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Suppression of pepper *SGT1* and *SKP1* causes severe retardation of plant growth and compromises basal resistance

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SGT1 associates with suppressor of kinetochore protein (Skp1)-Cullin-F-box (SCF)–ubiquitin-ligase complexes playing important roles in controlling developmental processes and defense responses in plants, yeast, and human. In this study, full-length cDNAs of *Sgt1* and *Skp1* orthologues were isolated from pepper (*Capsicum annuum* L.) to characterize their functions. Protein sequences of CaSgt1 and CaSkp1 showed high degrees of similarities with their homologues in other plant species. Southern blot analyses revealed that *CaSgt1* was a single copy gene in the pepper genome, whereas *CaSkp1* corresponded to multi copy genes. Levels of *CaSgt1* and *CaSkp1* mRNAs increased in pepper leaves in response to incompatible pathogen challenge or salicylic acid treatment. Silencing of *CaSgt1* or *CaSkp1* using Tobacco rattle virus-based virus-induced gene-silencing (VIGS) system resulted in severe dwarfism and final damping-off symptom in the greenhouse. To identify factors determining damping-off symptom in *CaSgt1*- or *CaSkp1*-silenced plants, we employed VIGS under sterile conditions. Under such conditions, damping-off symptom was not observed suggesting that *CaSgt1* and *CaSkp1* play an essential role in plant growth and development as well as basal disease resistance in pepper plant.

Introduction

Protein degradation is an essential mechanism regulating cell-cycle progression, signal transduction, and developmental processes in plants. The ubiquitin-mediated protein degradation pathway appears to be a major system for selective protein degradation in eukaryotic cells (Herschko and Ciechanover 1998). This pathway consists of three steps: E1, E2, and E3. The suppressor of kinetochore protein (Skp1)-Cullin-F-box (SCF) complex is a component of E3 enzyme that has been extensively studied in yeast, mammals, and plants. The SCF complex included four subunits, Skp1, Cullin/CDC53, and Rbx1/Roc1/Hrt1 as three constant components, and an F-box protein, which is a variable component in different organisms (Patton et al. 1998, Tyers and Jorgensen 2000). This complex mediates the degradation of multiple proteins involved in diverse signaling pathways through an ubiquitin-proteasome pathway (Deshaies 1999).

In yeast, SKP1, an essential component of the SCF complex has been demonstrated to be essential for

Abbreviations – cDNA, DNA complementary to DNA; RT–PCR, Reverse transcriptase-polymerase chain reaction; ESTS, Expressed sequence tags; TRV, *Tobacco rattle virus*.

cell-cycle progression at both the G1/S and G2/M transitions (Bai et al. 1996, Connelly and Hieter 1996). The SKP1 protein acts as a scaffold to link the Cullin-RING box protein dimer to the F-box protein. Originally, yeast SGT1 was identified as a suppressor of the G-two allele of SKP1 and has been known to be associated with Skp1 in the centromere-binding factor 3 of kinetochore complex of chromosome (Kaplan et al. 1997, Kitagawa et al. 1999) and the SCF-type ubiquitin ligase complex (Matsuzawa and Reed 2001). These findings imply that the coordination between SKP1 and SGT1 is important for regulating cellular functions in yeast.

In *Arabidopsis*, comparative genomics revealed the existence of 21 *SKP1*-like genes (Arabidopsis Genome Initiative 2000). *Arabidopsis* SKP1-like (ASK1) has been shown to interact with AtCUL1 as a component of the SCF^{TIR1} and SCF^{COI1} complexes and to function as an important element in auxin- and jasmonate-signaling pathway (Gray et al. 1999, Xu et al. 2002). ASK1 and ASK2 also were shown to be involved in male meiosis and flower development (Zhao et al. 2003). Furthermore, *Arabidopsis ask1 ask2* double mutant was shown to be defective in cell division, cell expansion/elongation, developmental delay on embryogenesis, and finally lethality in seedling growth (Liu et al. 2004).

Similar to SKP1, SGT1 has been reported to play a crucial role in developmental processes (Gray et al. 2003). *Arabidopsis* ETA3/SGT1b was shown to be required for the SCF^{TIR1}-mediated degradation of Aux/IAA proteins (Gray et al. 2003). SGT1 has unique domains necessary for protein functions: tetratricopeptide repeat domain (TPR), CHORD and SGT1 motif (CS) and SGT1-specific motif (SGS motif) (Blatch and Lassle 1999). The TPR domain has been known to mediate protein–protein interactions among multicomplex proteins functioning as chaperone, cell cycle, transcription, or protein transport complexes. For example, the TPR domain of SGT1 was shown to bind to heat-shock protein 70 (HSP70) (Liu et al. 1999). However, the CS domain of SGT1 is similar to the one in the human p23 protein, which is known to interact with HSP90 and participate in the folding of different regulatory proteins (Garcia-Ranea et al. 2002, Lee et al. 2004a).

Besides the critical role of plant SGT1 and SKP1 in development processes, a number of studies have reported that SGT1 and SKP1 contribute to pathogen defense responses including resistance (*R*) gene-mediated and non-host defense responses in plants (Liu et al. 2002, Peart et al. 2002). Silencing of *Skp1*, a key component of the SCF complexes abolished N-mediated resistance against TMV in *Nicotiana benthamiana* (Liu et al. 2002). Mutational screening identified *Arabidopsis SGT1b* as a regulator of *R* gene-mediated defenses against the downy mildew pathogen *Peronospora parasitica* (Austin et al. 2002, Tör et al. 2002). Subsequently, knockout of SGT1 reduced resistance triggered by R proteins against diverse types of pathogens in plants (reviewed by Shirasu and Schulze-Lefert 2003) and non-host resistance in *N. benthamiana* (Peart et al. 2002). Although the *Sgt1* and *Skp1* genes are involved in ubiquitination during defense responses against pathogen in plants, the precise molecular function of SGT1 and SKP1 contributing to defense remains to be elucidated.

To gain further insights into the functions of *Sgt1* and *Skp1* in plants, we determined the whole cDNA sequences of the pepper *SGT1* and *SKP1* orthologues and RNA expression profiles of *CaSgt1* and *CaSkp1* during incompatible pathogen inoculation and salicylic acid (SA) treatment. Silencing *CaSgt1* and *CaSkp1* using Tobacco rattle virus (TRV)-based virus-induced gene-silencing (VIGS) system resulted in severe phenotypes such as dwarfism and damping-off in pepper.

Materials and methods

Plant, pathogen, and chemical preparations

Chili peppers (*Capsicum annuum* L. cv. Bukang) were grown in a growth chamber at 25°C under a 16-h/8-h light/dark cycle. Leaf, stem, root, closed flower, and open flower organs were collected from the healthy pepper plants and immediately frozen in liquid nitrogen for RNA blot analysis. Healthy leaves from about 1-month-old plants were used for pathogen inoculation and SA treatment and subsequent nucleic acid extraction. For the pathogen challenge, incompatible bacterial pathogen *Xanthomonas axonopodis* pv. *glycines* 8ra (*Xag*; 0.4 of OD_{600} in 10 m*M* $MgCl_2$) was pressure-infiltrated to pepper leaves with needless syringe (Park and Hwang 1999). For the SA treatment, 5 m*M* of SA was sprayed to the whole plants and then leaves were harvested at the indicated time and immediately frozen in liquid nitrogen for total RNA extraction.

Isolation and sequencing of *CaSgt1* and *CaSkp1*

Chili pepper cDNA libraries were constructed from seven different plant tissues (leaves infiltrated with *Xag* suspension cells, main and axillary root, flower, fruit, placenta, and anther), and the cDNAs were amplified in *Escherichia coli* after in vivo excision, as previously described (Choi et al. 1996). The 5′ partial nucleotide sequences and deduced polypeptides of cDNA clones

are shown in the chili pepper EST cDNA database (http://genepool.kribb.re.kr; Lee et al. 2004b). The database was searched to identify cDNA clones corresponding to *Sgt1* and *Skp1*. *CaSgt1* and *CaSkp1* cDNAs in the pBluescript SK (-) were sequenced with T7 primer to determine the full-length cDNA sequences.

Genomic DNA and RNA blot analysis

Genomic DNA of chili pepper plants was prepared according to the method of Dellaporta et al. (1983). Twenty microgram of total DNA was digested with *Eco*RI, or *Xba*I. Digested DNAs were separated on 0.8% (w/v) agarose gel. Southern transfer was carried out according to the standard method (Sambrook et al. 1989). Probe labeling, blot hybridization, and washing conditions were previously described (Chung et al. 2003).

Total RNAs were extracted from pepper leaves using the LiCl-phenol extraction method of Prescott and Martin (1987). The RNA was fractionated on denaturing formaldehyde 1.0% (w/v) agarose gels according to Sambrook et al. (1989). RNA blots were hybridized and washed as previously described (Church and Gilbert 1983). Polymerase chain reaction (PCR) products covering the entire cDNA of *CaSgt1* and *CaSkp1* were used as probes. Probe labeling, blot hybridization, and washing conditions were as described earlier (Chung et al. 2003). Hybridized membranes were exposed to imaging plate and scanned using BAS-1800 (Fujifilm, Minato-ku, Japan).

Plasmid construction and *Agro*-infiltration

The 1130-bp *Bam*HI and *Xho*I DNA fragment of *CaSgt1* cDNA (GenBank accession number AY899280) was cloned into the pTRV2 vector, yielding pTRV2-*CaSgt1*. The 670-bp *Eco*RI/*Xho*I DNA fragment of *CaSkp1* cDNA (GenBank accession number AY899281) was cloned into the pTRV2 vector in a sense orientation to produce pTRV2-*CaSkp1*. The control TRV2 vector, TRV2-*GFP* was constructed as follows. The soluble-modified green fluorescent protein gene (David and Vierstra 1996) was amplified using *Pfu* DNA polymerase with 5-GFP primer (TGCCGACAGAATTCCCAAAGATGGACC) and 3-GFP primer (ATCATCGCAAGACCGGCAACAG AATTCA). The resulting PCR product was digested with *Eco*RI enzyme and cloned into *Eco*RI sites of pTRV2 vector. TRV2-*CaPDS* clone was used as previously described (Chung et al. 2004). *Eco*RI and *Xho*I DNA fragments of *CaChl H* cDNA (GenBank accession number DQ073919) was cloned into the pTRV2 vector, yielding pTRV2-*CaChl H*.

Chili pepper seeds were germinated on 3S media (MS salt and B5 vitamin, 3% sucrose, 0.8% plant agar), and the germinating seedlings were transferred to a pot of soil in a growth chamber at 25°C with a 16-h light and 8-h dark photoperiod cycle. *Agro*-infiltration procedure was carried out as previously described (Chung et al. 2004). For *Agro*-infiltration, pTRV1, pTRV2-*GFP*, and the recombinant plasmids (pTRV2-*CaPDS,* pTRV2-*CaChl H*, pTRV2-*CaSgt1*, and pTRV2-*CaSkp1*) were transformed into *Agrobacterium tumefaciens* GV2260 via a freeze-thaw method (An et al. 1988). Each strain was grown in 2 ml YEP (50 μg ml^{-1} of kanamycin and 50 μg ml^{-1} of rifampicin) overnight at 30°C. The overnight culture was inoculated into 20 ml of YEP medium and grown at 30°C overnight. The cells were collected by centrifugation (2000 *g*, 20 min, 10°C), resuspended in infiltration medium (10 m*M* MES, pH 5.6, 10 m*M* $MgCl_2$), and adjusted to $O.D_{600} = 0.5$. For the virulence induction of *Agrobacterium*, acetosyringone was added to the resuspended bacterial culture at a final concentration of 200 μ*M*, and then the culture was agitated at 22–25°C for 3–5 h. The induced *Agrobacterium* cells carrying pTRV1 and pTRV2-*GFP*, *pTRV2-CaPDS*, pTRV2-*CaChl H*, pTRV2-*CaSgt1*, or pTRV2-*CaSkp1* (1:1 ratio) were pressure-infiltrated with a needleless 1-ml syringe into cotyledons of pepper seedlings. The *Agro*-infiltrated pepper plants were transferred to 16°C for 1 day for enhancing *Agro*-transformation and grown in a growth chamber at 25°C with a 16-h light and 8-h dark photoperiod cycle (Ryu et al. 2004).

For in vitro VIGS, germinating seedlings were immersed in the acetosyringone-induced *Agrobacterium* mixtures of pTRV1 and pTRV2-*GFP*, pTRV2-*CaChl H*, pTRV2-*CaSgt1*, or pTRV2-*CaSkp1* (1:1 ratio) and then vacuum-infiltrated for 5 min at 20 Pound per square inch. The treated seedlings were transferred to 3S media containing cefotoxime (250 mg l^{-1}) to inhibit growth of any bacteria and placed in the tissue culture room under light.

Reverse transcription-PCR analyses of *CaSgt1*- and *CaSkp1*-silenced plants

Total RNA was extracted from the leaves of infected pepper plant using the TRIZOL solution (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using 2 μg of total RNA, oligo d (T) primer, and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer instructions. Semi-quantitative reverse transcription (RT)-PCR was performed as previously described (Burton et al. 2000). Samples from each reaction (4 μl) were used in a 20-μl premix PCR mixture containing Taq polymerase

(Bioneer, Daejeon, Republic of Korea). For RT-PCR, amplification was performed for 15, 18, 21, 25, and 30 cycles at 94°C for 50 s, 55°C for 50 s, and 72°C for 30 s. Amplification of the pepper actin gene sequences was performed as a control with F-actin (5′-TTG GAC TCT GGT GAT GGT GTG-3′) and R-actin (5′-AAC ATG GTT GAG CCA CCA CTG-3′) primers. For the detection of *CaSgt1* transcripts, PCR was carried out with 5-CaSgt1 (5′-TTC ACT GAA GCT GTT GTT GAT GCG-3′) and 3-CaSgt1 (5′-ATC AAG AGA TGT CCA GTG CAA TGG-3′) primers. To monitor the transcript level of *CaSkp1*, we performed amplification with 5-*CaSkp1* (5′-TGC ACG GGT GTT TCT TTC TCT TGC-3′) and 3-*CaSkp1* (5′-TAG CAG CCA GAA TGA GAT CGA AGA G-3′) as primers. The intensities of PCR-generated fragments were analysed using Digi Doc-It Version 1.1.25 (Ultra-Violet Products; Upland, CA).

Statistical analysis

Data were subjected to analysis of variance using JMP software (SAS Institute Inc., Cary, NC). Significance of *CaSgt1*- and *CaSkp1*-silenced plants on plant survival rate was determined by the magnitude of the *F*-value at $P = 0.05$. When a significant *F*-value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) at $P = 0.05$.

Results

Sequence analyses of *CaSgt1* and *CaSkp1*

In an attempt to study functions of *Sgt1* and *Skp1* in pepper plants, a pepper EST database (http://genepool.kribb.re.kr) was searched to identify pepper homologues of *Sgt1* and *Skp1*. Full-length cDNA clones of pepper *Sgt1* and *Skp1* were identified and designated as *CaSgt1* and *CaSkp1*, respectively. The deduced amino acid sequences for CaSgt1 and CaSkp1 of the pepper in this study and other plant species were aligned and compared (Figs 1 and 2).

The polypeptide encoded by *CaSgt1* contained 370 amino acid residues with predicted molecular masses of 41.3 kDa with isoelectric point (pI) of 5.3. The CaSgt1 protein sequence shows high degrees of similarity to SGT1 proteins from other plant species (Fig. 1A). Sequence conservation is not uniform throughout the protein but is localized in three regions (Fig. 1A): three TPRs at the N-terminus, CS of the junction domain, and SGS of the C-terminus. Phylogenetic analysis was carried out with CaSgt1 and six deduced Sgt1 amino acid sequences from other plant species (Fig. 1B). The pepper CaSgt1 was most similar to the *N. benthamiana* NbSgt1 (82%). The open-reading frame of *CaSkp1* was predicted to encode a protein of 156 amino acid residues with a molecular mass of 17.5 kDa and pI of 4.67. The pepper CaSkp1 was shown to share a high degree of sequence similarity with other homologues due to the presence of the conserved tetramerization and dimerization domains in the N-terminus part of the protein (Fig. 2A). The CaSgt1 was shown to share a high degree of sequence similarity to tobacco NtSkp1 (84%), *Nicotiana clevelandii* NcSkp1 (83%), *N. benthamiana* NbSkp1 (82%), and alfalfa MsSkp1 (82%) (Fig. 2B).

Genomic organizations and expression of *CaSgt1* and *CaSkp1*

Genomic DNA gel blot analyses were performed to determine the copy number of *CaSgt1* and *CaSkp1* in pepper genome (Fig. 3A). A single band was detected using the full-length cDNA probe of the *CaSgt1* gene (Fig. 3A). On the other hand, multiple bands with varying degrees of sequence similarity were detected with the *CaSkp1* probe suggesting that *CaSgt1* is a member of a gene family (Fig. 3A).

Northern blot analyses were carried out to determine whether the expression of *CaSgt1* or *CaSkp1* is developmentally regulated (Fig. 3B). *CaSgt1* was rarely expressed in leaves and flowers but moderately expressed in the roots and stem organs (Fig. 3B). In contrast, *CaSkp1* mRNA was moderately expressed in leaves, and high levels of *CaSkp1* mRNAs were detected in the root and stem organs (Fig. 3B). *CaSkp1* was most abundantly expressed in the floral organs (Fig. 3B). Taken together, expression of *CaSgt1* and *CaSkp1* is developmentally regulated in pepper plants distinctively.

Expression of *CaSgt1* and *CaSkp1* in response to incompatible pathogen infection and SA treatment

We examined the mRNA expression patterns of the *CaSgt1* and *CaSkp1* genes during non-host (incompatible) pathogen interaction (Fig. 4A). mRNA levels of *CaSgt1* and *CaSkp1* started to be increasing 6 h after inoculation with *X. axonopodis* pv. *glycines* 8ra (*Xag*), which elicited hypersensitive response (HR) on pepper leaves within 24 h after infiltration into the pepper leaves (Fig. 4A). *PR-1a* expression was specifically induced after pathogen infection (Fig. 4A).

Other sets of Northern blot analyses were carried out to find out whether SA application affects mRNA

A

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                                  TPR                            TPR
CaSGT1  : ----MASDLETRAKEAFIDDHFELAVDLYTQAISLSPKNPELFADRAQANIKLNYFTEAVVDANKAIELDPYMSKAY :  73
NbSGT1  : ----MASDLETRAKEAFIDDHFELAVDLYTQAIAMTPKNAELFADRAQANIKLNYFTEAVVDANKAIELDPSMSKAY :  73
HvSGT1  : MAAAAASDLESKAKEAFVDDDFELAAELYTQAIEAGPATAELYADRAQAHIKLGSYTEAVADANKAIELDPSMHKAY :  77
OsSGT1  : MATAAASDLESKAKAAFVDDDFELAAELYTQAIEASPATAELYADRAQAHIKLGNYTEAVADANKAIELDPSMHKAY :  77
BoSGT1  : ----MASELAEKAKEAFLEDDFDVAVDLYSRAIDLDPNCAAFFADRAQANIKILNFTEAVADANKAIELEPTLSKAY :  73
AtSGT1b : ----MAKELAEKAKEAFLDDDFDVAVDLYSKAIDLDPNCAAFFADRAQANIKIDNFTEAVVDANKAIELEPTLAKAY :  73
AtSGT1a : ----MAKELAEKAKEAFLDDDFDVAVDLYSKAIDLDPNCAAFFADRAQANIKIDNFTEAVVDANKAIELEPTLAKAY :  73

              TPR
CaSGT1  : LRKGLACMKLEEYQTAKAALETGASLAPGESRFTKLMKECDESIAEEAGELPNISLE-KPSANAVASS-ASELSDNV : 148
NbSGT1  : LRKGLACMKLEEYQTAKAALETGASLAPAESRFTKLIKECDERIAEEAGELPNQSVD-KTSGNVVAPP-ASESLDNV : 148
HvSGT1  : LRKGSACIKLEEYQTAKAALEVGSSYASGDSRFTRLMKECDDRIAEEASQAPVKNAA-AAVAPATSSG-ATTVV--- : 149
OsSGT1  : LRKGAACIRLEEYQTAKAALELGYSFASGDSRFTRLMKECDERIAEELSEVPVKKAEDGAAAPSVAS------F--- : 145
BoSGT1  : LRKGTACMKLEEYSTAKAALQKGASVAPNESKFNKLIDECNLHIAEEEKDLAQQMPP-TLPSSSTTPPLATAADSPP : 149
AtSGT1b : LRKGTACMKLEEYSTAKAALEKGASVAPNEPKFKKMIDECDLRIAEEEKDLVQPMPP-SLPSSSTTP-LATEADAPP : 148
AtSGT1a : LRKGTACMKLEEYSTAKAALEKGASVAPNEPKFKKMIDECDLRIAEEEKDLVQPMPP-SLPSSSTTP-LATEADAPP : 148

                                                       CS
CaSGT1  : AIAPRD-PQSTVTLSH--QESAAKPKYRHEFYQKPEEVVVTIFAKGIPAKNVVVDFGEQILSVSIDLPGGETYSFQP : 222
NbSGT1  : AVAPKD-AQPTVNLSY--QGSAARPKYRHEFYQKPEEVVVTIFAKGIPAKNVVVDFGEQILSVSIDVPGDETYSFQP : 222
HvSGT1  : -TEAEDQDGENMENAQPTVEVPSKPKYRHDYYNTPTEVVLTIFAKGVPADSVVVDFGEQMLSVSIELPGEEPYHFQP : 225
OsSGT1  : -VEEKD-DAANMDNTP--PMVEVKPKYRHDFYNSATEVVLTIFAKGVPAENVVVDFGEQMLSVSIEVPGEEPYHFQP : 218
BoSGT1  : -------------------AAPAKPMFRHEFYQKPEEVVVTVFAKGIPKQNLNVEFGDQILSVVIDVAGEEAYHFQP : 207
AtSGT1b : V---------------PIPAAPAKPMFRHEFYQKPEEAVVTIFAKKVPKENVTVEFGEQILSVVIDVAGEEAYHLQP : 210
AtSGT1a : V---------------PIPAAPAKPMFRHEFYQKPEEAVVTIFAKKVPKENVTVEFGEQILSVVIDVAGEEAYHLQP : 210

                CS                                                         SGS
CaSGT1  : RLFGKITPAKCRYEVMSTKIEIRLAKAEPLHWTSLDYTREPVVIHRPVVSSA--APRPSYPSSKLRNVDWDKLEAQV : 297
NbSGT1  : RLFGKITPAKCRYEVMSTKIEIRLAKAEPLHWTSLEYTRASAVVQRPNVSSD--APRPSYPSSKLRHVDWDKLEAEV : 297
HvSGT1  : RLFSKIVPDKCKYTVLSTKVEIRLAKAEPVTWTSLDYTGKPKAPQKINVPAES-AQRPSYPSSKSKK-DWDKLEAEV : 300
OsSGT1  : RLFSKIIPEKSRYQVLSTKVEIRLAKAEQITWTSLDYDKKPKAVPQKIIPPAESAQRPSYPSSKSKK-DWDKLEAEV : 294
BoSGT1  : RLFGKIIPDKCRYEVLSTKVEIRLAKAEIITWASLEYVKGQALLPKPNVASAV-SQRPVYPSSKPAK-DWDKLEAEV : 282
AtSGT1b : RLFGKIIPEKCRFEVLSTKVEIRLAKAEIITWASLEYGKGQSVLPKPNVSSAL-SQRPVYPSSKPAK-DWDKLEAEV : 285
AtSGT1a : RLFGKIIPEKCRFEVLSTKVEIRLAKAEIITWASLEYGKGQSVLPKPNVSSAL-SQRPVYPSSKPAK-DWDKLEAEV : 285

                                     SGS
CaSGT1  : KKEEKDEKLDGDAALNKFFRDIYKDADEDTRRAMMKSFVESNGTVLSTNWKEVGTKKVEGSPPDGMELKKWEI : 370
NbSGT1  : KKEEKDEKLDGDAALNKFFRDIYKDADEDTRRAMMKSFVESNGTVLSTNWKEVGTKKVEGSPPDGMELKKWEI : 370
HvSGT1  : KKQEKDEKLDGDAALNKFFREIYSDADEDMRRAMMKSFVESNGTVLSTNWKDVGKKTVEGSPPDGMELKKWEY : 373
OsSGT1  : KKEEKEEKLEGDAALNKFFRDIYSDADEDMRRAMMKSFVESNGTVLSTNWKDVGSKKVEGSPPDGMELKKWEY : 367
BoSGT1  : KKQEKDEKLDGDAAMNKFFSDIYQSADEDMRRAMNKSFAESNGTVLSTNWKEVGTKKVESTPPDGMELKKWEY : 355
AtSGT1b : KKQEKDEKLDGDAAMNKFFSDIYSSADEDMRRAMNKSFAESNGTVLSTNWKEVGTKKVESTPPDGMELKKWEY : 358
AtSGT1a : KKQEKDEKLDGDAAMNKFFSDIYSSADEDMRRAMNKSFAESNGTVLSTNWKEVGTKKVESTPPDGMELKKWEY : 358
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B

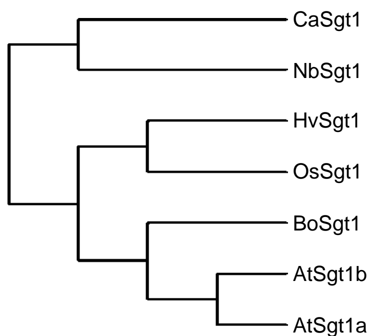


Fig. 1. Alignment of deduced amino acid sequences and phylogenetic analysis of CaSgt1 with Sgt1 sequences from other plant species. (A) Alignment of CaSgt1 and Sgt1 sequences from other plant species was performed using the T-COFFEE program (http://www.ch.embnet.org/software/TCoffee.html; Notredame et al. 2000). Overlines indicate each consensus domain: tetratricopeptide repeat domain (TPR) domain, CHORD and SGT1 (CS) domain, and SGT1-specific (SGS) domain. Black boxes denote the identical amino acids, and gray boxes denote highly conserved amino acids. Dashes indicate gaps in the sequence to allow for the maximal alignment. The peptide sequences are Sgt1 from *Nicotiana benthamiana* (NbSgt1; AA085509), barley (HvSgt1; AAL33610), rice (OsSgt1; AAF18438), cabbage (BoSgt1; CAF06580), and *Arabidopsis* (AtSgt1b; AAL33612, AtSgt1a; AAL33611). (B) The tree was constructed using the PhyloDraw v0.82 software after a multiple sequence comparison following the Clustal method of the CLUSTALW program.

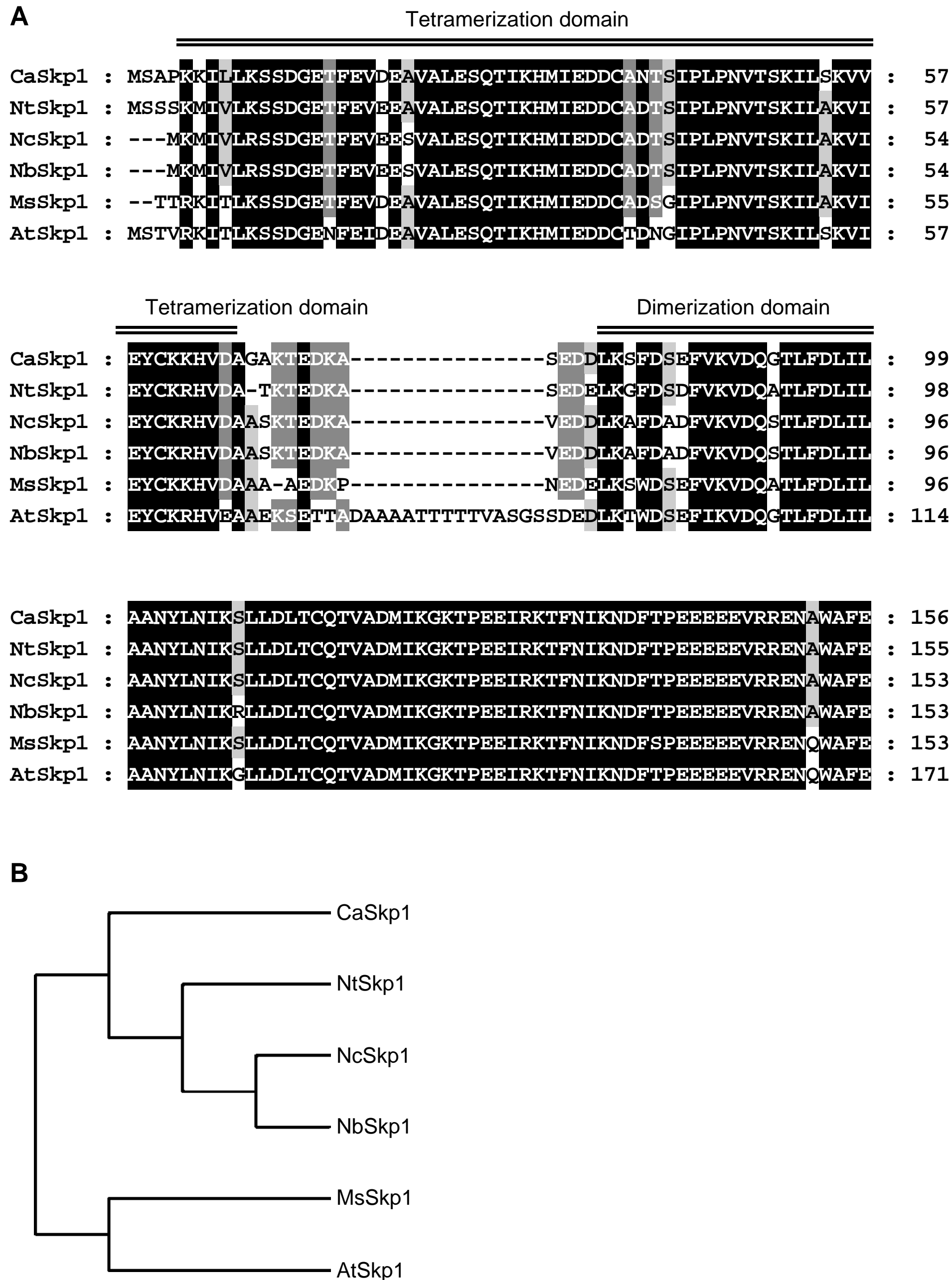


Fig. 2. Alignment of deduced amino acid sequences and phylogenetic analysis of CaSkp1 with Skp1 sequences from other plant species. (A) Alignment of CaSgt1 and Skp1 sequences from other plant species was performed using the T-COFFEE program (Notredame et al. 2000). Overlines indicate each consensus domain: tetramerization domain and dimerization domain. Black boxes denote the identical amino acids, and gray boxes denote highly conserved amino acids. Dashes indicate gaps in the sequence to allow for the maximal alignment. The peptide sequences are Skp1 from tobacco (NtSkp1; AAT99735), *Nicotiana clevelandii* (NcSkp1; AF070967), *Nicotiana benthamiana* (NbSkp1; AA085510), *Medicago sativa* (MsSkp1; AAD34458), and *Arabidopsis* (AtSkp1; AAC63110). (B) The tree was constructed as described in Fig. 1B.

expression levels of *CaSgt1* and *CaSkp1* in the pepper plants (Fig. 4B). Expression of *CaSgt1* and *CaSkp1* was slightly increased by SA (Fig. 4B). PR-1a expression was used as positive control after challenging of pathogen (Fig. 4A). SA was adequately applied to the plants, because *PR-1a* expression was induced from 1.5 h after SA treatment (Fig. 4B). Collectively, *CaSgt1* and *CaSkp1* were upregulated by the inoculation of a non-host pathogen or by application of SA.

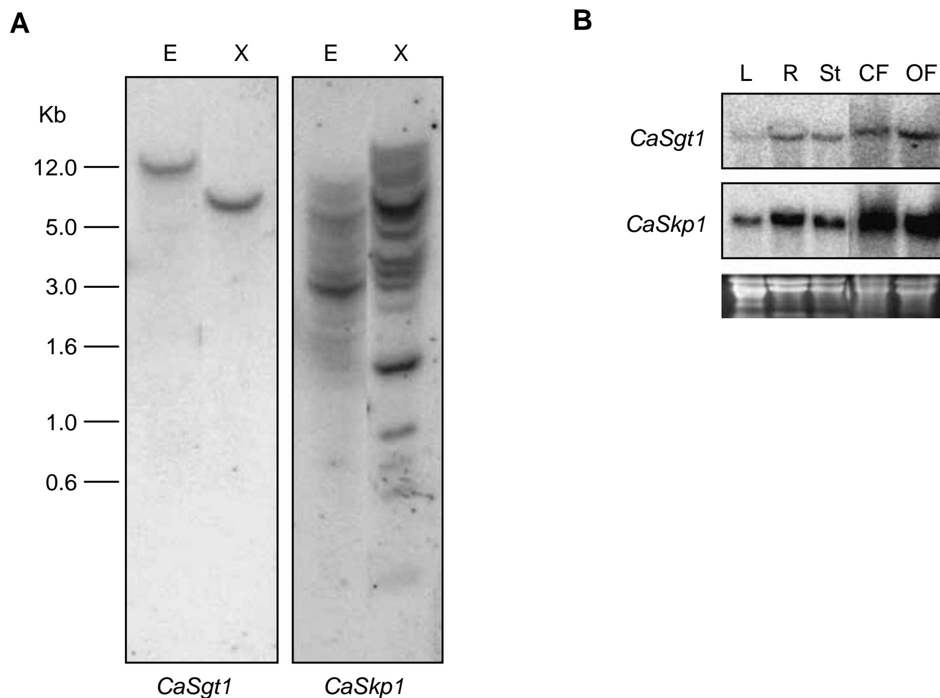


Fig. 3. Genomic DNA gel blot and tissue-specific expression analyses of *CaSgt1* and *CaSkp1*. (A) Genomic DNA (20 μg) purified from chili pepper was digested with *Eco*RI (E) or *Xba*I (X), separated on a 0.8% (w/v) agarose gel, and hybridized with the ^{32}P-labeled probe corresponding to *CaSgt1* or *CaSkp1*. (B) *CaSgt1* and *CaSkp1* RNA levels were monitored in chili pepper leaves (L), roots (R), stems (St), closed flowers (CF), and open flowers (OF) as described in *Materials and methods*.

VIGS of *CaSgt1* and *CaSkp1* in pepper plants

Sgt1 and *Skp1* have been shown to be required for defense responses including *R* gene-mediated (host resistance) and non-host resistance in plants (Liu et al. 2002, Peart et al. 2002). VIGS was employed to determine the role of *CaSgt1* and *CaSkp1* during non-host defense response in pepper (Fig. 5). VIGS of the pepper *CaPDS* or *CaChl H* gene was used as a visual phenotypic control for estimating on silencing frequency and efficiency (Hiriart et al. 2002, Ryu et al. 2004). TRV2 vector containing GFP, a non-endogenous gene, was used as a negative control of VIGS. Around 3–4 weeks after VIGS, the *CaSgt1*- or *CaSkp1*-silenced pepper plants displayed damping-off symptoms similar to those caused by soil-borne pathogens such as *Pythium* spp., *Rhizoctonia solania*, *Sclerotinia sclerotiarum*, or *Fusarium oxysporum* (Fig. 5A). However, we could not isolate any damping-off-causing fungi such as *Pythium* spp., *Rhizoctoinia* spp., *Sclerotinia* spp., and *Fusarium* spp. from the symptomatic tissues (data not shown). The symptoms progressed worse, leading to plant death. Photobleaching and chlorosis phenotypes, caused by silencing of *CaPDS* or *CaChl H*, were obvious with high efficiency (nearly 100% silencing frequency)

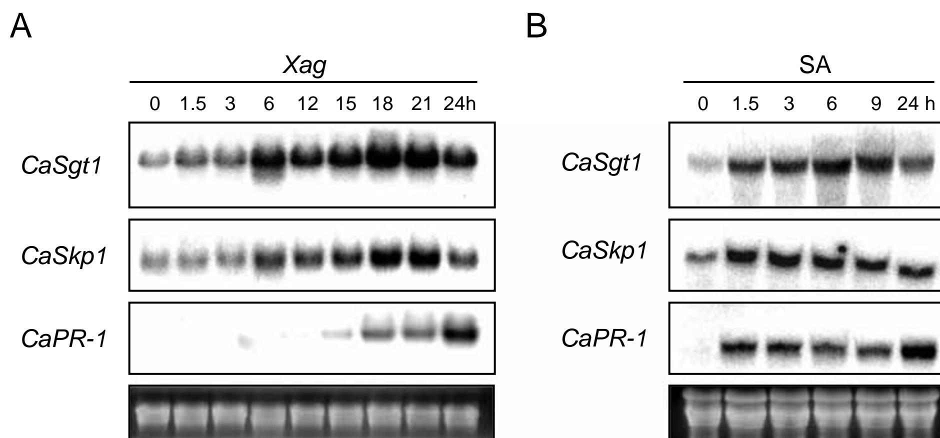


Fig. 4. mRNA expressions of *CaSgt1* and *CaSkp1* in response to incompatible bacterial pathogen inoculation and salicylic acid (SA) treatments. (A) Leaves were syringe-infiltrated with *Xag* suspension cells and harvested at each time interval. (B) Whole plants were sprayed with 5 m*M* of SA solution and prepared for RNA sampling at the indicated time. RNA blot analyses were performed as described in *Materials and methods*.

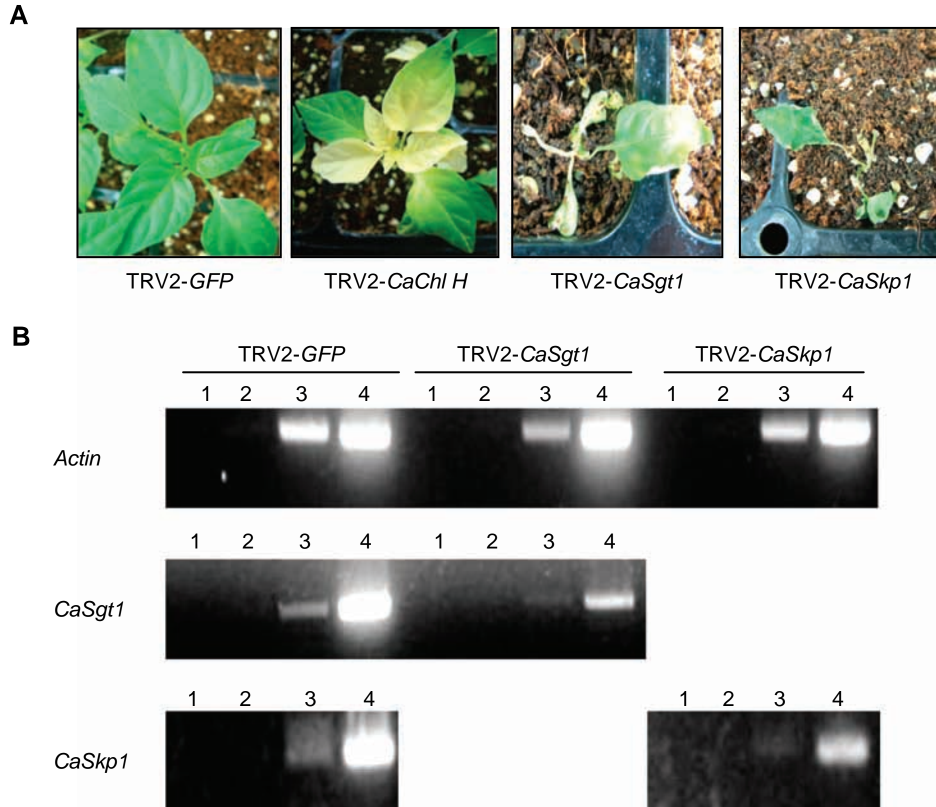


Fig. 5. Silencing phenotypes and reverse transcription-polymerase chain reaction (RT-PCR) analysis of TRV2-*GFP*, TRV2-*CaSgt1*, and TRV2-*CaSkp1* pepper plants. (A) Pepper plants were inoculated with *Agro*-mixtures of TRV1 and TRV2-*GFP*, TRV2-*CaChl H*, TRV2-*CaSgt1*, or TRV2-*CaSkp1*, and the photographs were taken 4 weeks post-inoculation (A). A photograph of each TRV2-*GFP*, TRV2-*CaChl H*, TRV2-*CaSgt1*, or TRV2-*CaSkp1* plant was taken 4 weeks post-inoculation. (B) RT-PCR products of *actin*, *CaSgt1*, or *CaSkp1* were compared by ethidium bromide-stained agarose gels. The first strand cDNA was generated from total RNA isolated from silenced plants using an oligo (dT) primer and reverse transcriptase. This first strand cDNA was used in a PCR reaction using gene-specific primers corresponding to *actin*, *CaSgt1*, or *CaSkp1*. Lanes 14 correspond to products from PCR cycle number 15, 20, 25, and 30, respectively.

suggesting that silencing of the *CaSgt1* or *CaSkp1* gene was responsible for the lethal phenotype. To examine whether *CaSgt1* and *CaSkp1* were effectively down-regulated in the silenced plants, we carried out semi-quantitative RT-PCR analysis (Fig. 5B). *CaSgt1* and *CaSkp1* transcript levels were dramatically reduced in silenced plants compared with TRV2-*GFP* plants (Fig. 5B).

In addition, the growth of survived *CaSgt1*- or *CaSkp1*-silenced pepper plants was significantly retarded when compared with TRV2-*GFP* and TRV2-*CaChl H* plants (Fig. 6A). We examined the survival rate of TRV2-*CaSgt1*, TRV2-*CaSkp1*, TRV2-*CaChl H*, or TRV2-*GFP* plants after 25 or 45 days of VIGS. As shown in Fig. 6B, only 57% of *CaSgt1*-silenced and 13% of *CaSkp1*-silenced plants survived at 25 days after VIGS, whereas 100% of plants treated with TRV2-*CaChl H* or TRV2-*GFP* survived at 25 and 45 days after VIGS. At the day 45 of VIGS, survival rates of *CaSgt1*- and *CaSkp1*-silenced plants were 17 and 13%, respectively.

VIGS of *CaSgt1* and *CaSkp1* in the gnotobiotic condition

To test involvement of phytopathogens on the lethality of *CaSgt1*- and *CaSkp1*-silenced plants, we attempted to silence pepper in the gnotobiotic (in vitro) condition (Fig. 7). It was estimated that silencing was performed very efficiently, because apparent chlorosis phenotype was observed with 95% frequency in the *CaChl H*-silenced plants. In contrast to lethal phenotype of *CaSgt1*- and *CaSkp1*-silenced plants in the soil, *CaSgt1*- and *CaSkp1*-silenced plants grown in vitro did not show any damping-off phenotypes but displayed arrested growth and leaf development compared with those of TRV2-*GFP* plants (Fig. 7). Especially, *CaSkp1*-silenced plants were significantly disturbed in

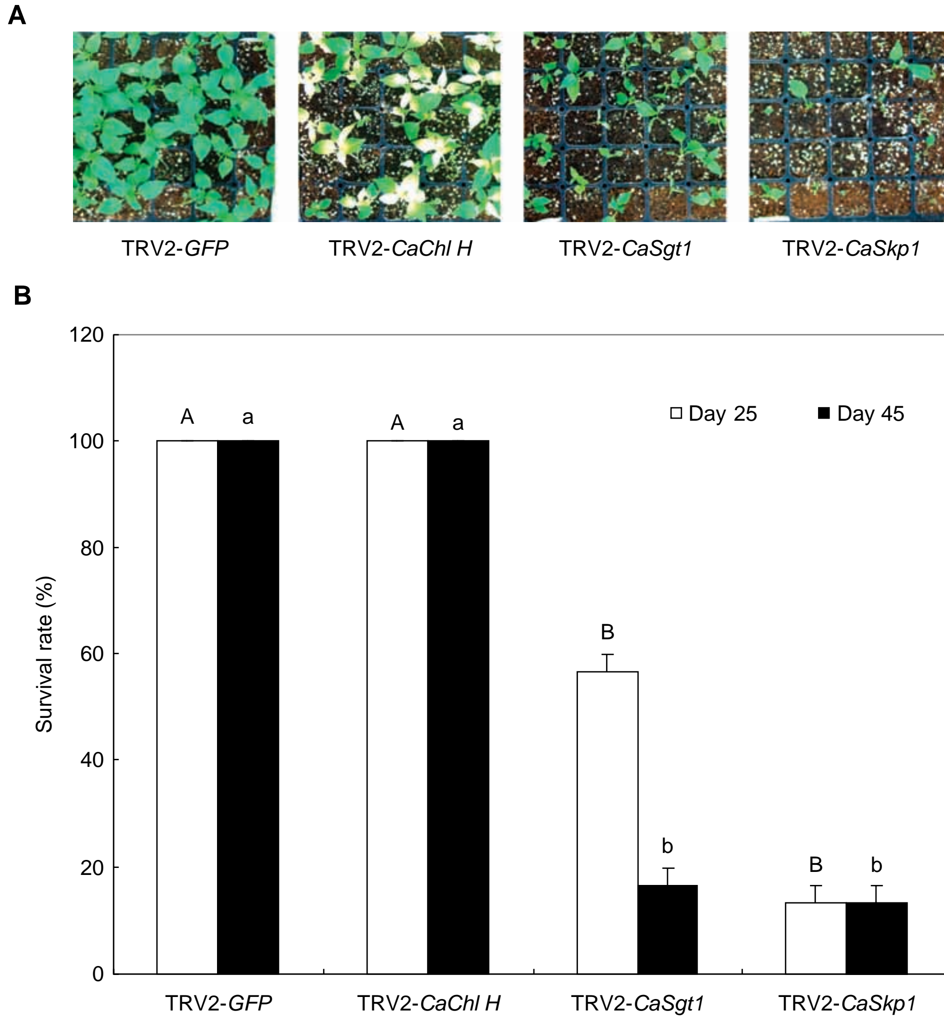


Fig. 6. Silencing effect of *CaSgt1* and *CaSkp1* and survival rate of TRV2-*GFP*, TRV2-*CaChl H*, TRV2-*CaSgt1*, and TRV2-*CaSkp1* pepper plants. (A) Lethal silencing phenotypes of *CaSgt1*- and *CaSkp1*-silenced pepper plants were compared with normal phenotypes of TRV2-*GFP* plants 4 weeks after VIGS. *CaChl H*-silenced phenotypes were included to check the VIGS efficiency. (B) Statistical analysis of survival rate was carried out with TRV2-*GFP*, TRV2-*CaChl H*, TRV2-*CaSgt1*, and TRV2-*CaSkp1* pepper plants 25 and 45 days after VIGS. Data were subjected to analysis of variance using JMP software. Significance of *CaSgt1*- and *CaSkp1*-silenced plants on plant survival rate was determined by the magnitude of the *F*-value at $P = 0.05$. When a significant *F*-value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference at $P = 0.05$.

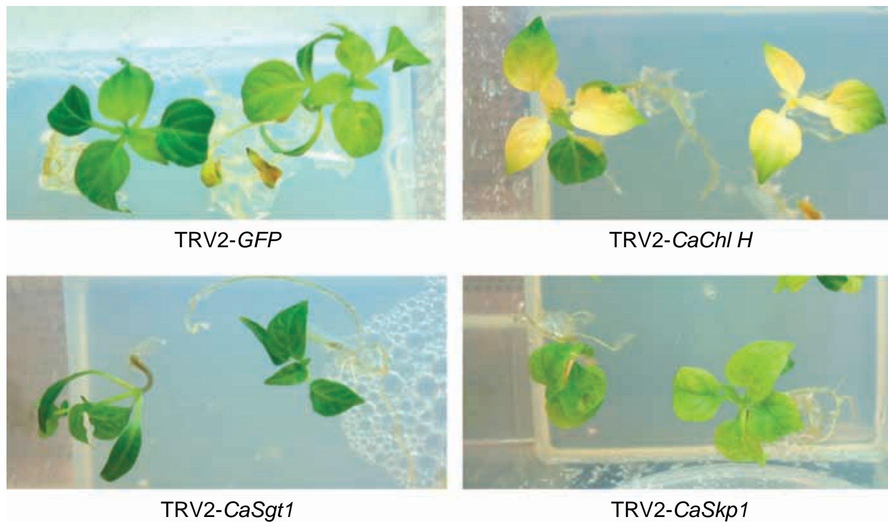
development of the apical meristem when compared with *CaSgt1*-suppressed plants (Fig. 7B). Taken together, lethality of *CaSgt1*- and *CaSkp1*-silenced plants grown in the soil can be caused by the presence of soil micro-organisms such as saprophytic fungi or bacteria, which normally do not attack pepper plant in natural growth condition.

Discussion

Plant *Skp1* and *Sgt1* have been reported as the essential components of SCF-complex playing a central role in development and host and non-host resistance (Austin et al. 2002, Gray et al. 2003, Musket and Park 2003, Peart et al. 2002, Shirasu and Schulze-Lefert 2003). In this study, we have studied the functions of pepper *Sgt1* and *Skp1* using VIGS. Amino acid sequences of CaSgt1 and CaSkp1, orthologues of Sgt1 and Skp1 in pepper, showed high degrees of similarities with Sgt1 and Skp1 in other plant species (Figs 1 and 2).

mRNA expression of *CaSgt1* and *CaSkp1* appeared to be positively affected by the treatments of an incompatible pathogen, *Xag* or a chemical or a chemical inducer, SA in pepper (Fig. 4). These results indicate that *CaSgt1* and *CaSkp1* are transcriptionally regulated during defense responses against pathogen or chemical

A



B

Fig. 7. Silencing phenotypes of in vitro TRV2-*GFP*, TRV2-*CaChl H*, TRV2-*CaSgt1*, and TRV2-*CaSkp1* pepper plants. (A) Pepper seeds were surface sterilized and germinated on sterile growth media (*Materials and methods*). Just after germination, pepper seedlings were vacuum-infiltrated with an *Agro*-mixture of TRV1 and TRV2-*GFP*, TRV2-*CaChl H*, TRV2-*CaSgt1*, or TRV2-*CaSkp1*, and the photographs of each plant were taken 4 weeks post-inoculation. (B) Whole seedlings of each TRV2-*GFP*, TRV2-*CaChl H*, TRV2-*CaSgt1*, or TRV2-*CaSkp1* plant were photographed at 4 weeks post-inoculation. Experiment was conducted two times with 30 replications for each experiment.

inducer in pepper. In contrast, protein level of the *Arabidopsis* SGT1b was not affected by the inoculation of an oomycete fungal pathogen, *P. parasitica* (Austin et al. 2002).

Silencing of *CaSgt1* or *CaSkp1* resulted in lethality in pepper when the pepper was grown in the soil (Figs 5 and 6). However, silencing of *Sgt1* or *Skp1* in *N. benthamiana* did not show any lethal phenotypes but showed minor leaf curling (Peart et al. 2002). In the line of data, *AtSgt1b Arabidopsis* mutant displayed normal phenotype regardless of the alteration in auxin signaling (Gray et al. 2003). Lethality of *CaSgt1*- and *CaSkp1*-silenced plants can be explained by the fact that *CaSgt1*- and *CaSkp1*-mediated protein degradation pathway is essential in integrity of pepper. Survival rates of *CaSgt1*- and *CaSkp1*-suppressed plants were 57 and 13%, respectively, 25 days after VIGS. At 45 days after VIGS, survival rates were as low as 17 and 13% in *CaSgt1*- and *CaSkp1*-silenced pepper plants, respectively. This result indicates that the damping-off symptom in *CaSkp1*-silenced plants was more severe than that in *CaSgt1*-silenced plants (Fig. 6B). Moreover, silencing of *CaSgt1* did not disrupt maintenance of the apical meristem of pepper.

Severe developmental defects of *CaSkp1*-silenced plants can be due to silencing of suppression of multiple members of the gene family homologues to *CaSkp1* (Fig. 3A). In addition, their mRNAs are abundant in all tested plant tissues (Fig. 3B). We hypothesize that other gene family members can be silenced because gene silencing can be initiated by the presence of 21nt perfect match between any target genes in plant during VIGS. These data are agreeable to previous results from *Arabidospis ask1 ask2* double mutants showing developmental delay during embryogenesis and lethality in seedling growth (Liu et al. 2004). *AtSgt1b* mutant did not exhibit any significant phenotype (Gray et al. 2003). *ask1* or *ask2-1* mutant of *Arabidopsis* was morphologically similar to wild-type plants under normal growth conditions (Liu et al. 2004, Zhao et al. 1999, 2001), but *ask1* and *ask2* double mutation affected cell division and elongation resulting in lethality in seedlings (Liu et al. 2004). In the SCF complex, mutation of *Skp1* or *cullin* in *Arabidopsis* caused significant plant developmental defect mainly due to defects in embryogenesis process (Thomann et al. 2005). These previous data and our result presented indicate that SCF complex-mediated protein degradation is crucial for plant development.

However, we still could not exclude the possibility that the lethal phenotype of *CaSgt1*- and *CaSkp1*-silenced plants is caused by lack of basal resistance against non-pathogenic soil-borne fungi or bacteria. We found several saprophytic bacteria that did not show any hypersensitive cell death on the leaves (data not shown). This indicates that the lethal phenotype in *CaSgt1*- and *CaSkp1*-silenced plants may be derived from non-phytopathogenic microorganisms. Thus, we attempted silencing of *CaSgt1* or *CaSkp1* under gnotobiotic condition in which any other biological effect such as soil microorganisms could be excluded (Fig. 7). Surprisingly, no lethal phenotype but dwarfism resulted from the silencing of *CaSgt1* or *CaSkp1* grown under gnotobiotic condition (Fig. 7). Similar results were obtained when we conducted the same experiment in the sterile soil (unpublished data). Due to lack of damping-off symptoms of *CaSgt1*- or *CaSkp1*-silenced pepper plants grown under gnotobiotic condition, we were able to conclude that *CaSgt1* and *CaSkp1* play a critical role in basal resistance and development. Previous results demonstrated that *Sgt1* is implicated in *N* gene-mediated or *Rx*-mediated resistance signaling against TMV or Potato virus X (Liu et al. 2004, Peart et al. 2002). Similar to results of viral resistance, silencing or mutation of *Sgt1* abolished resistance against pathogenic bacteria and fungi (Austin et al. 2002, Muskett and Parker 2003, Peart et al. 2002). To our knowledge, this is the first report that SGT1 and SKP1 are involved in basal resistance other than host or non-host resistance.

Taken together, our results suggest that *CaSgt1* and *CaSkp1* are transcriptionally regulated in pepper inoculated by *Xag*-inducing HR and treated by SA. Pepper silencing of *CaSgt1* or *CaSkp1* caused damping-off and dwarfism phenotypes only when they grew in soil but not under gnotobiotic condition. These results provide new evidence that *CaSgt1* and *CaSkp1* are crucial for proper developmental control and general defense mechanisms in pepper.

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