A novel BSD domain-containing transcription factor controlling vegetative growth, leaf senescence and fruit quality in tomato

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Abstract

BSD (mammalian BTF2-like transcription factors, Drosophila synapse-associated proteins and yeast DOS2-like proteins) is a domain existing in a variety of organisms but its function has not been well studied. In this study, we identified a novel BSD domain-containing protein (SlBSD1) in tomato. Biochemical and subcellular assay indicated SlBSD1 is a functional transcription factor and predominantly localized in the nucleus. The genetic analyses suggested that SlBSD1 is a novel negative regulator of vegetative growth and leaf senescence in tomato. The SlBSD1-knockdown tomato plants exhibited retarded vegetative growth and precocious leaf senescence, whereas SlBSD1-overexpression tomato plants displayed the opposite phenotypes. The negative role of SlBSD1 in leaf senescence was also supported by RNA-Seq analysis on the SlBSD1-knockdown tomato leaf in comparison with the wild type tomato leaf. Moreover, altered soluble solids contents in fruits were detected in the SlBSD1-knockdown and SlBSD1-overexpression tomato plants. Taken together, our data suggested that the novel transcription factor SlBSD1 plays important roles in controlling fruit quality and other physiological processes in tomato, including vegetative growth and leaf senescence.

Key words

leaf senescence, SlBSD1, tomato, transcription factor, vegetative growth

Introduction

Given the fact that massive reprogramming of gene expression is required during plant development and growth, many transcription factors act as key regulators due to their potential activation or repression on many genes' expression cooperatively. Thus, identification and characterization of novel transcription factors would help us better understand the complicated processes of plant growth and development. Nearly two decades ago, a novel domain, BSD (mammalian *B* TF2-like transcription factors, Drosophila *s* ynapse-associated proteins and yeast *D* OS2-like proteins), was found present in basal transcription factors, synapse-associated proteins and several hypothetical proteins (Doerks, Huber, Buchner, & Bork, 2002). The BSD domain is featured with three α-helices likely involved in DNA binding, with two highly conserved adjacent tryptophan and phenylalanine residues located at the C-terminus (Doerks et al., 2002). Limited studies have suggested BSD-containing proteins are involved in diverse physiological processes in a variety of species ranging from primal protozoan to human. For example, yeast DELOCALIZATION OF SWI6 (DOS2) plays an important role in heterochromatic histone modification and RNA interference (Li et al., 2005); drosophila SYNAPSE-ASSOCIATED PROTEIN OF 47 KDA (SAP47) is required for association function and short-term plasticity of synapses (Reichmuth et al., 1995); mammalian BTF2H1 and its yeast homologue TFB1 are the component of the general transcription and DNA repair factor IIH core complex, which play a role in transcription initiation and nucleotide excision repair of damaged DNA (Iben et al., 2002; Wang et

al., 1995). In plants, BSD-containing transcription factors have been identified in Arabidopsis and banana (*Musa acuminata*) (Ba et al., 2014; Park, Kim, Jung, & Suh, 2009). However, the functionality of such BSD-containing transcription factors in plants has not been well studied, despite that it has been speculated the BSD-containing transcription factor plays a role in cell proliferation during somatic embryogenesis and is involved in ethylene-mediated fruit ripening in banana (Ba et al., 2014).

Leaf senescence, as the last stage of leaf development, is a highly regulated developmental process associated with the degradation of chlorophyll and macromolecules, subsequent mobilization of nutrients to actively growing organs (new buds, young leaves, developing seeds and fruits), and eventually massive programmed cell death (Lim, Kim, & Nam, 2007). Senescence of leaves occurs in an age-dependent manner under optimal conditions, whereas when plants are exposed to nutrient deficiency or environmental stresses, leaf senescence can be induced precociously as an adaptive response to promote survival and reproduction (Guo & Gan, 2005; Woo, Kim, Lim, & Nam, 2019). Global transcriptome analysis has revealed that reprogramming of gene expression occurs during developmental or dark-induced leaf senescence in various plant species, including Arabidopsis (Breeze et al., 2011; V. Buchanan-Wollaston et al., 2005), wheat (Gregersen & Holm, 2007), maize (Zhang et al., 2014) and aspen (Andersson et al., 2004). In general, in a senescing leaf, genes essential for chloroplast activity, including those involved in photosystems, carbon fixation, chlorophyll biosynthesis and amino acid metabolism, are down-regulated, while a subset of genes, mainly involved in chlorophyll and macromolecule catabolism and generally referred to as senescence-associated genes (SAGs), are up-regulated (Woo et al., 2019).

Transcription factors have been demonstrated to act as key regulators of senescence by activating or repressing senescence-related genes' expression cooperatively. In Arabidopsis, NAM/ATAF/CUC (NAC) is the major transcription factor family controlling leaf senescence. At least four NAC transcription factors, ANAC092/ORESARA1 (ORE1) (J. H. Kim et al., 2009), ANAC029/Arabidopsis NAC-LIKE Activated by AP3/PI (AtNAP) (Guo & Gan, 2006), ANAC059/ORESARA1 SISTER1 (ORS1) (Salma Balazadeh et al., 2011) and ANAC016 (Y. S. Kim, Sakuraba, Han, Yoo, & Paek, 2013), act as positive regulator of leaf senescence, whereas two NAC family members, ANAC042/JUNGBRUNNEN1 (JUB1) (Wu et al., 2012) and ANAC083/VND-INTERACTING2 (VNI2) (Yang, Seo, Yoon, & Park, 2011), function as negative regulator. Among them, ORE1 is a master positive regulator controlling the expression of numerous SAGs by directly binding to their promoters (S. Balazadeh et al., 2010; Matallana-Ramirez et al., 2013). In tomato (*Solanum lycopersicum*), the orthologs of ORE1 (SlORE1S02, SlORE1S03, and SlORE1S06) positively regulate leaf senescence (Lira et al., 2017); Two additional NAC transcription factors, NOR and SlNAP2, act as a positive regulator module for leaf senescence: SlNAP2 activates *NOR* expression by directly binding to its promoter and, together with NOR, it jointly regulates two senescence-related genes *SlSAG113* and *SlSGR1* ; and SlNAP2 also directly regulates at least four other senescence-related genes,*SlSAG15* , *SlPPH* , *SlKFB20* , and *SlYLS4* (Ma, Balazadeh, & Mueller-Roeber, 2019; Ma et al., 2018).

In this study, we identified a novel BSD-containing protein termed SlBSD1 in tomato that plays significant roles in diverse aspects of plant development and growth. SlBSD1 is a functional transcription factor and predominantly localized in the nucleus. Significantly, SlBSD1 negatively regulates vegetative growth and senescence, as manifested by opposite pleiotropic phenotypes in transgenic tomato plants with knockdown or overexpression of SlBSD1. These phenotypes included retarded or promoted vegetative growth, precocious or delayed leaf senescence, and altered soluble solids content in fruits.

Materials and Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicu* m cv. Ailsa Craig), obtained from the C. M. Rick Tomato Genetics Resource Center (University of California, Davis, USA) and *Nicotiana benthamiana* were grown under natural light supplemented with high-pressure sodium bulbs (100 μmol m^{-2} s^{-1}) on a long-day light photoperiod (16-h light/8-h dark). Daytime and nighttime temperatures were 24 to 28 and 16 to 20 , respectively. The relative humidity was 50% to 70%.

Generation of transgenic tomato plants

The binary vectors pBI121 were transformed into tomato by Agrobacterium tumefaciens-mediated transformation (Van Eck, Kirk, & Walmsley, 2006). After in vitro regeneration of transgenic plants, well-rooted plants were transplant into moistened soil in plastic pots. Plants were covered with transparent plastic cups and maintained in a shaded area for acclimatization. 1 week later, the plants were moved to greenhouse and the plastic cups were removed.

Yeast two-hybrid and transcription activation assays

The LexA-based yeast two-hybrid (Y2H) system was used to determine the interactions between SlSINA1 and SlBSD1. The full-length cDNAs of*SlBSD1* and *SlSINA1* genes were cloned into the bait vector pEG202 and the prey vector pJG4-5, respectively. The yeast (*Saccharomyces cerevisiae*) strain EGY48 harboring the *LacZ*mark gene was transformed with the bait and prey constructs in the appropriate combinations. The transformed yeast cells were streaked onto X-Gal plates to assess the interaction between SlBSD1 and SlSINA1. For transcription activation assays, the full-length cDNA of *SlBSD1*was cloned into pEG202 in frame with the LexA-coding region. The construct was introduced into yeast stain EGY48 containing the lacZ reporter plasmid pSH18-34 to test the possible activation of the reporter genes. Photographs were taken after incubation at 30 °C for 2 days.

Transient expression experiments

Agrobacterium-mediated transient expression on *N. benthamiana*leaves was performed as described previously (Sessa, D'Ascenzo, & Martin, 2000). *A. tumefaciens* cells carrying appropriate constructs were syringe-infiltrated into *N. benthamiana* leaves. Agroinfiltrated leaf tissues were collected 36 h after agroinfiltration. Proteins were extracted from leaf tissues or protoplasts, resolved by SDS-PAGE and analyzed by immunoblotting. All transient expression experiments were repeated at least three times with similar results.

Total chlorophyll content measurement

Leaves was extracted with 80% acetone at 4 for 14 h in darkness. Total chlorophyll per fresh weight of leaves was calculated as described previously (Arnon, 1949).

Trypan blue staining

The trypan blue staining assays were conducted as described (Fernandez-Bautista, Dominguez-Nunez, Moreno, & Berrocal-Lobo, 2016) with minor modifications. Leaves were detached and submerged in 0.2% lactophenol trypan blue solution (0.2% trypan blue, 25% lactic acid, 25% water-saturated phenol, and 25% glycerol). The samples were washed in 95% ethanol to reduce background staining.

RT-qPCR analysis

Total RNA was isolated from leaf tissues using TRIzol reagent (Ambion, Carlsbad, CA, USA) and cDNA was synthesized using SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, Vilnius, Lithuania). Real-time PCR was performed using SYBR Green (Life Technologies, Warrington, UK) and an ABI 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). *Actin-41*(*Solyc04g011500*) served as reference gene for data analysis. Statistical significance was determined using student's t -test. Primers used for in RT-qPCR are listed in Table S1.

RNA-Seq analyses

RNA was extracted from *SlBSD1-KD* , *SlSINA1-OX* and WT leaves using SPLIT RNA Extraction Kit (Lexogen, Greenland, NH, USA) following the manufacturer instructions. RNA samples were quantified and quality-checked on a Fragment Analyzer Automated CE System (Advanced Analytical, Ankeny, IA, USA). Libraries for sequencing were prepared using mRNA-Seq Library Prep Kit for Illumina (Lexogen, Greenland, NH, USA) following the manufacturer instructions. Libraries were sequenced at the University of Oregon on a SE100 run using an Illumina HiSeq 4000 platform. Approximately 5 million reads were generated for each sample library. High-quality trimmed reads were mapped to the genome of*S. lycopersicum* (genome version

SL4.0 and annotation ITAG4.0) (Tomato Genome Consortium, 2012) and quantified using Salmon (Soneson, Love, & Robinson, 2016). Differential expression analysis was then carried out using DESeq2 (Love, Huber, & Anders, 2014).

Accession Numbers

Sequence data from key genes in this article can be found in the GenBank/EMBL/Solgenomics databases under the following accession numbers: *SlBSD1* , *Solyc04g077600* ; *SlSAG12* ,*Solyc02g076910* ; *SlSAG15* , *Solyc03g117950* ;*SlSGR1* , *Solyc08g080090* ; *SlPPH* ,*Solyc01g088090* .

Results

Phylogenetic analysis of BSD domain-containing proteins

A novel BSD domain-containing protein family, existing in a variety of organisms ranging from protozoans to humans, was newly identified by computational analysis (Doerks et al., 2002). In order to investigate the BSD domains found in plant proteins, we retrieved the amino acid sequences of BSD domains from 9 representative species (http://www.ebi.ac.uk/interpro/), including 3 plant species and 6 non-plant species. Highly similar sequences in the BSD domain were not included (for example the majority of BSD domains of human and mouse proteins are identical) and the remaining 63 unique sequences were used for multiple sequence alignment and phylogenetic analyses. This collection contained 16 unique BSD domain sequences in Arabidopsis, 12 in rice, 12 in tomato and 23 in other species. The multiple sequence alignment indicated that BSD domains are evolutionarily conserved and two less common amino acids, Phe and Trp, are adjacent and immutable (Fig. **S1**).

We utilized the Neighbor-Joining (NJ) method to construct a phylogenetic tree with 5 main clades, supported by high bootstrap values (Fig.**1a**). Clade I and II were named the BTF2-like transcription factors, as they included BSD domain a and b in the well-studied BTF2H1/TFB1 transcription factors. Mammalian BTF2H1 and its yeast homolog TFB1 are the component of the general transcription and DNA repair factor IIH core complex, which plays a role in transcription initiation and nucleotide excision repair of damaged DNA (Iben et al., 2002; Wang et al., 1995). The third clade included BSD domain from Drosophila SAP47 was designated as the synapse-associated protein clade, despite many BSD domains in this Clade are also found in plant proteins without synapse activity. Clade IV, the DOS2-like clade, included BSD domain from yeast DOS2 which is required for heterochromatic histone modification and RNA interference (Li et al., 2005). Clade V contained BSD domains of plant proteins, including the BSD domain of the Arabidopsis BSD1 protein that has been characterized as a transcription activator (Park et al., 2009), suggesting this clade may evolve later with plant-specific functions.

We selected the tomato gene *Solyc04g077600* for further analysis. The phylogenetic analysis revealed that Solyc04g077600 and Soly07g022920 are orthologs of AtBSD1 (Fig. **1b**). The Soly04g077600 contained a central BSD domain and was designated as SlBSD1 (Fig. **1c**), as it is the closest ortholog to AtBSD1. Although SlBSD1 and Soly07g022920 were paralogs, they were related to each other distantly (˜50% identity). Moreover, most BSD proteins, including SlBSD1 and Soly07g022920 protein, cluster in pairs (Figure **1b**), suggesting genome duplications for possible neofunctionalization.

SlBSD1 is a functional transcription factor

The presence of BSD domain in BTF2H1/TFB1 (a transcription factor and DNA repair factor subunit) and DOS2 (a heterochromatic histone modification-related protein) suggest that the BSD domain could have a role in chromatin-associated processes, which take place in the nucleus (Iben et al., 2002; Li et al., 2005; Wang et al., 1995). To analyze the subcellular localization of SlBSD1 in plant cells, a fusion construct of SlBSD1 fused with GREEN FLUORESCENCE PROTEIN (GFP) driven by the CaMV 35S promoter was generated and transiently expressed in *N. benthamiana* protoplasts, followed by the fluorescent microscopy. As shown in Fig. **2a** , the fluorescence signal of free GFP protein was distributed evenly in both cytoplasm and nucleus, whereas the vast majority of SlBSD1-GFP-derived fluorescence signal was observed in the nucleus, suggesting SlBSD1 protein is predominantly localized in the nucleus.

The identification as a transcriptional activator of AtBSD1 (Park et al., 2009), the Arabidopsis ortholog of SlBSD1, suggests SlBSD1 may possess potential transcriptional activity as well. To test this notion, a trans-activation assay was conducted in yeast. Expression of the LexA-DBD (DNA binding domain of the LexA operator) construct alone in yeast did not activate the *lacZ* marker gene. In contrast, expression of the LexA-DBD-SlBSD1 fusion construct rendered the activation of the *lacZ* gene, as manifested by the beta-galactosidase activity of yeast cells grown on the X-Gal plate (Fig. **2b**), suggesting SlBSD1 is a functional transcriptional activator.

Transcription factors with distinct functions are often expressed in a tissue-specific manner, stimulating or depressing the transcription of associated genes in specific cell types. To determine whether the expression profiling of SlBSD1 is tissue-specific in tomato, we investigated the expression of SlBSD1 in different tissues. qRT-PCR analysis indicated SlBSD1 is expressed in all tested tissues (root, stem, leaf, bud, flower and fruit), with relatively lower expression level in leaves (Fig. **2c**).

SlBSD1 is essential for vegetative growth

To determine the physiological function of SlBSD1 in tomato, a loss-of-function approach was implemented using the RNA interference technique. Transgenic tomato lines with *SlBSD1* knockdown were generated, of which two lines (*SlBSD1-KD1* and *SlBSD1-KD2*) with most reduced *SlBSD1* mRNA levels (70% and 85% reduction in*SlBSD1-KD1* and *SlBSD1-KD2* respectively, Fig. **S2a**) were selected for further analysis. The mRNA level of Soly07g022920, the ortholog of SlBSD1, was unaffected in *SlBSD1-KD1* or*SlBSD1-KD2* (no statistically significant change, Fig.**S2b**), indicating the specific silencing of the SlBSD1 target gene. One dramatic phenotypic change we observed was the vegetative growth of *SlBSD1-KD* plants is significantly affected. We examined the height of SlBSD1-KD plants in comparison with the wild type (WT) tomato plants through the growth at 4-week, 6-week and 13-week stage. The height of SlBSD1-KD plants was reduced 40.80%, 44.24% and 21.98% compared to that of WT plants, respectively, indicating knockdown of the *SlBSD1* gene results in significant growth retardation in tomato (Fig. **3**).

Next, we sought to verify the function of SlBSD1 in growth by the gain-of-function approach, via generating transgenic tomato constitutively over-expressing *SlBSD1* under the control of the CaMV 35S promoter. Two transgenic lines with highest *SlBSD1*expression (29- and 40-fold overexpression in *SlBSD1-OX1* and*SlBSD1-OX2* respectively, Fig. **S2c**) were selected for analysis. We found *SlBSD1-OX* plants display opposite vegetative growth phenotype as *SlBSD1-KD* plants did: at 4-week, 6-week and 13-week growth stages, SlBSD1-KD plants were 38.79%, 25.15% and 10.84% taller the WT plants at the same age, respectively (Fig.**3**). Thus, we concluded that SlBSD1 plays a positive role in vegetative growth in tomato.

SlBSD1 negative regulates leaf senescence in tomato

SlBSD1-KD plants did not exhibit any other morphological alterations except retarded vegetative growth at the early developmental stages (8-week-old). However, knockdown of *SlBSD1* caused yellowing of mature green leaves in the 8-week-old *SlBSD1-KD*tomato plants, whereas the WT tomato leaves were still green at the same growth stage (Fig. **4a**). Since leaf yellowing due to the preferential degradation of chlorophyll over carotenoids is the most obvious sign of senescence (Vicky Buchanan-Wollaston, 1997), we examined the senescence-associated characteristics of leaves at different growth ages in SlBSD1-KD tomato plants. Accelerated loss of chlorophyll content in the leaves at a later age (7^{th} and 4^{th} true leaves) was observed in *SlBSD1-KD*plants (Fig. **4b**). In addition, senescence-associated cell death was also enhanced in *SlBSD1-KD* plants, as shown by local patches of trypan blue-stained early-dying cells in the aged leaflets (7^{th} and 4^{th} true leaves, Fig.**4c**).

To further elucidate the regulation of the early senescence phenotype of the *SlBSD1-KD* plants at the transcription level, we examined the expression of SAGs in these plants in comparison with the WT plants. Four senescence-related genes were examined. *SlSAG12*(*Solyc02g076910* , encoding a cysteine protease), *SlSAG15*(*Solyc03g117950* , encoding a Clp ATPase), *SlSGR1*(*Solyc08g080090* , encoding a stay-green protein involved in chlorophyll degradation) and *SlPPH* (*Solyc01g088090* , encoding a pheophytinase involved in chlorophyll degradation were induced during developmental senescence in the WT tomato leaves (Fig.**S3a-d**

). Significantly, these 4 genes were significantly up-regulated in the 3rd true leaf of 8-week-old*SlBSD1-KD* plants compared to the WT leaves (Fig. **4d**), suggesting knockdown of *SlBSD1* leads to precocious leaf senescence in tomato.

There was no significant difference in leaf senescence between the 8-week-old *SlBSD1-OX* and the WT plants (Fig. **S4**). However, at the 12-week-old age, the aged leaves of WT tomato plants began turning yellow, whereas the leaves of the same age of*SlBSD1-OX* plants stayed green (Figure **5a**). Moreover, analyses of senescence-associated characteristics in the 12-week-old*SlBSD1-OX* and the WT plants indicated that leaf senescence was significantly delayed in *SlBSD1-OX* plants, as shown by delayed loss of chlorophyll content (Fig. **5b**), the absence of trypan blue-stained dying cells (Fig. **5c**), and the delayed induction of senescence marker genes (Fig. **5d**). Taken together, these results suggest that constitutive over-expression of *SlBSD1*renders delayed leaf senescence in tomato. Thus, based on our loss-of-function and gain-of-function analyses, we conclude that SlBSD1 functions as a negative regulator of leaf senescence in tomato.

SlBSD1 is involved in dark-induced leaf senescence

Leaf senescence occurs in an age-dependent manner but is also regulated by a variety of endogenous and exogenous factors. Darkness is often considered as an exogenous inducer of leaf senescence and has been commonly used to induce synchronous senescence in detached leaves (Nooden, 1988). Although the dark-induced senescence in detached leaves shares many common features with developmental senescence, the underlying molecular mechanisms might not be identical. In fact, incubation of detached leaves in darkness induce overlapping but different sets of SAGs compared to developmental senescence (Quirino, Noh, Himelblau, & Amasino, 2000). To test whether SlBSD1 is also involved in dark-induced leaf senescence, mature green leaflets from 6-week-old plants were incubated in the dark for up to 10 days. Our results indicate that, in comparison to those of the WT plants, detached leaflets of *SlBSD1-KD* plants showed enhanced yellowing under dark conditions, while leaflets detached from *SlBSD1-OX* plants remained relatively green (Fig. **6a,c**); the senescence-associated cell death was accelerated in *SlBSD1-KD*leaves but delayed in *SlBSD1-OX* leaves (Fig. **6b**); expression of senescence marker genes in *SlBSD1-KD* leaves was up-regulated but down-regulated in *SlBSD1-OX* leaves (Fig.**6d**). Taken together, our data suggest SlBSD1 is involved in both developmental and dark-induced leaf senescence.

RNA-seq analysis on the SlBSD1-KD and WT leaves

To further investigate the molecular basis of SlBSD1-mediated regulation of leaf senescence, we sought to identify genes that are regulated by SlBSD1 during leaf senescence by RNA-seq analysis. To this end, we examined the transcriptomes of *SlBSD1-KD* leaves with those of WT leaves to determine differentially expressed genes when the expression of *SlBSD1* is repressed, particularly genes involved in leaf senescence. As shown in Table 1 and Table S2, genes involved in degradation of macromolecules and mobilization were up-regulated in*SlBSD1-KD* leaves. In particular, increased transcript level was detected in genes encoding different types of proteases, including cysteine and aspartyl proteases, and genes encoding components of ubiquitin-proteasome pathway (such as ubiquitin-conjugating enzyme, F-box protein and proteasome subunit). In addition, genes encoding ribonuclease, which is involved in RNA degradation, were up-regulated, and one gene encoding chlorophyllase, a key enzyme in chlorophyll catabolic process, was up-regulated in *SlBSD1-KD* leaves as well. Moreover, many transporter genes exhibited increased transcript levels in *SlBSD1-KD* leaves, including genes coding for amino acid, peptide, sugar and cation transporters, indicating increased mobilization of nutrients from the senescing leaves to other parts of plants. On the other hand, many genes essential for chloroplast activity were down-regulated in *SlBSD1-KD* leaves, including genes encoding chlorophyll binding protein, photosystem I and II subunit, as well as genes involved in chlorophyll (tetrapyrrole) biosynthetic process. In addition, genes that encode enzymes involved in carbohydrate metabolic process were down-regulated in *SlBSD1-KD* leaves.

It is interesting that numerous genes involved in stimulus responses were also up-regulated in *SlBSD1-KD* leaves. Many of these genes encode chitinase, pathogenesis-related protein, detoxification and heat shock protein, indicating the protective steps which *SlBSD1-KD*tomatoes takes to respond to the stress generated

by the degradative and mobilization functions. Significantly, several genes involved in translation were up-regulated in *SlBSD1-KD* leaves, including genes encoding ribosomal protein, ribosome recycling factor and elongation factor. Taken together, our RNA-seq analysis results indicated numerous genes, of which many are tightly related to leaf senescence, are differentially expressed in *SlBSD1-KD* leaves. These results suggest that, as a transcription factor, SlBSD1 controls expression of large numbers of genes in leaves, including genes involved in leaf senescence.

SlBSD1 and fruit quality

Delaying leaf senescence and extending the duration of active photosynthesis could substantially increase the instant photoassimilate source and hence increase the grain yield and quality (Ali, Gao, & Guo, 2018). Sweetness, which results from total soluble sugar, is one of the most important traits of tomato fruits, especially for industrial processing. Given the fact that SlBSD1 plays a negative role in leaf senescence, we next sought to determine whether altered expression of*SlBSD1* has effect on fruit sweetness. We measured Brix values of*SlBSD1-KD* , *SlBSD1-OX* and the WT tomato fruits. Compared to the WT plants, *SlBSD1-KD* tomatoes showed lower levels (4.61% decrease) of soluble solids content in ripe fruits, whereas*SlBSD1-OX* tomatoes showed higher levels (16.25% increase) of soluble solids content, as manifested by the Brix indexes of the ripe fruits (Fig. **7**). Collectively, these data suggest that SlBSD1 plays an important role in leaf senescence and fruit quality in tomato.

Discussion

It has been found that the BSD domain-containing proteins, including transcription factors, exist in a variety of organisms ranging from protozoans to humans. However, not all BSD proteins function as transcription factor, suggesting the BSD domain could possess other conserved functions and be involved more general processes (Doerks et al., 2002). In plants, the first studied BSD protein was Arabidopsis BSD1 (AtBSD1) that is the closest homolog of tomato SlBSD1. AtBSD1 has been characterized as a functional transcription factor localized in the nucleus but its function is unknown (Park et al., 2009). Additionally, banana possesses at least four BSD domain-containing transcription factors namely MaBSD and MaBSD1/2/3, among which MaBSD is involved in fruit ripening, presumably via, at least partially, directly regulating two cell wall modification-related genes, *MaEXP1* and*MaEXP2* (Ba et al., 2014), whereas MaBSD1, MaBSD2 and MaBSD3 might be involved in cell proliferation during somatic embryogenesis (Maldonado-Borges, Ku-Cauich, & Escobedo-Graciamedrano, 2013; Shivani et al., 2017). The tomato BSD-containing transcription factor SlBSD1 identified in this study appears to play an important role in vegetative growth and leaf senescence, as manifested by retarded growth and precocious leaf senescence in the *SlBSD1-KD* plants, in which the*SlBSD1* gene is specifically repressed (Fig. **3,4**). It is notable SlBSD1 and the four banana BSD proteins do not share much similarity and are in different nodes by the phylogenetic analysis (Fig.**S5**), which could explain the difference of their predicted functionalities. In addition, the Arabidopsis T-DNA knockout line of*AtBSD1* does not exhibit any growth retardation or early senescence phenotype, which is likely due to functional redundancy of other BSD1 homologs in Arabidopsis (Park et al., 2009). However, this could also suggest BSD transcription factors may have distinct functions in different plant species.

It appears that one of the most important roles of SlBSD1 is controlling vegetative growth and leaf senescence in tomato, which is supported by both loss-of-function and gain-of-function assays. Knockdown of*SlBSD1* in tomato resulted in retarded vegetative growth, whereas overexpression of *SlBSD1* rendered the opposite effect (Fig.**3**), suggesting SlBSD1 is essential for plant growth. The genetic analyses also implicate SlBSD1 negatively regulates leaf senescence. The *SlBSD1-KD* tomato plants, in which the*SlBSD1* gene was specifically repressed, displayed precocious leaf senescence, including yellowing of mature green leaves in the 8-week-old plants (Fig. **4a**), accelerated loss of chlorophyll content in the leaves at a later age (7th and 4th leaves, Fig. **4b**), senescence-associated cell death in aged leaflets (7th and 4th leaves, Fig. **4c**), and up-regulation of senescence marker genes (*SlSAG12* , *SlSAG15* , *SlSGR1* and *SlPPH*) in the third leaf of 8-week-old plants (Fig. **4d**). Significantly, consistent with these observations, the *SlBSD1-OX* plants overexpressing *SlBSD1* exhibited opposite senescence-related phenotypes (Fig. **5**). In addition, the negative role of SlBSD1 in leaf senescence was also supported by our RNA-Seq analysis on the*SlBSD1-KD* plants in a

comparison to the WT plants. We found large number of genes involved in macromolecular degradation are up-regulated, whereas genes involved in chloroplast activity are down-regulated, in*SlBSD1-KD* leaves. Leaf senescence is a developmental process reflecting aging of leaves but can be triggered precociously by internal cues, such as disruption of homeostasis of phytohormones, and external environmental factors, including abiotic stress and nutrition starvation. Thus, the onset and progression of senescence must be dynamically controlled by the synergistic and/or antagonistic effects of regulatory factors. Several transcription factors have been identified as positive senescence regulators in tomato, including NAC transcription factors SlNAP2 (Ma et al., 2018), NOR (Ma et al., 2019), and the orthologs of Arabidopsis ORE1 (SlORE1S01/02/03) (Lira et al., 2017). The identification of the novel transcription SlBSD1 as a negative regulator has now added a new member on the list of leaf senescence regulators in tomato. It is interesting that, like SlBSD1, the NAC transcription factors NOR and SlNAP2 also appears possessing pleiotropic functions in plant development, including vegetative growth, age- and dark-induced leaf senescence, and fruit ripening and yield (Ma et al., 2019; Ma et al., 2018).

Positive and negative regulators of leaf senescence could be up-regulated and down-regulated during leaf senescence, respectively. For example, in tomato, both positive regulators *NOR* and*SlNAP1* are up-regulated during leaf senescence (Ma et al., 2019; Ma et al., 2018). Interestingly, the expression of *SlBSD1* is not significantly affected during senescence, as indicated by no significant changes of SlBSD1 expression detected by the real-time PCR analysis at different developmental stages: young leaves (YL), mature leaves (ML), senescent leaves (SL), and late senescent leaves (LS). (Fig.**S3e**). We speculate that the SlBSD1 transcription factor mainly functions to control the onset and/or progression of senescence during plant growth and development to prevent precocious senescence, which could be critical for plant productivity and quality by avoiding premature aging. However, it is equally possible that SlBSD1 could be down-regulated at the protein level, such as through ubiquitination-mediated degradation, upon the onset of senescence, which awaits further investigation.

Although *SlBSD1-KD* plants exhibited retarded growth and precocious leaf senescence, it is unlikely that the retarded growth is the consequence of precocious senescence, because precocious leaf senescence did not occur until reaching the 8-week-old stage when growth arrest had already taken place. This phenotype doesn't resemble the one of the tomato *slsbpase* mutant, in which the growth arrest is likely due to premature leaf senescence occurring in early developmental stage that leads to severe photosynthesis defect-triggered chlorosis, thereby affecting the overall vegetative growth of the plant (Ding, Wang, & Zhang, 2018).

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Gene class	Num	Example
Up-regulated	Up-regulated	Up-regulated
Proteolysis	12	cysteine proteinase, aspartyl protease, ubiquitin conjugating enzyme,
Nucleic acid catabolic process	1	ribonuclease
Chlorophyll catabolic process	1	chlorophyllase
Transport	11	amino acid transporter, amino acid permease, sugar transporter, ion
Response to stimulus	15	chitinase, pathogenesis-related protein, detoxification protein, heat sh
Down-regulated	Down-regulated	Down-regulated
Photosynthesis	13	chlorophyll binding protein, photosystem I subunit, photosystem II su
Tetrapyrrole biosynthetic process	5	protoporphyrinogen oxidase, porphobilinogen deaminase, uroporphyri
Carbohydrate metabolic process	8	fructose-1,6-bisphosphatase, sucrose synthase, hexokinase 2, glucose-6
Translation	26	ribosomal protein, elongation factor

Table 1 Potential senescence-associated genes showing altered transcript abundance in *SlBSD1-KD* leaves compared to WT

Figure legends

Fig. 1. Phylogenetic analysis of BSD domain and BSD domain-containing proteins.

(a) Unrooted tree of BSD domains with the main clades highlighted in different colors. The phylogenetic tree was constructed using the NJ method and a bootstrap test with 1000 replicates. Bootstrap values are marked for the branches that separate the main clades. Names of representative BSDs and locus of tomato BSDs are present at the leaves. Multiple domains in the same protein are labelled a and b.**(b)** Subtree of BSD domain-containing proteins. The phylogenetic tree was constructed using the NJ method. The numbers listed on each node represent the bootstrap support value associated with that node after running 1000 replicates. **(c)** SlBSD1 protein (440 amino acids) contains one conserved BSD domain in its center. Black shading indicates the neighboring Phe and Trp residues. Abbreviations: At,*Arabidopsis thaliana* ; Ce, *Caenorhabditis elegans* ; Dm,*Drosophila melanogaster* ; Hs, *Homo sapiens* ; Mm, *Mus musculus* ; Os, *Oryza sativa* ; Sc, *Saccharomyces cerevisiae* ; Sl, *Solanum lycopersicum* ; Sp, *Schizosaccharomyces pombe* .

Fig. 2 SlBSD1 is a functional transcription factor.

(a) Subcellular localization of SlBSD1 in *N. benthamiana*protoplasts. scale bars, 10 μm. **(b)** Transactivation activity of SlBSD1 in yeast, as indicated by blue coloration of yeast cells (transformed with construct of SlBSD1 fused to the DNA binding domain of LexA operator) grown on the selective medium containing X-Gal.**(c)** qRT-PCR analysis of *SlBSD1* transcript in various tomato tissues. The expression level in leaf was defined as "1". Data are mean ± 95% CL (n=3).

Fig. 3 SlBSD1 plays in important role in vegetative growth in tomato.

(a) 6-week-old *SlBSD1* knockdown (*SlBSD1-KD*),*SlBSD1* overexpression (*SlBSD1-OX*) and wild type (WT) plants. Scale bar, 10 cm. **(b)** Plant height of *SlBSD1*knockdown (*SlBSD1-KD*), *SlBSD1* overexpression (*SlBSD1-OX*) and wild type (WT) plants at 4-week, 6-week and 13-week stage.

Fig. 4 *SlBSD1* knockdown (*SlBSD1-KD*) plants display an early senescence phenotype.

(a) Phenotype of WT and *SlBSD1-KD* plants. Upper panel, 8-week-old whole plants, scale bars, 10 cm; lower panel, detached leaflets from the 3rd true leaf of 8-week-old plants, scale bars, 1 cm. **(b)** Total chlorophyll content of the 10th, 7th and 4thtrue leaves of 8-week-old WT and *SlBSD1-KD* plants. FW, fresh weight. **(c)** Trypan blue staining of detached leaflets from the 10th, 7th and 4thtrue leaves of 8-week-old WT and *SlBSD1-KD* plants. Blue-colored patches indicate areas of dead or dying cells. Scale bars, 1 cm.**(d)** Expression level

of senescence marker gene *SlSAG12* ,*SlSAG15* , *SlSGR1* and *SlPPH* in the 3rd true leaves of 8-week-old WT and *SlBSD1-KD*plants. Data presented In **(b)** and **(d)** were means ± SD (n=3). Asterisks indicated statistically significant difference compared with WT. *P <0.05, **P <0.01; two-tailed Student's t -test.

Fig. 5 Developmental leaf senescence is delayed in*SlBSD1* overexpression (*SlBSD1-OX*) plants.

(a) Phenotype of WT and *SlBSD1-OX* plants. Upper panel, 12-week-old whole plants, scale bars, 10 cm; lower panel, detached leaflets from the 3rd true leaf of 12-week-old plants, scale bars, 1 cm. **(b)** Total chlorophyll content of the 10th, 7th and 4thtrue leaves of 12-week-old WT and *SlBSD1-OX* plants. FW, fresh weight. **(c)** Trypan blue staining of detached leaflets from the 10th, 7th and 4thtrue leaves of 12-week-old WT and *SlBSD1-OX* plants. Blue-colored patches indicate areas of dead or dying cells. Scale bars, 1 cm.**(d)** Expression level of senescence marker gene *SlSAG12* ,*SlSAG15* , *SlSGR1* and *SlPPH* in the 3rd true leaves of 12-week-old WT and *SlBSD1-OX*plants. Data presented In **(b)** and **(d)** were means ± SD (n=3). Asterisks indicated statistically significant difference compared with WT. *P <0.05, **P <0.01; two-tailed Student's t -test.

Fig. 6 SlBSD1 control dark-induced senescence.

(a) Young detached leaflets of 6-week-old plants as indicated before (0 DDI) and after 10 days of dark incubation (10 DDI). Scale bars, 1 cm. **(b)** Trypan blue staining of leaflets after 10 days of dark incubation. Blue-colored patches indicate areas of dead or dying cells. Scale bars, 1 cm. **(c)** Total chlorophyll content in the leaflets before and after 10 days of dark incubation. FW, fresh weight.**(d)** Expression level of senescence marker genes *SlSAG12* ,*SlSAG15* , *SlSGR1* and *SlPPH* in the leaflets after 10 days of dark incubation. Data presented In **(b)** and **(c)**were means ± SD (n=3). Asterisks indicated statistically significant difference compared with WT. *P <0.05, **P <0.01; two-tailed Student's t -test.

Fig. 7 Soluble solids content in *SlBSD1* knockdown (*SlBSD1-KD*), *SlBSD1* overexpression (*SlBSD1-OX*) and wild type (WT) ripe fruits.