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Ectopic Expression of *Capsicum*-Specific Cell Wall Protein *Capsicum annuum* Senescence-Delaying 1 (CaSD1) Delays Senescence and Induces Trichome Formation in *Nicotiana benthamiana*

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Secreted proteins are known to have multiple roles in plant development, metabolism, and stress response. In a previous study to understand the roles of secreted proteins, *Capsicum annuum* secreted proteins (CaS) were isolated by yeast secretion trap. Among the secreted proteins, we further characterized *Capsicum annuum senescence-delaying 1* (*CaSD1*), a gene encoding a novel secreted protein that is present only in the genus *Capsicum*. The deduced CaSD1 contains multiple repeats of the amino acid sequence KPPIHNHKPTDYDRS. Interestingly, the number of repeats varied among cultivars and species in the *Capsicum* genus. *CaSD1* is constitutively expressed in roots, and *Agrobacterium*-mediated transient overexpression of *CaSD1* in *Nicotiana benthamiana* leaves resulted in delayed senescence with a dramatically increased number of trichomes and enlarged epidermal cells. Furthermore, senescence- and cell division-related genes were differentially regulated by *CaSD1*-overexpressing plants. These observations imply that the pepper-specific cell wall protein CaSD1 plays roles in plant growth and development by regulating cell division and differentiation.

INTRODUCTION

Plants have developed unique structures and mechanisms to control growth and development or to overcome biotic or abiotic stresses. One of the unique features of plant cells is the presence of a cell wall, which is a dynamic structure and varies according to developmental stage and environmental conditions. The plant cell wall is primarily composed of polysaccharides, such as cellulose, hemicelluloses, and pectin, which together make up to 90% of the dry weight of the plant (Cosgrove, 2005; Lerouxel et al., 2006). However, the plant cell wall also contains proteins that often play crucial roles in maintaining the structure and function of the cell wall (Agrawal et al., 2010; Showalter, 1993).

The group of proteins exported through the secretory pathway and localized in the cell wall or extracellular space is called the secretome. Members of plant secretomes have various roles in plant cell expansion, differentiation, cell-to-cell communication, and defense against pathogens (Humphrey et al., 2007; Lee et al., 2004). Structural proteins, such as hydroxyproline-rich glycoproteins, proline-rich proteins, glycine-rich proteins, and arabinogalactan proteins, act on polysaccharides and are primarily involved in plant growth and development. For example, LRR/extensin-1 (LRX1) is a hydroxyproline-rich glycoprotein that is linked to root hair morphogenesis and elongation (Baumberger et al., 2001). In addition, expansins, xyloglucan endotransglucosylase/hydrolases, and endo-(1,4)-β-D-glucanases are wall-associated proteins that play roles in cell extensibility and differentiation. In particular, expansins have been extensively studied and are known to control leaf initiation and shape (Cho and Cosgrove, 2000; Fleming et al., 1997; Pien et al., 2001). Other secreted proteins are known to be involved in cell wall strengthening and signal transmission in the defense against pathogens (Hematy et al., 2009; Huckelhoven, 2007; Oh et al., 2005; Yeom et al., 2012). However, the roles of a large portion of the plant secretome remain to be elucidated.

Senescence is the final stage of growth and development for all living organisms. In plants, the degradation of chlorophyll, proteins, and nucleic acids is the main symptom in senescing leaves (Lim et al., 2007; Quirino et al., 2000). To date, genetic and molecular approaches have been performed to elucidate the senescence mechanism. Using T-DNA insertion or chemical-induced mutagenesis, a number of mutants showing delayed senescence were identified. For example, mutation of *ORE9,* which contains an F-box motif and 18 leucine-rich repeats, delayed overall senescence symptoms in *Arabidopsis* (Woo et al., 2001). A mutant produced by T-DNA insertion into the *AtATE* gene encoding arginyl-tRNA:protein arginyltransferase also delayed age-dependent and dark-induced senescence (Yoshida et al., 2002). In a molecular approach, DNA microarray analysis was used to profile the expression of genes in senescing leaves (Buchanan-Wollaston et al., 2005; van der

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Graaff et al., 2006). Over 800 genes were upregulated more than 3-fold during senescence and were referred to as senescence-associated genes (SAGs).

In previous research, 101 unique proteins in the secretome that may be related to plant cell defense and development were isolated from peppers using the yeast secretion trap (YST) system (Yeom et al., 2011). One of the members of the secretome, Capsicum annuum senescence-delaying 1 (CaSD1), has a novel feature and was selected for in-depth study. Here, we functionally characterize the *CaSD1* gene, which is present only in the genus *Capsicum* and has a unique repeat region with variable length among *Capsicum* species. Gain-of-function studies using ectopic transient overexpression in *Nicotiana benthamiana* revealed that *CaSD1* plays roles in the delay of senescence and trichome formation.

MATERIALS AND METHODS

Plant materials

Pepper plants were used for cloning, gene expression, and loss-of-function analysis. *Capsicum annuum* L. cv. 'Bukang' was used to clone the full-length *CaSD1* cDNA and to evaluate organ-specific expression. Four cultivars or germplasms of each of five different *Capsicum* species were randomly selected from RDA-Genebank information center, and genomic DNAs of each germplasm were also provided for polymerase chain reaction (PCR) amplification of the repeat region (Supplementary Table 1). *Nicotiana benthamiana* plants were used for subcellular localization and transient overexpression (TOE) of *CaSD1*. All plants were grown in a walk-in chamber maintained at 22-25°C and subjected to a 16-h photoperiod for 4-6 weeks.

Cloning and DNA sequence analysis

The full-length sequence of *CaSD1* was isolated from a bacterial artificial chromosome (BAC) 529L23 sequence of *C. annuum* 'CM334' (Yoo et al., 2003). Both the gene-coding region and the 3′ untranslated region were predicted using the FGENESH program (http://linux1.softberry.com/berry.phtml). Gene-specific primers were designed for PCR amplification of *CaSD1* based on the BAC sequence (Supplementary Table 2). PCR was performed to amplify *CaSD1* using 'Bukang' cDNA, which was cloned into the pJET vector using the pJET™ PCR Cloning Kit (Fermentas Canada Inc., Canada). The *CaSD1* sequence was confirmed by DNA sequencing using the ABI 3730 XL (Applied Biosystems Inc, USA) at the National Instrumentation Center for Environmental Management (NICEM, Korea). The sequence analyses were performed using the ExPasy translation tool (http://us.expasy.org/tools/dna.html), National Center for Biotechnology Information Blast search (Altschul et al., 1997), SignalP signal peptide prediction (http://www.cbs.dtu.dk/services/SignalP/), and the TCoffee multi-alignment tool (http://www.ebi.ac.uk/Tools/msa/tcoffee/).

RNA extraction and gene expression analysis

For gene expression analysis, total RNA was extracted from frozen pepper and *N. benthamiana* leaves using the TRIzol reagent (Molecular Research Center, USA). First-strand cDNA was synthesized from 5 μg total RNA using Oligo (dT) and SuperScript II reverse transcriptase (Invitrogen, USA). Quantitative or semi-quantitative RT-PCR was performed using gene-specific primers designed using Primer3 software (http://frodo.wi.mit.edu/primer3, Supplementary Table 2). For quantitative gene expression analysis, quantitative reverse transcription (RT)-PCR was performed with a Rotor-Gene 2000 (Qiagen, USA) using Syto 9 (Invitrogen). Fluorescence was measured at 72°C for 60 cycles, and expression levels of each sample were normalized to those of actin. Semi-quantitative RT-PCR was carried out using a MyCycler (Biorad, USA), and PCR products were electrophoresed in 1% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light.

Subcellular localization of CaSD1-soluble modified form of green fluorescent protein (smGFP) or smGFP

Both pMBP1:CaSD1-smGFP (pBI121-Modified) and pMBP1:smGFP were constructed using the ligation-independent cloning method (Oh et al., 2010) and transformed into *Agrobacterium tumefaciens* strain GV2260. The transformed cells were cultured overnight in YEP medium at 30°C with shaking (200 rpm), centrifuged, and resuspended in infiltration buffer containing 10 mM MES (pH 5.5)/10 mM $MgCl_2$. The cell suspension ($O.D_{600}$ = 0.5) was incubated with 200 μM acetosyringone for 3 h at room temperature. The cell suspension was then pressure-infiltrated into the backsides of *N. benthamiana* leaves using a needleless syringe. One day after infiltration, the abaxial epidermal cell layer was peeled off, and GFP fluorescence was observed by confocal laser scanning microscopy (LSM510, Carl Zeiss, Germany).

Transient overexpression of *CaSD1* in *N. benthamiana*

The *CaSD1* open reading frame (ORF) containing the 3′ untranslated region was amplified with primers containing *Bam*HI and *Sac*I recognition sequences at the 5′ and 3′ ends, respectively. The PCR product was cloned into a pMBP1 vector treated with *Bam*HI and *Sac*I (Suh et al., 1998). The cloning product was transformed into *Agrobacterium* strain GV2260. *Agrobacterium* transformed with pMBP1:CaSD1 and the pMBP1 control were cultured overnight in YEP medium at 30°C with shaking (200 rpm). The cells were centrifuged and resuspended in infiltration buffer containing 10 mM MES (pH 5.5)/10 mM $MgCl_2$. The resuspended bacterial suspension ($O.D_{600}$ = 0.5) was incubated with 200 μM acetosyringone for 3 h at room temperature. After incubation, the bacterial suspension was pressure-infiltrated into the backsides of *N. benthamiana* leaves using a needleless syringe. The infiltrated leaves were used for RNA extraction and microscopic observation at the indicated time points.

Light and field emission scanning electronic microscopy

The surfaces of *N. benthamiana* leaves transiently overexpressing *CaSD1* were monitored and photographed 10 days after TOE by light microscopy (Siwon Optical Technology, Korea) to observe the trichomes. For analysis of trichome density and cell size, field emission scanning electron microscopy (SUPRA 55VP, Carl Zeiss, Wetzlar, Germany, NICEM at Seoul National University) was performed 2, 7, and 10 days after TOE without processing samples in low-vacuum mode. The samples were monitored with a 15-kV accelerating voltage, and pictures were digitally captured.

RESULTS

CaSD1 is a novel protein present only in species of the *Capsicum* genus

To identify secreted proteins involved in growth, development, and plant-pathogen interactions, *C. annuum* secretome (CaS) genes were isolated from pepper roots following *P. capsici* infection using YST (Yeom et al., 2011). Among them, *CaS113* was selected for further study. Using the partial sequence of *CaS113*, a putative full-length cDNA was identified from *C. annuum* 'CM334' BAC sequences (Yoo et al., 2003). The pre-

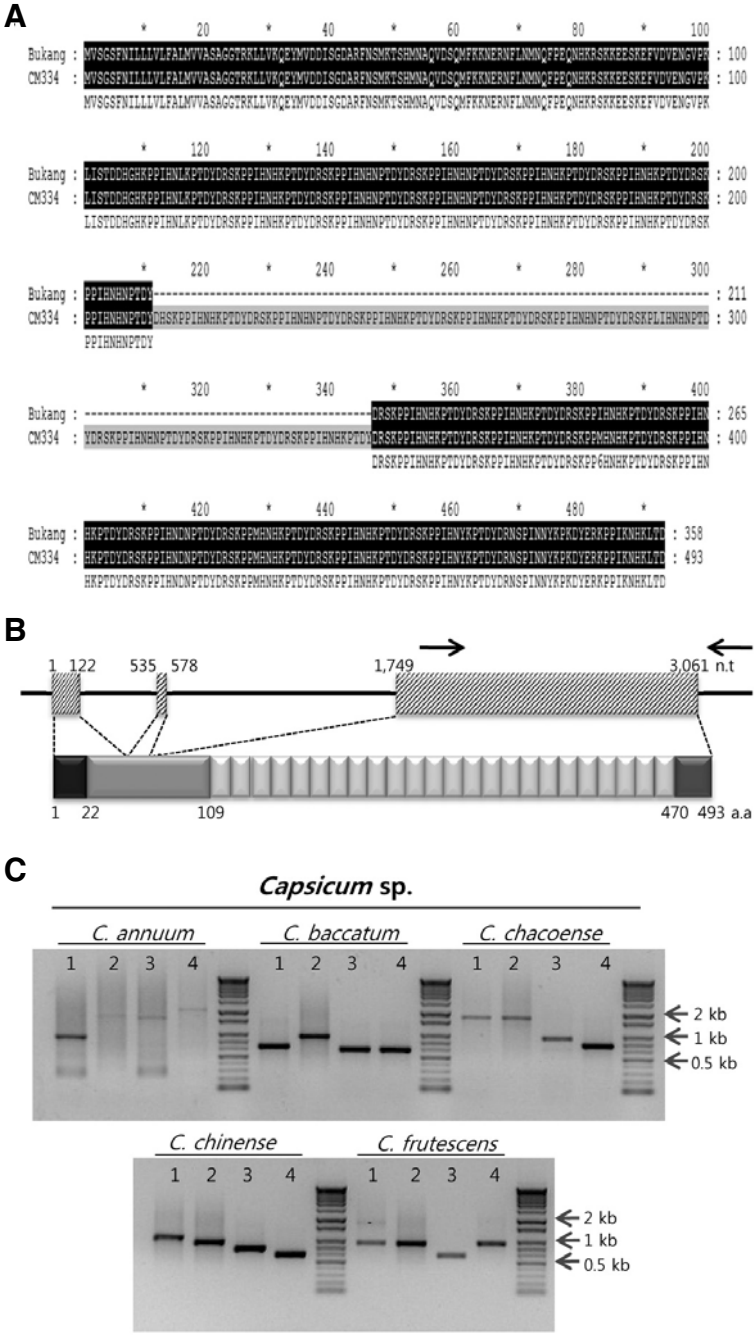

Fig. 1. Sequence analysis of CaSD1. (A) Sequence alignment of CaSD1 in *C. annuum* L. cv. 'Bukang' and 'CM334.' Black box denotes sequence match between 'Bukang' and 'CM334.' (B) Schematic structure of *CaSD1* genomic DNA region and amino acid sequence of CaSD1. The black line indicates the 5′ and 3′ untranslated regions and introns. The box with diagonal line indicates exons of *CaSD1*. Black arrow designates primers for PCR amplification of the repeat region. Black box: signal peptide, light gray: non-repeat region, white: repeat region, dark gray: another non-repeat region. n.t, nucleotide. a.a, amino acid. (C) Four genomic DNA samples from each *Capsicum* species were used for PCR amplification. PCR products were electrophoresed in 1% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light.

dicted gene encodes a 493-amino acid protein with a molecular mass of 58 kDa composed of a signal peptide (SP), N-terminal non-repeat region, multiple repeat region, and C-terminal non-repeat region (Fig. 1B). The gene was thereafter referred to as *C. annuum senescence-delaying gene 1* (*CaSD1*). In the multiple repeat regions, each repeat unit was 15 amino acids in length (KPPIHNHKPTDYDRS), often with one or two amino acids differing among the repeat units. *CaSD1* from the pepper cultivar 'Bukang' was cloned, and the number of repeats was fewer than that of the 'CM334' cultivar. Other regions, except for the repeat number, were identical (Fig. 1A). Further investigation using PCR amplification of genomic DNA and sequence analysis revealed that the number of repeat units was variable among species and cultivars in the *Capsicum* genus (Fig. 1C). We cloned the full-length *CaSD1* homologs in two *Capsicum chinense* cultivars, 'Jolokia' and 'Numex Suave Orange,' in which the SPs and non-repeat regions of the deduced proteins showed 92% sequence similarity with CaSD1. However, these homologs had 9 and 12 repeat units, respectively (Supplementary Fig. 1). Additionally, two genes predicted from the BAC sequence around *CaSD1* had conserved SPs and non-repeat regions but no repeat regions (Fig. 2). Comparative genomic analysis revealed that genes lacking a repeat region exist in the Solanacea family but that *CaSD1* homologs with a repeat region are only present in the *Capsicum* genus (Fig. 2). Additional searches in the GenBank database and Interpro-

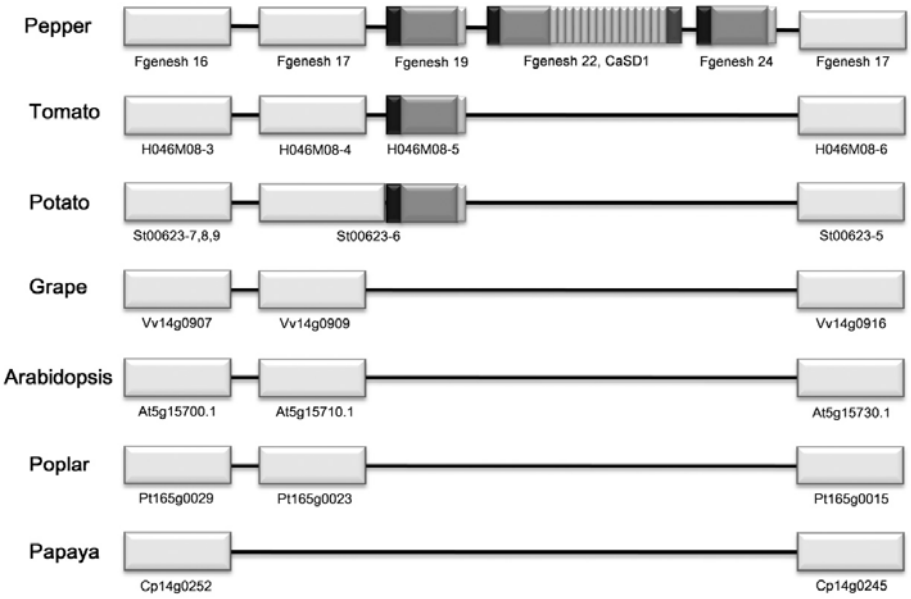

Fig. 2. Comparative genomic analysis of syntenic segments of known plant genomes. Syntenic segments including *CaSD1* were compared to those of other known plant genomes. The ORFs of pepper BAC 529L23 were predicted using the FGENESH program, and each ORF was presented according to the order of FGENESH number. White boxes represent other proteins in syntenic regions. Proteins with sequence similarity were placed at the same vertical position.

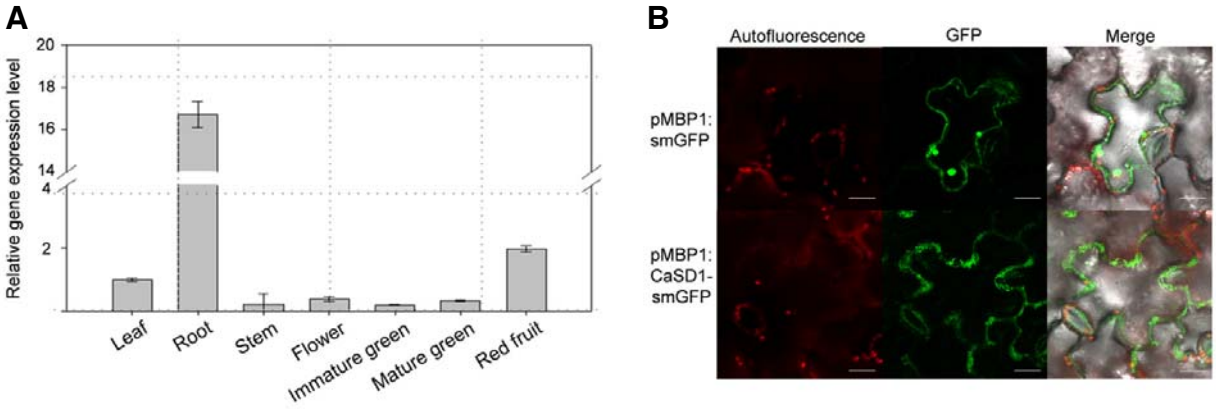

Fig. 3. Organ-specific expression of *CaSD1* and localization of CaSD1 in the cell wall. (A) Organ-specific expression of *CaSD1* in pepper plants. Real-time PCR was performed with *CaSD1*-specific primers, and the values were normalized to *CaActin* and calculated relative to expression levels in leaves. Bars represent standard deviation of four replicates. (B) *Agrobacterium* containing pMBP1: smGFP or pMBP1: CaSD1-smGFP was infiltrated into *N. bentha-miana* leaves. Pictures were taken 1 day after infiltration by confocal laser scanning microscopy. White bars, 20 μm.

Scan could not retrieve any significantly matched genes or domains except for the signal peptide (data not shown). These results suggest that *CaSD1* is a novel *Capsicum*-specific gene.

Organ-specific expression and subcellular localization of CaSD1

To investigate the organ-specific expression of *CaSD1*, real-time RT-PCR was performed using RNA samples from different pepper organs. *CaSD1* was constitutively expressed in roots, where the transcript levels were 16.7-fold higher than those of leaves (Fig. 3A). To determine the cellular localization of CaSD1, targeting experiments in plants were performed by TOE using *Agrobacterium* carrying *CaSD1* fused to a *smGFP* as a fluorescent marker (Davis and Vierstra, 1998). *Agrobacterium* suspensions carrying pMBP1:CaSD1-smGFP or pMBP1:smGFP were pressure-infiltrated into *N. benthamiana* leaves. One day after TOE, abaxial epidermal cell layers of the leaves were observed by confocal laser scanning microscopy. GFP fluorescence of pMBP1:CaSD1-smGFP was observed in the cell wall and extracellular matrix, while pMBP1:smGFP was localized throughout the cells, including the nucleus (Fig. 3B). These observations indicate that CaSD1 is secreted and localized to the extracellular space, including the plasma membrane or cell wall, in *N. benthamiana* plants.

Transient overexpression of *CaSD1* causes delay of senescence

To investigate the function of *CaSD1* in plants, we adopted loss-of-function and gain-of-function approaches. Virus-induced gene silencing (VIGS) in pepper was conducted for loss-of-function study. However, *CaSD1*-silenced pepper plants showed no significant difference compared to those of control plants. To investigate gain-of-function of *CaSD1* in plants, *Agrobacterium*-mediated TOE of pMBP1 or pMBP1:CaSD1 was performed in *N. benthamiana* leaves. Significant differences between *CaSD1*- and control vector-expressing plants were observed about 5-7

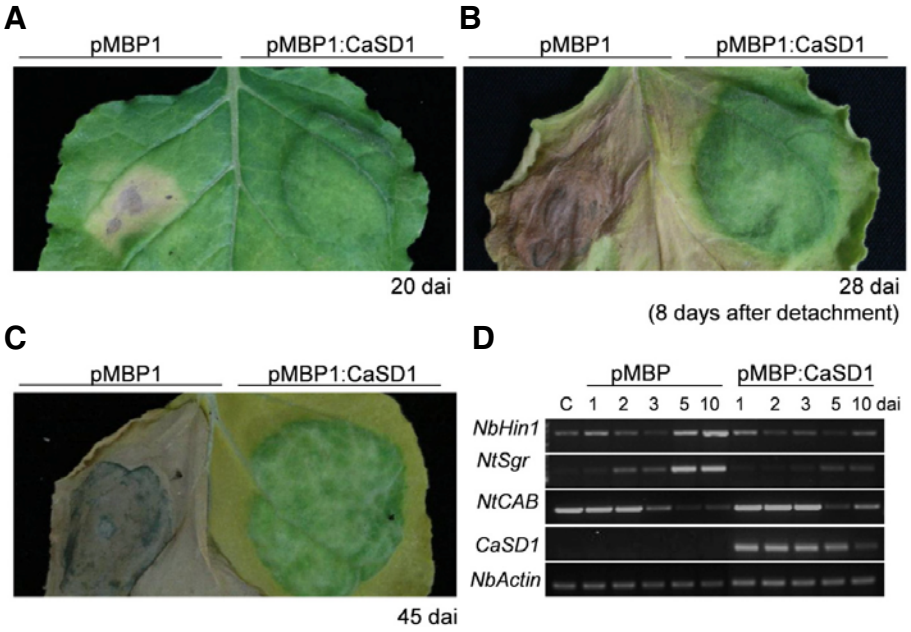

Fig. 4. Transient overexpression of *CaSD1* delays senescence and regulates expression of senescence-related genes. *Agrobacterium*-mediated overexpression of pMBP1 or pMBP1:CaSD1 in *N. benthamiana* leaves. Photographs were taken of the same leaf (A) 20 days after inoculation (dai) and (B) 8 days after detachment. (C) Photograph was taken 45 dai. Similar results were obtained more than three times of independent experiments. (D) Total RNA was isolated from leaves at the indicated time points after TOE, and semi-quantitative RT-PCR was performed using gene-specific primer sets. *NbActin* gene was the control. C, control plants. *NbHin1, N. benthamiana harpin-induced 1; NtSgr, N. tabaccum Staygreen; NtCAB, N. tabaccum chlorophyll a/b binding protein gene.*

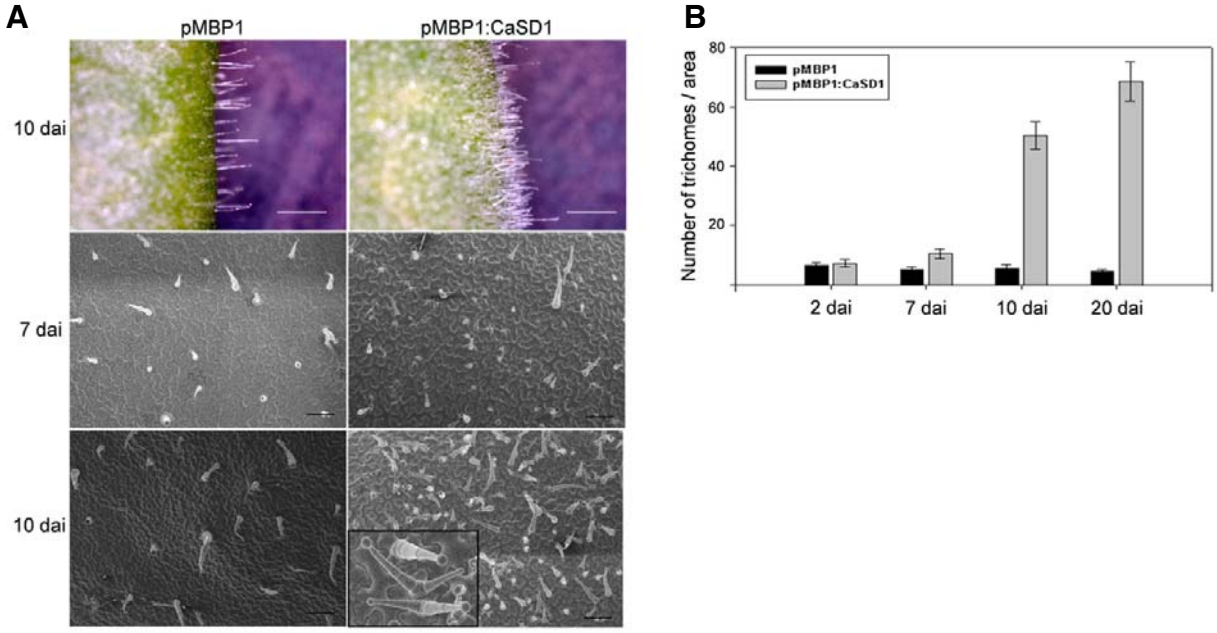

Fig. 5. Increase in trichome density in *CaSD1*-overexpressing *N. benthamiana*. (A) Surfaces of pMBP1 vector control (left) or *CaSD1*-overexpressing (right) *N. benthamiana* leaves were observed at the indicated time points after TOE by light microscopy or field-emission scanning electron microscopy. White bars, 1 mm. The bottom right hand black box is magnified for clusters of adjacent trichomes. Black bars, 200 μm. (B) pMBP1 vector control (Black) or *CaSD1*-overexpressing leaves (grey) were comparted into 2 mm × 1.4 mm regions, and trichomes in the fixed regions were counted at the indicated time points after TOE. Bars represent standard deviation ($n = 5$).

days after inoculation (dai). *CaSD1*-overexpressing regions showed delayed senescence, while senescence was accelerated in empty vector pMBP1-expressing regions (Fig. 4A). Furthermore, the senescence-delayed region remained as a green island even after the leaf was detached (Fig. 4B). The *CaSD1*-expressing region of intact plants remained green until the pMBP1-control region withered away (Fig. 4C). To better understand the effects of *CaSD1* on senescence, gene profiling experiments were conducted. *Sgr* (*Staygreen*) is a typical senescence-associated (SAG) gene which regulates chlorophyll degradation and *sgr* mutant rice show senescence-delayed phenotype (Park et al., 2007). Also, *Harpin-induced 1 (Hin1*) is a marker gene of senescence as well as cell death in plants (Pontier et al., 1999). Expression levels of *Hin1* and *Sgr* were downregulated in *CaSD1*-overexpressing plants compared to control plants (Fig. 4D). On the other hand, the transcript levels

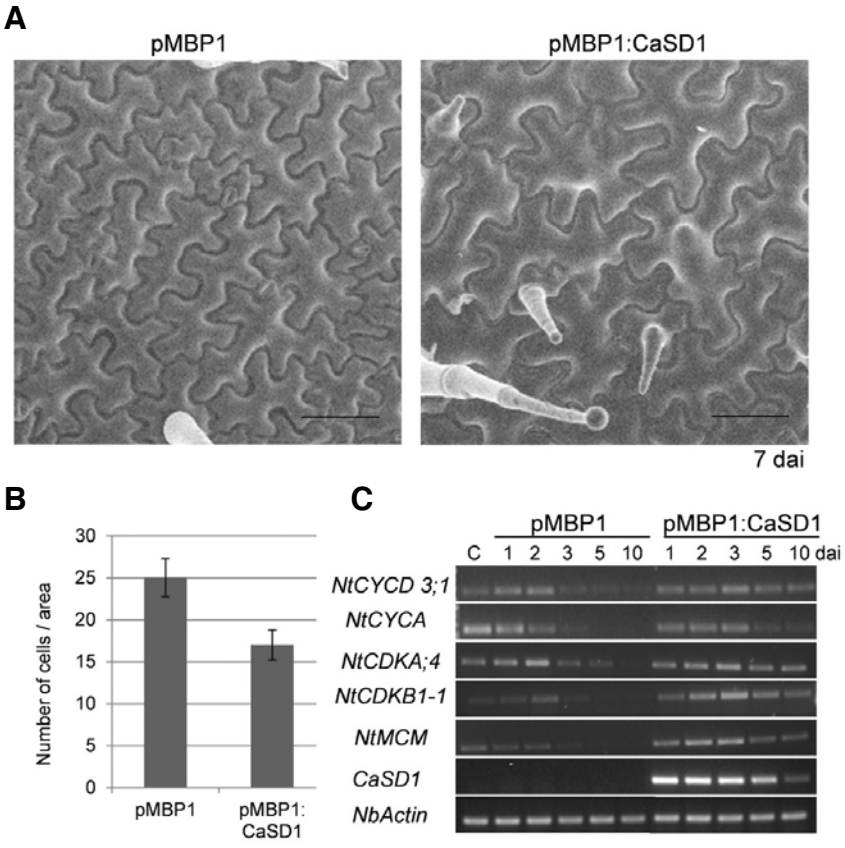

Fig. 6. Cell size and expression levels of cell division-related genes in *CaSD1*-overexpressing *N. benthamiana*. (A) Surfaces of pMBP1 vector control or *CaSD1*-overexpressing leaves were observed using field-emission scanning electron microscopy 7 days after inoculation. Black bars, 100 μm. (B) pMBP1 vector control or *CaSD1*- overexpressing leaves were comparted into 500 μm × 500 μm regions, and cells in the fixed regions were counted. Bars represent standard deviation (n = 40). (C) Expression patterns of cell division-related genes in *CaSD1*-overexpressing plants. *NtCYCD, N. tabaccum D-type cyclin; NtCYCA, N. tabaccum cyclin A; NtCDKA, N. tabaccum cyclin-dependent kinase A; NtCDKB, N. tabaccum cyclin-dependent kinase B; NtMCM, N. tabaccum mini chromosome maintenance.*

of *CAB* (*Chlorophyll a/b binding protein gene*), which is related to the progression of photosynthesis and a representative control for leaf senescence (Lohman et al., 1994; Weaver et al., 1998), were higher in *CaSD1*-overexpressing plants than in vector control-expressing plants during senescence. These reults indicate that overexpression of *CaSD1* suppressed senescence by affecting the expression levels of senescence-related genes and chlorophyll-associated genes.

Transient overexpression of *CaSD1* accelerates trichome formation with enlarged cell size

Another peculiar phenotype of *CaSD1*-overexpressing plants is the induction of trichome formation (Fig. 5). Trichomes are specialized epidermal cells that are regularly distributed on plant leaves (Hulskamp et al., 1994). Similar to the kinetics of *CaSD1*-induced senescence alterations, a significant difference was observed about 7 days after TOE. Trichome initiation sites (TIS) were significantly increased, and approximately 10-fold more trichomes were observed 10 days after *CaSD1* overexpression in *N. benthamiana* leaves compared to those of control plants. In contrast to a single trichome emerging from one cell on control leaves, *CaSD1*-overexpressing leaves showed more than one trichome per TIS, forming clusters of adjacent trichomes (Fig. 5A).

Furthermore, the *CaSD1*-overexpressing region bulged out, so we speculated that *CaSD1* overexpression also affects cell size or number. The *CaSD1*-overexpressing *N. benthamiana* leaves were monitored by field-emission scanning electron microscopy. The size of epidermal cells in CaSD1-overexpressing plants was significantly increased compared to the size of those in control plants before trichome cells developed (Fig. 6A). We counted the number of cells in fixed areas and found that there were 30% fewer cells in *CaSD1*-overexpressing regions than in control regions (Fig. 6B). Trichome development and cell size are related to cell cycle regulation, including endoreduplication (Dewitte et al., 2007; Schnittger et al., 2002; Sugimoto-Shirasu and Roberts, 2003). To elucidate the molecular mechanism of cell cycle regulation in *CaSD1*-overexpressing plants, semi-quantitative RT-PCR of cell cycle-related genes, such as cyclins, cyclin-dependent kinases, and mini chromosome maintenance (MCM) members, was conducted. The expression levels of those genes were down-regulated during senescence in pMBP1-control plants, but these genes were still expressed in *CaSD1*-overexpressing plants (Fig. 6C). These results suggest that *CaSD1* may play roles in cell fate determination and regulation of cell division.

DISCUSSION

In previous work, we performed YST to isolate secreted proteins related to interactions between *C. annuum* CM334 and *P. capsici* (Yeom et al., 2011). Using YST, we isolated a novel gene, *CaSD1*. We further investigated the biological function of the *CaSD1* gene by expression analysis and TOE in *N. benthamiana*. The predicted CaSD1 protein has repeat sequences that are variable among *Capsicum* species and cultivars (Figs. 1A and 1C). Indeed, two CaSD1 homologs in *C. chinenese* have shorter repeat regions than *C. annuum* 'CM334' or 'Bukang.' In addition, genes that had a partially conserved sequence with *CaSD1* were predicted around the *CaSD1* genomic region and in the syntenic segments of tomato and potato but lack repeat regions (Fig. 2). In peppers, duplication of

the entire genome did not occur during evolution (Wu et al., 2009). Therefore, *CaSD1* may be formed by partial duplication and may thus acquire additional functions.

The other interesting feature of CaSD1 is that there are no known conserved domains or motifs except the signal peptide. However, the repeat unit of CaSD1 shares some amino acid homology with root growth factor 1 (RGF1) from *Arabidopsis*. RGF1 recovers root meristem activity in short-root mutants as a small signaling peptide that is cleaved from pre-RGF (Matsuzaki et al., 2010). The pre-RGF sequence has no similarity with CaSD1, but the small peptide sequence is similar to a repeat unit of CaSD1. Accordingly, it is possible that CaSD1 has a function during growth and development like RGF1, and the repeat region may play a crucial role in these processes. Indeed, TOE of the *CaSD1⊿repeat* construct lacking the repeat and C-terminal non-repeat regions did not result in the phenotype observed under conditions of *CaSD1* overexpression (data not shown).

In addition, we found that *CaSD1* is related to leaf senescence using gain-of-function study. Senescence is controlled by a genetic program, and various internal or external factors are involved (Buchanan-Wollaston et al., 2003; Lim et al., 2007). To date, genes related to senescence have been isolated using mutagenesis or transcriptomic analyses (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006). For example, certain proteases, dehydrogenases, proteins acting on chlorophyll, etc. are known to be involved in senescence (Buchanan-Wollaston et al., 2005; Lara et al., 2004; Zapata et al., 2005; Zhou et al., 2011). In this study, *CaSD1*-overexpressing regions maintained greenness, while senescence and cell death were accelerated by *Agrobacterium* in control regions (Fig. 4). Furthermore, the fact that expression of senescence-associated genes was altered in *CaSD1*-overexpressing plants lends further support to a role for *CaSD1* in regulating critical steps of leaf senescence. Since the greenness of *CaSD1*-overexpressing regions remained after the leaf was detached, *CaSD1* may be involved in the maintenance or production of chlorophyll.

We also observed that *CaSD1* overexpression accelerated trichome formation. Trichomes are specialized epidermal cells that respond to positional cues (Hulskamp et al., 1994). However, *CaSD1*-overexpressing plants produced a large number of trichomes, some even in clusters (Fig. 5). From these data, we hypothesized that *CaSD1* might alter cell fate. In *Arabidopsis*, research on trichome formation is well known (Hulskamp, 2004; Ishida et al., 2008). A cell that is destined to become a trichome enlarges, grows, and forms branches. Although *N. benthamiana* trichomes have multicellular stalks but no branches, a similar mechanism is likely to operate at the initiation of trichome formation. As shown in Figs. 5 and 6, at the beginning of trichome formation, epidermal cells of *CaSD1*-overexpressing plants are enlarged, and this enlargement may indicate early trichome formation. This enlargement of epidermal cells caused *CaSD1*-overexpressing regions to bulge.

Furthermore, *sim* (SIAMESE) mutants and the misexpression of CYCD3;1 in *Arabidopsis* lead to trichome phenotypes similar to those of *CaSD1*-overexpressing plants, showing multicellular trichome clusters (Schnittger et al., 2002; Walker et al., 2000). However, TIS were not increased in *sim* mutants. SIAMESE controls endoreduplication, and CYCD3;1 is related to both DNA replication and cell division. The expression of CYCD3;1 remains stable in *CaSD1*-overexpressing plants as do the expression levels of other genes related to cell division. These results indicate that *CaSD1* may be involved in cell division and endoreduplication. The expression level stability of these genes in *CaSD1*-overexpressing plants may also contribute to the delay in senescence by maintaining cell division and viability. However, in the loss-of-function approach, we observed no significant difference between *GFP*- and *CaSD1*-silenced leaves because the transcript levels of *CaSD1* in leaves was low in normal condition.

Even though we could not show the mechanistic relationship between *CaSD1* and senescence, we clearly show functions of *CaSD1* in senescence and trichome formation. Overexpression of CaSD1, a novel secreted protein, delays senescence and induces trichome formation, possibly through changes in the expression of a subset of genes common to both senescence and cell division in *N. benthamiana*. We suggest that CaSD1, as a *Capsicum*-specific protein, has roles in growth and development by regulating cell division and differentiation.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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