, I found that O_2^- and H_2O_2 also participated in over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced plants under light condition and heat stress tolerance (Figs. 46, 47, 49, 50). Over-expressed *SITIL1*, *SITIL2* and *SICHL* under light condition and heat stress treated showed lower oxidative damage, O_2^- and H_2O_2 in normal and stress condition compared control (wild-type) leaf plants (Figs. 45, 46, 47) and increased activities of *SISODs* (Fig. 41,42,43). Furthermore, I used pTRV-based VIGS system in these study to silence *PDS*, *SITILs*, *SICHL* and *CHRC*. Under light condition and heat stress treated showed increased oxidative damage, O_2^- and H_2O_2 in normal and stress condition (Figs. 48, 49, 50) and decreased activities of *SISODs* (Fig. 44). These result indicated that O_2^- and H_2O_2 could up-regulate the antioxidant defense system that ultimately lead to improved thermo tolerance in transgenic *SITILs* and *SICHL* over-expressed tomato plants, and the other side decreased thermo tolerance in silenced plants. These results suggest that lipocalins play an important role in abiotic oxidative stress tolerance in tomato plants.

CONCLUSION

Plastid proteomic data showed that lipocalins are related to differentiation of chromoplasts and ripening in tomato fruits. Lipocalins are a group of proteins and distributed in bacteria, invertebrate, and vertebrate animals. Lipocalin has various functions including those related to environmental stress response, apoptosis induction, membrane formation and fixation, regulation of immune response, cell growth, and metabolism adjustment. However, very little is known about plant lipocalins. To better understand the function of lipocalins, I made over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced plants. The over-expressed *SITILs* and *SICHL* plants compared with wild-type plants under $405\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ showed their leaves with curling, longer terminal leaflet, bullwhip phenotype in leaves, early flowering, increasing number of flowers and inflorescences, and bigger peduncle and fruits. The over-expressed *SITIL1* showed longer of leaves with curling, over-expressed *SITIL2* showed earlier flowering, and over-expressed *SICHL* showed dark greening in seedling and mesocarp of mature green fruit and earlier ripening compared others. Moreover pericarp transversal cell structures were expanded in over-expressed *SITIL1*, *SITIL2* and *SICHL* fruits. I also suppressed *lipocalins* expression in tomato using VIGS-system to observe their phenotype. The suppressed expression of *SITILs* induced aberrant shapes of leaves, such as yellowing and curling, and yellowing in pericarps and

mesocarps of fruits. The suppressed expression of *SICHL* induced yellowing in pericarps and mesocarps, and greening in endocarps of fruits. Moreover subcellular localization analyses of SITILs and SICHL using a particle bombardment showed that the fusion proteins of SITILs-sGFP and sGFP-SITILs were located around the plasma membrane, plastid, nuclear and in reticulate structure. SICHL-sGFP was localized in the chloroplast. Their promoters that 1000 bp upstream from the start codons of lipocalins are included many light-responsive *cis*-elements in promoters. *SISODs* (*SISOD1*, *SISOD3* and *SISOD6*) were highly expressed in seedling, leaf, flower and fruit of over-expressed *SITIL1*, *SITIL2* and *SICHL* plants, on the other hand very low expression of them in their gene silenced plants. I found that O_2^- and H_2O_2 also participates in over-expressed *SITIL1*, *SITIL2* and *SICHL* and gene silenced plants under light stress ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress (37°C ; 5 days) tolerance. Over-expressed *SITIL1*, *SITIL2* and *SICHL* under light stress ($405 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) and heat stress (37°C ; 5 days) showed lower oxidative damage (O_2^- and H_2O_2) in normal and stress condition compared control (wild-type) leaf plants and increased level activities of *SISODs*. Furthermore, I used pTRV-based VIGS system in these study to silence *PDS*, *SITIL1*, *SITIL2*, *SICHL* and *CHRC*. Under light stress ($405 \mu\text{mol m}^{-2} \text{s}^{-1}$) and heat stress (37°C ; 5 days) showed increased oxidative damage (O_2^- and H_2O_2) and decreased level activities of *SODs*. These result indicate that O_2^- and H_2O_2 could up-regulate the antioxidant defense system that ultimately lead to improved thermo tolerance in over-expressed *SITILs* and *SICHL* tomato plants, and the other side decreased thermo tolerance in gene silenced plants. These result suggest that lipocalins play an important role in abiotic oxidative stress tolerance (eliminating ROS) in tomato plants.

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