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Original Paper

Comparative co-expression network analysis extracts the *SlHSP70* gene affecting to shoot elongation of tomato

Nam Tuan Vu[1,†], Ken Kamiya[1,†], Atsushi Fukushima[2], Shuhei Hao[1], Wang Ning[1,3], Tohru Ariizumi[1,3], Hiroshi Ezura[1,3], Miyako Kusano[1,2,3,*]

[1] Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; [2] RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro, Tsurumi, Yokohama 230-0045, Japan; [3] Graduate School of Life and Environmental Science, Tsukuba-Plant Innovation Research Center (T-PIRC), University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
* E-mail: kusano.miyako.fp@u.tsukuba.ac.jp Tel & Fax: +81-29-853-4809

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Abstract Tomato is one of vegetables crops that has the highest value in the world. Thus, researchers are continually improving the agronomical traits of tomato fruits. Auxins and gibberellins regulate plant growth and development. *Aux/indole-3-acetic acid 9* (*SlIAA9*) and the gene encoding the DELLA protein (*SlDELLA*) are well-known genes that regulate plant growth and development, including fruit set and enlargement by cell division and cell expansion. The absence of tomato *SlIAA9* and *SlDELLA* results in abnormal shoot growth and leaf shape and giving rise to parthenocarpy. To investigate the key regulators that exist up- or downstream of *SlIAA9* and *SlDELLA* signaling pathways for tomato growth and development, we performed gene co-expression network analysis by using publicly available microarray data to extract genes that are directly connected to the *SlIAA9* and *SlDELLA* nodes, respectively. Consequently, we chose a gene in the group of *heat-shock protein* (*HSP*)*70*s that was connected with the *SlIAA9* node and *SlDELLA* node in each co-expression network. To validate the extent of effect of *SlHSP70-1* on tomato growth and development, overexpressing lines of the target gene were generated. We found that overexpression of the targeted *SlHSP70-1* resulted in internode elongation, but the overexpressing lines did not show abnormal leaf shape, fruit set, or fruit size when compared with that of the wild type. Our study suggests that the targeted *SlHSP70-1* is likely to function in shoot growth, like *SlIAA9* and *SlDELLA*, but it does not contribute to parthenocarpy as well as fruit set. Our study also shows that only a single *SlHSP70* out of 25 homologous genes could change the shoot length.

Key words: co-expression network, *DELLA*, *HSP70*, *IAA9*, tomato.

Introduction

Tomato (*Solanum lycopersicum*) is an important commercial vegetable that belongs to the Solanaceae family (Tomato Genome Consortium 2012). The tomato genome consists of 12 chromosomes with a genome size of 950 Mb that has been completely sequenced by the Tomato Genome Consortium (2012). This consortium provides a huge amount of data for genomic researches and reference genomes for more than 3000 species (Tomato Genome Consortium 2012). One cultivar of tomato, namely Micro-Tom, is considered to be a great model because of its relatively short life cycle, small size, prolific seed production, and small genome size with the availability of genetic and genomic resources (Meissner et al. 1997). In particular, tomato is a typical climacteric fleshy fruit that shows special characteristics from flowering to ripening that cannot be gained from model plants such as *Arabidopsis* and rice (Koornneef and Meinke 2010; Shimamoto and Kyozuka 2002). Understanding the gene functions in tomato can increase the understanding of the principle and dynamics of molecular plant physiology, which can be used to create new valuable agronomic traits for vegetable and fruit plants (Aoki et al. 2013).

Aux/indole-3-acetic acid 9 (*SlIAA9*) and *SlDELLA* are two well-known genes that are involved in plant growth and development, including fruit set and enlargement through cell division and cell expansion (Fuentes et al. 2012; Fujita et al. 2012; Sun 2010; Wang et al. 2005). *SlIAA9* is a member of the *Aux*/*IAA* gene family consisting of 26 genes in tomato plants. *SlIAA9* acts as a transcription factor in the regulation of the expression of auxin-responsive factors through auxin signaling

Abbreviations: HSP70, heat shock protein 70; OE, over expressing; WT, wild type.
† These authors contributed equally to this work.
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(Audran-Delalande et al. 2012). Tomato *Aux/IAA9* acts as a negative regulator of the auxin response involved in the controlling of the fruit set by repressing the transcription of the auxin-responsive and fruit developmental genes by interacting with *Auxin response factor 7* (*ARF7*) (de Jong et al. 2009b). Downregulation in the transcription level of *SlIAA9* can simplify the leaf shape and elongate shoot parts, leading to changes in the plant height. The downregulation also induces parthenocarpy (Mazzucato et al. 2015; Okabe et al. 2011; Wang et al. 2005). *SlDELLA* is a negative regulator of gibberellin signaling by combining with the gibberellin receptor GID1 (Yoshida et al. 2014). DELLA has been suggested to function as a transcriptional activator and as the fundamental component of the gibberellin-GID1-DELLA signaling pathway. *Procera*, a *Sldella* mutant, has been reported to show morphological changes in plant elongation, branching architect, reproductive organ development, and parthenocarpy (Bassel et al. 2008; Carrera et al. 2012; Lombardi-Crestana et al. 2012; Martí et al. 2007). The parthenocarpy development in both *Sliaa9* and *Sldella* mutants has been elucidated previously by their downstream activities that stimulate phytohormone-related fruit development through their direct or indirect crosstalk (Hu et al. 2018). Changes in the plant architecture in these mutants were investigated. However, these mechanisms remain unknown.

The rapid development of biotechnology with the introduction of microarray and RNA sequencing (RNA-seq) has brought a huge amount of information from thousands of genes that are obtained from one or multiple experimental conditions (Horvath et al. 2003; Schadt et al. 2010; Wang et al. 2009). Gene co-expression analysis shows potential candidate genes that are related to plant growth and development (Aoki et al. 2007; Rhee and Mutwil 2014; Saito et al. 2008; Usadel et al. 2009). For example, co-expression network analysis has been utilized to discover genes in enriched co-expression module(s) related to the flavonoid biosynthetic pathway and the modules of metabolites associated with fruit ripening-related traits in tomato (DiLeo et al. 2011; Ozaki et al. 2010). Besides, the Tomato Functional Genomics Database (TFGD) provides not only the fundamental database for functional genomics research but also a huge resource for microarray, metabolite, and small RNA-seq data sets for co-expression analysis based on computational applications (Kudo et al. 2017; Ohyanagi et al. 2015).

In this study, we conducted gene co-expression network analysis by using publicly available microarray data. Potential candidate genes that were directly connected with *SlIAA9* and *SlDELLA* were investigated. Consequently, a *heat-shock protein SlHSP70-1* could be extracted in each network. To understand the mechanisms underlying tomato growth, development, and fruit set such as parthenocarpy, lines overexpressing the targeted *SlHSP70*-coding gene were generated. Phenotyping using the lines were performed to evaluate the gene functions of the targeted gene by comparing the growth and development of transgenic plants to that of wild type (WT) as the control when grown under no-stress conditions. Overexpression of the *SlHSP70-1* promoted internode elongation, leading to the production of leggy plants. The findings of our study show the roles of *SlHSP70-1* in tomato growth and development processes in association with *SlIAA9* and *SlDELLA* regulations.

Materials and methods

Construction of gene co-expression networks

The data of 307 Affymetrix Tomato GeneChip was acquired from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/), ArrayExpress (https://www.ebi.ac.uk/arrayexpress/), and TFGD (http://ted.bti.cornell.edu/), which are the gene expression databases (Fukushima et al. 2012). To generate co-expression networks, R (CRAN ver. 3.5.1) software was used (Team RC and Computing RFfs 2019). The method of normalization and probe sets removal was performed as reported by Fukushima et al. (2012). For the ID conversion of Affymetrix microarray's probeset-ID and ITAG ID, we used information in the Sol Genomics Network (ftp://ftp.solgenomics.net/genomes/Solanum_lycopersicum/microarrays_mapping-/A-AFFY-87_AffyGeneChipTomatoGenome.compositeelements_ITAG2.3-GeneID_map-ping.txt). From this conversion, IDs with one-to-one correspondence were extracted, and 5228 genes were used for network construction. The mrnet function from the minet package (Meyer et al. 2008) was used for the construction, and the threshold was set to 0.05. MRNet generated a network using a feature selection method called the minimum Redundancy Maximum Relevance (mRMR) (Meyer et al. 2007).

Network diagram construction, gene ontology analysis, and distance measurement

Cytoscape ver. 3.7.0 was used for preparing the network diagram (Shannon et al. 2003). BiNGO ver. 3.0.3, an application of Cytoscape, was used for gene ontology (GO) analysis of the neighboring genes (Maere et al. 2005). Pesca ver. 3.0, a Cytoscape application, was used to measure the distances between each gene in the network. The product name of each gene was acquired using Panther (http://pantherdb.org/) (Mi et al. 2013; Thomas et al. 2003).

Sequence analysis

For the molecular description and the phylogenetic analysis of the *SlHSP70-1* gene, genomic and amino acid sequences of the target gene were download from the Phytozome database ver.12.1 (available on https://phytozome.jgi.doe.g.,ov/pz/portal.html) by using a search tool with the keyword "HSP70" for

gene and "tomato" for species. Amino acid multiple sequence alignments were performed using ClustalW (https://www.genome.jp/tools-bin/clustalw). The phylogenic analysis was inferred using the neighbor-joining method (Saitou and Nei 1987). A phylogenetic tree was constructed using the MEGA7 software, with 1000 replicates for bootstrap test (Kumar et al. 2016). The *SlHSP70-1* gene features were visualized with intron, exon, and UTR compositions using the Gene Structure Display Server 2.0 (Hu et al. 2015), which is available online on the website http://gsds.cbi.pku.edu.cn/. The genomic sequences, CDS sequences, and the amino acid sequences of tomato family genes are assembled in Supplementary Figure S1.

Plasmid construction and Agrobacterium introduction

The binary vector pDEST_35S_3fstop_BCKH/BCKK carried the full-length cDNA of gene *SlHSP70* (*Solyc06g076020.2.1*) and the kanamycin-resistance gene (*neomycin phosphotransferase II*, *NPTII*) for selection was driven by the CaMV 35S promoter. The plasmid was introduced into the *Rhizobium radiobacter* (*Agrobacterium*) strain LBA4404 using the electroporation method. Bacterial cells were cultured in liquid Luria Broth (LB) at 28°C for 1 h, and then the cells were collected by centrifugation using the centrifuge TOMY MX-305 (TOMY, Japan) at 1,000×*g* for 5 min. Thereafter, the cells were spread in 1% (w/v) LB agar with 50 mg/l kanamycin. The colony in each petri dish was validated by polymerase chain reactions (PCRs) in the Bio-rad T100 Thermal Cycler (BIO-RAD, US) with a specific primer of *NPTII* for genotyping. The colony showing positive signal for *NPTII* was re-cultured in the liquid LB medium with 100 mg/l kanamycin at 28°C in a shaking incubator (Bioshaker BR-21FP, TAITEC, Japan) until OD_{600} of 0.6–0.8 was achieved for co-cultivation with tomato explants. *Agrobacterium* in glycerol stock was stored in −80°C until use.

One day before the inoculation, *Agrobacterium* was taken from the colony or glycerol stock of *Agrobacterium* harboring the binary vector for 24 h at 28°C in 3 ml of LB medium containing 100 mg/l kanamycin until the OD_{600} reached 0.6–0.8. Samples of the bacterial culture were centrifuged at 1,000×*g* for 5 min at room temperature and then the supernatant was discarded. The obtained pellet was re-suspended in an infection medium consisting of 1.2 g sucrose, 100 μM acetosyringone, and 10 μM mercaptoethanol at pH 5.8. The bacterial suspension was poured into the petri dish for inoculation.

Transformation

The *Agrobacterium* strain LBA4404 carrying the *SlHSP70-1* gene was transformed into tomato (*S. lycopersicum* cv. Micro-Tom) through cotyledon explants. Cotyledons of 7-day-old seedlings were cut into two halves, which were dipped and soaked in the infection medium for 10 min without shaking. Thereafter, the explants were taken out and excess bacteria were removed by absorption on a sterilized paper towel for 5 min. The explants were placed in a co-cultivation petri plate containing 30 mg/ml sucrose, 10 μM acetosyringone, 1.5 mg/l zeatin, and 3 mg/ml Gelrite in a pH 5.8 medium in the dark for 2 days at 24°C. After 2 days of co-cultivation, calli were transferred to a petri plate with Murashige–Skoog (MS) basal medium containing 30 mg/ml sucrose, 1.5 mg/l zeatin, 100 mg/l kanamycin, 375 mg/l Augmentin, and 3 g/l Gelrite at pH 5.8 to induce callus formation at 24°C (16 h light/8 h dark condition, 16/8 (light/dark), hereafter). The medium was renewed every 10 days. After 3 weeks, 5–7 calli were transferred to a petri plate with a shoot regeneration medium containing MS basal medium with 30 mg/ml sucrose, 1 mg/l zeatin, 100 mg/l kanamycin, 375 mg/l Augmentin, and 3 g/l Gelrite at pH 5.8. Shoots (2-cm long) were transferred to a rooting medium containing half-strength MS basal medium, 15 mg/ml sucrose, 50 mg/l kanamycin, 375 mg/l Augmentin, and 3 g/l Gelrite at pH 5.8 at 24°C (16/8 (light/dark)). Shoots with lateral roots (approximately 3–4 cm long) were transferred to rock wool supplied with 1/500 Hyponex 6-10-5 nutrient (HYPONeX Corp., Japan) for the growth of transgenic plants. The process of plant transformation was followed, as described previously (Shikata and Ezura 2016).

Plant growth conditions

Tomato seeds were sterilized in 0.5% (v/v) sodium hypochlorite solution for 10 min. Seeds were washed three times in sterilized deionized water (each rinse lasted 10 min). Seeds were germinated in sterilized deionized water for 2 days before sowing in a magenta box containing MS basal medium with 30 mg/ml sucrose and 3 g/l gelrite (pH 5.7). Thereafter, they were placed in the plant growth chamber (BiOTRON Type LH-350SP, NK System, Taiwan) under fluorescent light with 120 μmol/m^2/s irradiance with 16/8 (light/dark) at 24°C for 1 week. Transgenic and WT plants were grown in soil in a growth chamber under the condition of 120 μmol/m^2/s light density and 16/8 (light/dark) duration at 24°C. Water and 1/500 Hyponex 6-10-5 nutrients were supplied every 2 days.

Genotyping

Genotyping of transgenic plants was performed using PCR in a Bio-rad T100 Thermal Cycler (BIO-RAD, US) with genomic DNA from leaves of transgenic plants and that of WT samples with specific primers of a selective gene (*NPTII*). Genomic DNA was isolated from the leaves, using cetyl trimethylammonium bromide (CTAB) method (Doyle 1991). Primers used for PCR amplification of the selective gene are shown in Supplementary Table S1. The expected sizes of the PCR products were 700 bp. PCR conditions were as follows: pre-incubation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were analyzed on 1.5% agarose gel and visualized under UV light of 254 nm wavelength.

RNA isolation and real-time quantitative PCR analysis

Total RNA from frozen tomato plant tissues was extracted using

TRIzol Reagent (Ambion, US) and treated with TURBO DNA-free Kit (Invitrogen, US). Samples were collected from young leaves and stems of 17-day-old plants and ovaries at −2, 0, 2 and 4 days after flowering (DAF), respectively. The young leaves and stems of *Sliaa9* mutant plants were collected at the same stage with those from transgenic plants and WT plants for RNA extraction by the same method. The quality of RNA was assayed by the electrophoresis of total RNA on 1.2% agarose gel. The cDNA was obtained from 1 μl total DNA-free RNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan).

For real-time PCR analysis, 2.0 μl of 10-fold diluted cDNA was added to the PCR mixture containing 10 μM of each primer and 5.0 μl of Fast SYBR™ Green Master Mix (Thermo Fisher Scientific, US). To investigate the expression level of *SlHSP70-1* in leaves, stems, and fruits of tomato, quantitative amplifications were performed with specific primers of HSP70 for tomato, while the ubiquitin gene was used as the reference gene. To investigate the relationships among *SlIAA9*, *SlDELLA*, and *SlHSP70*, the expression levels of these genes were evaluated in transgenic plants of *SlHSP70, Sliaa9*, and WT and then compared by real-time PCR using specific primers for each gene. The primers were designed using the computer program OLIGO 7 Primer Analysis Software (Molecular Biology Insights, Inc., US). Primer sequences are shown in Supplementary Table S1. The amplification was conducted as follows: preheat at 95°C for 3 min, 40 cycles of denaturation at 95°C for 10 s, synthesis at 60°C for 20 s, and final extension at 72°C for 3 min in the StepOnePlus™ Real-Time PCR System (Applied Biosystems, US). The melting temperature of the product was determined to verify the specificity of the amplified fragment at the end of PCR. The expression level of the targeted gene was analyzed by the comparative Ct method (ΔΔCt) using the reference gene (Livak and Schmittgen 2001). The relative expression of the target gene was compared with that of WT in the same tissue.

Phenotyping

The length of the whole plant, internodes, and leaves of transgenic *SlHSP70-1* tomato and WT at 60 days after sowing were measured and compared together in pairs. The number of leaves and internodes were counted from the ground to the first inflorescence of each plant. The fruit sizes of the transgenic tomatoes were measured and compared with that of WT. To measure the fruit size at 0, 2, 4, 12, and 30 DAF, flowers were labeled and hand pollinated on the day of anthesis. Then, all the petals, sepals, and stamens were carefully eliminated to leave the pistil and the ovary. The morphology of the fruit was observed under the optical microscope Stemi 2000-CS (Zeiss, Germany) with 50 folds of magnification and the photos were taken using the camera AxioCam ERc5s (Zeiss). The size of the fruits was analyzed and processed using the AxioVision software (Zeiss).

Statistical analysis

The significance of differences in the expression level of *SlHSP70-1* between overexpressing (OE) and WT plants were assayed by Student's *t*-test using Graphpad Prism 5.04 software (GraphPad Software, San Diego California, US) on Windows 10 (64-bit). Significant difference by mean values between genotypes for the evaluation of phenotypes was assayed according to the *p*-value (significant, $p \leq 0.05$, 0.01, 0.001). Data were graphically represented as mean value ± standard deviation (SD) of each category for each genotype.

Results

Gene-to-gene correlation networks using publicly available microarray data

SlIAA9 and *SlDELLA* are the two key genes that are involved in auxin and gibberellin signaling. These genes play important roles in the growth and development of tomato (de Jong et al. 2009a; Wang et al. 2005). To investigate the up- or downstream of *SlIAA9* and *SlDELLA*, networks around *SlIAA9* and *SlDELLA* were cut out from each network constructed in the publicly available microarray data (Figures 1 and 2). The gene co-expression network comprising 26 genes were directly connected to *SlIAA9* as the main hub (Figure 1), while the network of *SlDELLA* had direct connection with seven different genes (Figure 2).

Next, we investigated the neighboring genes of *SlIAA9* and *SlDELLA*. Genes directly connected with *SlIAA9* and *SlDELLA* in the co-expression networks are listed in Supplementary Table S2 and Table 1, respectively. Among the 26 neighboring genes in the *SlIAA9* network, nine genes have been characterized by their functions. The most prominent one is the gene encoding *SlAGAMOUS-LIKE 11* (*AGL11*) transcription factor, which is also known as the member of the *MADS* box transcription factors that plays an important role in the process of plant growth and development, especially in the timing of flowering and fruit development (Becker and Theißen 2003; Puranik et al. 2014; Smaczniak et al. 2012). In tomato, overexpressing *SlAGL11* results in abnormal stamens with poorly viable pollen (Daminato et al. 2014). The gene coding for *Ubiquitin-conjugating enzyme E2 8* (*UBC8*) was reported to participate in the protein modification process of protein ubiquitination (Kraft et al. 2005), while two other genes are involved in the sugar metabolism in tomato (Cai et al. 2018; Wong et al. 1990).

The analysis of the neighboring genes in the *SlDELLA* network showed seven genes that were directly connected to the *SlDELLA* node. The gene *SAMDC3* encoding *S*-adenosylmethionine decarboxylase proenzyme 3 (EC 4.1.1.50) is vital for the biosynthesis of polyamines in the *S*-adenosylmethionine biosynthesis pathway (Majumdar et al. 2017). The gene encoding constitutive photomorphogenesis 9 (COP9) signalosome complex subunit 4, a component of the COP9 signalosome complex, is involved in various cellular and developmental processes related to phytohormone auxin responses

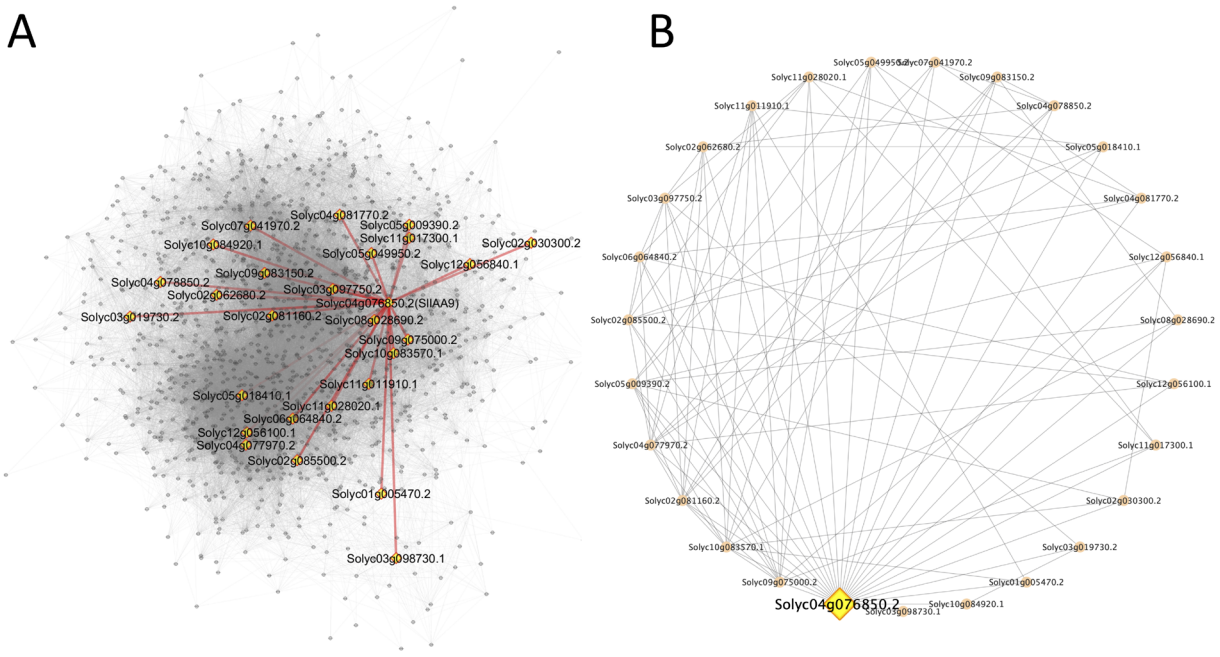

Figure 1. The co-expression network of the *SlIAA9*-neighboring genes. A) The first and second neighboring genes of *SlIAA9* (*Solyc04g076850*.2.1) are drawn. B) The nearest neighboring genes of *SlIAA9* in the network. *SlIAA9* (*Solyc04g076850*.2.1) is located at the bottom of network with yellow color.

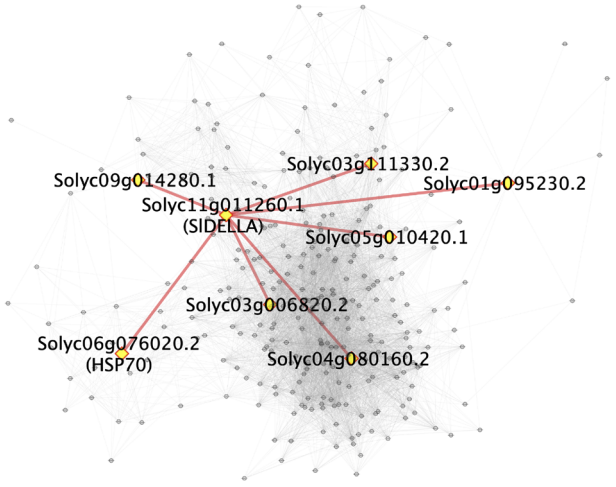

Figure 2. The co-expression network of the *SlDELLA*-neighboring genes The first and second neighboring genes of *SlDELLA* were drawn using the same method of the *SlIAA9*-network generation.

(Wang et al. 2003). The gene *Solyc06g076020.2.1* that was annotated as *heat-shock protein 70 SlHSP70-1* appears to have a direct connection with *SlDELLA*. In general, genes of the HSP70 family are often expressed in response to stresses such as heat or drought stresses (Zhang et al. 2015). The direct connection between *SlHSP70-1* and *SlDELLA* generates a new hypothesis that *SlHSP70-1* in association with *SlDELLA* might be involved in tomato plant growth and development.

Next, we investigated the distance between the targeted *SlHSP70-1* and *SlIAA9* and between *SlHSP70-1* and *SlDELLA*, respectively. *SlDELLA* was directly connected to the *SlHSP70-1* gene, although the distance from *SlIAA9* to *SlHSP70-1* was three hops (Supplementary Table S3). Moreover, the distance from *SlIAA9* to *SlDELLA* was three hops. As the average of the gene-to-gene distance in the network was 2.615 in this network, the distance between *SlHSP70-1* and *SlDELLA* was greater than the connectivity of *SlHSP70-1* and *SlIAA9* and *SlIAA9* and *SlHSP70-1*. Further, the overlaying of the *SlLAA9* and *SlDELLA* networks could extract the *SlHSP70-1* that was potentially useful because this was the only gene to show direct connection with *SlDELLA* within other SlHSP70s on the microarray chip (Supplementary Table S3). We thus focused on the *SlHSP70-1* gene for further analysis.

The results of GO enrichment analysis showed that the over-presented GO terms linked to *SlIAA9* neighboring genes were involved in various functions (Supplementary Table S4). In the *SlDELLA* neighboring genes, GO terms such as protein binding and proteasome complex were over-represented (Supplementary Table S5).

Molecular characterization of the targeted SlHSP70-1 on the tomato genome

Based on the genetic sequence of tomato from the Phytozome database, 25 genes were predicted to be in the *SlHSP70* gene family (Supplementary Table S6). Twenty-one genes were contained in the Interpro domain IPR013126, while four genes were contained in the Interpro domain IPR012725 for *Dna*K chaperone. This chaperone was a member of the homologous subfamily in *HSP70*s that was often expressed in bacteria (Genevaux et al. 2007). The targeted *SlHSP70-1* gene, *Solyc06g076020.2.1*, was located on chromosome six in

Table 1. The nearest neighbour gene group of *SlDELLA* gene. Gene names are at Planther (http://pantherdb.org/). IDs are base on iTAG 2.3.

Mapped ID	Gene name/gene symbol	PANTHER family/subfamily	PANTHER protein class
Solyc03g006820	Uncharacterized protein	Fi16820p1-related (PTHR10869:SF123)	
Solyc09g014280	Uncharacterized protein	Subfamily not named (PTHR31896:SF5)	
Solyc05g010420	*S*-adenosylmethionine decarboxylase proenzyme	*S*-Adenosylmethionine Decarboxylase Proenzyme 3 (PTHR11570:SF15)	Decarboxylse (PC00089)
Solyc11g011260	DELLA protein GAI	DELLA protein RGL1-related (PTHR31636:SF47)	
Solyc03g111330	Uncharacterized protein	Subfamily not named (PTHR47525:SF1)	
Solyc06g076020	Uncharacterized protein	Subfamily not named (PTHR19375:SF255)	
Solyc04g080160	Uncharacterized protein	COP9 signalosome complex subunit 4 (PTHR10855:SF2)	

the tomato genome. The phylogenetic tree constructed by the alignment of the amino acid sequences of HSP70s showed that the protein of the targeted gene showed similarity with the other three genes in the gene family located on chromosome nine (*Solyc09g010630.2.1*), ten (*Solyc10g086410.2.1*), and eleven (*Solyc11g066060.1.1*). The tree showed that they might share their biological functions although the functions of these genes are still uncharacterized (Supplementary Figure S1A). The target gene is also named as *SlHSC70-1*, which was expressed under stress conditions such as heat stress (Duck et al. 1989). The *Solyc06g076020.2.1* gene was classified in heat-shock cognate 70 (*SlHSC70-1*) along with the gene *Solyc10g086410.2.1* (*SlHSC70-2*) and two isoforms of *SlHSC70-3* (*Solyc04g011440.2.1* and *Solyc09g010630.2.1*). The four gene structures had the same number of introns and exons and were of similar length (Supplementary Figure S1B).

Overexpression of the targeted SlHSP70-1 promoted tomato internode elongation but did not affect the leaf shape

To characterize the physiological functions of the targeted *SlHSP70-1* gene, a full-length cDNA of this gene was introduced into tomato cv. Micro-Tom. Thirteen transformants (T_0) regenerated from kanamycin-resistant calli contained the targeted *SlHSP70-1* inserted gene. T_0 transgenic lines with the introduced mRNA of the targeted *SlHSP70-1* were screened to choose the homozygous mutants (Supplementary Figure S2). Homozygous mutant plants of the T_3 generation of two transgenic lines (S10 and S13) were used for phenotyping during the vegetative and reproductive stages.

The main shoot of the overexpressing *SlHSP70*-OE was longer than that of the controls (WT plants) (Figure 3, Supplementary Figure S3A). After two months of cultivation, the average plant height of the control plants was 137.4±10.95 mm (mean±SD), while the average plant height of *SlHSP70*-OE (S13) was 183.46±46.26 mm (Figure 3A), suggesting that the *SlHSP70*-OE plant shoots were significantly higher (133.6%, $p=0.02$) than that of WT (Figure 3B). The transgenic line S10 was also higher (120%, $p=0.01$) than WT (Supplementary Figure S3B).

For further analysis, the length of the internodes (from

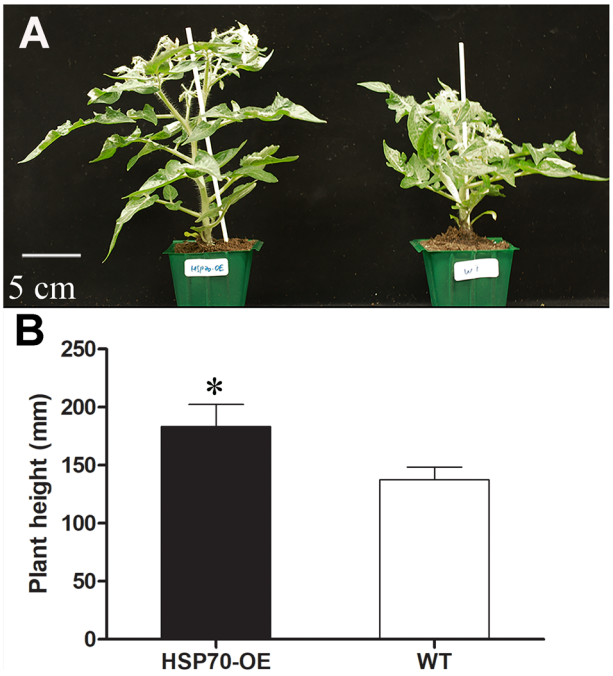

Figure 3. The shoot height of the *SlHSP70*-OE plant. A) The visible phenotype of the *SlHSP70*-OE and WT plants at 60 DAF. B) Mean values of plant height of the *SlHSP70*-OE and WT. Asterisks representative for significant difference with * $p<0.05$, ** $p<0.01$ according to Student's *t*-test carried out on raw data. Bar indicates mean values of six biological replicates±SD.

cotyledon to the first inflorescence) of six independent plants of *SlHSP70*-OE were measured and then compared with those of WT at the same position. The length of nine internode positions of each *SlHSP70*-OE (line S13) as well as those of WT was compared. The internodes of *SlHSP70*-OE tended to be longer than that of WT, and the most remarkable difference in the internode length between the *SlHSP70*-OE and WT was observed in the fifth and sixth internode length (Figure 4A–C). A comparison of the fifth and sixth internode lengths of the transgenic plants of line S13 showed 147.2% ($p=0.0023$) and 143.2% ($p=0.0065$) increase, respectively, over that of the WT plants in the same position (Figure 4C). Similarly, the transgenic line S10 showed longer internodes than WT (Supplementary Figure S4A, B); in particular, the eighth internode had 207% length of that in WT plants ($p=0.0004$) (Supplementary Figure S4D).

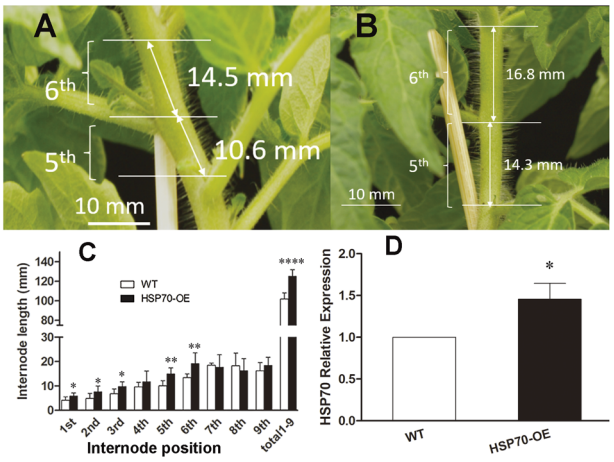

Figure 4. Comparison of the internode length of *SlHSP70-OE* and WT plants. A) Morphological phenotypes of 5th and 6th internodes of WT at 60 DAS. B) Morphological phenotypes of 5th and 6th internodes of *SlHSP70*-OE plant at 60 DAS. C) Comparison the length of internode of *SlHSP70*-OE and WT by each internode position. Internode position was set from cotyledon (the first) to top (the 9th) of the plant. Bar indicates the mean value of the internode length of the six independent plants for each genotype±SD. D) Comparison of the *SlHSP70-1* expression level in the internode of *SlHSP70*-OE and WT. Bar indicates the mean value of the three biological replications for each genotype±SD (*), (**), (****) representative for significant difference at *p*-value less than 0.05, 0.01 and 0.001 by Student's *t*-test, respectively.

The expression level of *SlHSP70*-OE in the stems of the transgenic mutants was higher than that of *SlHSP70*-OE in WT stems (Figure 4B, Supplementary Figure S4C). Since the high expression of *SlHSP70-1* gene leads to a longer internode in transgenic plants, it is suggested that this gene might promote internode elongation via cell division or elongation in tomato.

Next, we compared the morphological phenotypes of *SlHSP70*-OE and *Sliaa9* mutant plants. The shoot length of *Sliaa9* mutants was significantly longer than that of WT with 135% increase in length ($p=0.0014$) under the same condition, while the shoot length of *SlHSP70*-OE and *Sliaa9* mutants showed a similar trend ($p=0.0661$) (Supplementary Figure S5). The differences in the lengths generate a hypothesis that *SlHSP70-1* in association with *SlIAA9* might also be involved in phytohormone signals in controlling the growth and development of tomato plants (Goda et al. 2004)

To investigate the effects of *SlHSP70-1* on leaf size and structure, true leaves from the first to the ninth leaf node position of the mutants at 30 DAF were compared with those of WT. The mutants and WT showed considerable similarity in the appearance of leaf structures as well as leaf complexity at each leaf position (Supplementary Figure S6A, Supplementary Figure S7A). The leaf lengths of the mutants were also compared with those of WT. Although the relative expression level of the *SlHSP70-1* gene in the transgenic plants was double that of the *SlHSP70-1* gene of WT (Supplementary Figure S6B, Supplementary Figure S7B), there were no significant differences in the leaf sizes between the *SlHSP70*-OE and WT plants (Supplementary Figure S6C, Supplementary Figure S7C). Our study shows that the *SlHSP70-1* expression level did not have any considerable effect on tomato leaf morphology.

SlHSP70-1 showed no effect on fruit set, formation, and development

In order to investigate the effects of *SlHSP70-1* on tomato fruit set and the first flowering period, the number of flowers and fruits were observed to calculate the fruit set rate in the *SlHSP70-OE* and WT. Our findings showed that the blooming of transgenic plants started earlier than that of WT (Supplementary Figure S8A). However, though the number of flowers and fruits in WT plants was more than that in transgenic plants (Supplementary Figure S8B, C), there was no significant difference in the fruit set rate between SlHSP70-OE and WT (Supplementary Figure S8D).

For evaluating the effects of the targeted gene on fruit formation, the size of the transgenic fruits at 0, 2, and 4 DAF were measured and compared with that of WT (Supplementary Figure S9A). At four days after anthesis, the level of *SlHSP70-1* in the transgenic fruits was significantly higher than that of WT (Supplementary Figure S9B), while the sizes of the transgenic and WT fruits were not significantly different. This finding suggested that the high expression of the *SlHSP70-1* gene did not contribute much to the fruit formation. The mutant and WT fruits showed almost similar sizes at each time period (Supplementary Figure S9C, D). Fruit sizes of *SlHSP70*-OE plants and WT were compared at 12 DAF and 30 DAF periods. There were no clear differences in the fruit sizes of the *SlHSP70*-OE lines and WT. In summary, these results suggested that the high expression of the targeted *SlHSP70-1* did not clearly contribute to tomato fruit development (Supplementary Figure S9E, F).

mRNA content of SlIAA9, SlDELLA, and SlHSP70-1 in the SlHSP70-OE tomato

To assess the relationships among *SlIAA9*, *SlDELLA*, and *SlHSP70-1* in plant growth and development, we quantified the expression level of *SlIAA9* and *SlDELLA* genes in the leaves, stems, and fruit tissues of the transgenic plants of *SlHSP70-1* and WT. Our findings showed that the gene *SlIAA9* was highly expressed in the leaves of the transgenic plants compared to that in WT plants ($p=0.016$), while the mRNA content of *SlIAA9* showed no significant changes in the stem tissue ($p=0.063$; Figure 5A). Meanwhile, the *SlDELLA* gene was also highly expressed in the leaves of the transgenic plants ($p=0.0286$) but not in the stems of the transgenic plants ($p=0.0168$) (Figure 5B).

To evaluate the role of *SlIAA9* in the expression

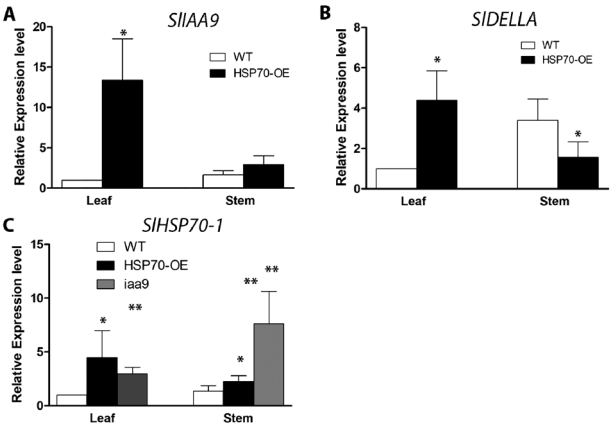

Figure 5. Quantification of mRNA content of *SlIAA9*, *SlDELLA* and *SlHSP70-1*. The expression levels of A) *SlIAA9* and B) *SlDELLA* in leaves and stems of *SlHSP70*-OE plants comparing to WT. C) The expression level of *SlHSP70-1* in leaves and stems of S*lHSP70*-OE, *Sliaa9* mutant and WT plants. Bar indicates the mean value±SD of the three biological replications±SD. (*), (**) showed the significance at $p<0.05$, $p<0.001$ by Student's *t*-test.

of *SlHSP70-1*, we quantified the expression level of *SlHSP70-1* gene in the leaves and stems of the *Sliaa9* mutant plants and subsequently compared the levels with that of WT. The *SlHSP70-1* gene in the *SlHSP70*-OE and that in *Sliaa9* were more highly expressed in both tissues than in WT (Figure 5C). This result shows the hidden relationships between *SlIAA9* and *SlHSP70-1* genes in the leaves and stems of tomato plants. These results also suggest that the expression level of the *SlHSP70-1* gene was likely to be suppressed in the presence of *SlIAA9* gene. This gene expression seemed to be up-regulated when the *SlIAA9* level was down-regulated (Figure 5C).

Discussion

Gene co-expression networks focusing on phytohormone-related genes can extract candidate genes related to tomato growth and development

Gene co-expression networks can be utilized to analyze a big dataset of DNA microarray or RNA-seq for several purposes such as discovering new candidate genes that have specific functions in a biological process, functional annotation, and identifying the regulating elements (van Dam et al. 2018). Therefore, gene co-expression networks are often used to clarify individual objectives for various plant species (Rao et al. 2019; Tai et al. 2018; Wisecaver et al. 2017). Depending on the different objectives, many internet-based packages have been built to analyze the gene expression data (Langfelder and Horvath 2008; Liu et al. 2010). In this study, the minet package was applied to construct the co-expression network of *SlIAA9* and *SlDELLA* from a public microarray dataset. MRNet utilized a method of maximum relevance/minimum redundancy feature selection to decide the candidate genes based on its highly relevant selection criteria (Meyer et al. 2007). GO enrichment analysis showed that genes connecting to *SlIAA9* and to *SlDELLA* have GO terms related to plant growth and development functions in both networks. The greater number of correlated genes with *SlIAA9* in the *SlIAA9* network than that of the *SlDELLA* indicated that *SlIAA9* may participate in more biological processes at the transcript levels than *SlDELLA*. Since the complex *SlIAA9* network contained many uncharacterized genes, it was difficult to find out which candidate gene(s) should be chosen for further analysis (Figure 1). However, the association between the targeted *SlHSP70-1* and *SlDELLA* with direct connection suggested that *SlHSP70-1* might have a strong correlation with *SlDELLA* that acts as a hub in the *SlDELLA* network (Figure 2). The integrated analysis of the networks of *SlIAA9* and *SlDELLA* highlighted *SlHSP70-1* as a potential target for further analysis. The targeted *SlHPS70* gene was directly connected with *SlDELLA*. As we mentioned above, the *SlIAA9* network showed complex connections. The integrated network approach described here suggests a possibility to find candidate genes that may act as key genes for phytohormones in tomato (He and Maslov 2016; Obayashi et al. 2018; Serin et al. 2016).

SlHSP70-1 may regulate internode elongation that might orchestrate with SlIAA9

The targeted gene *SlHSP70-1* belonging to the *HSP70* family in tomato was expressed in most of the organs with various expression levels (Koenig et al. 2013) (Supplementary Figure S10, Supplementary Table S7). The abundant expression of the targeted *SlHSP70-1* in both vegetative and reproductive tissues suggested that the gene family is likely to play roles in tomato growth, development, and fruit ripening (Duck et al. 1989). There are 18 genes in the *AtHSP70* family of which 14 genes are classified in the DnaK subfamily while 4 genes are classified in the Hsp110/SSE subfamily (Sung et al. 2001). Of these, two orthologous genes of *SlHSP70-1* in *Arabidopsis* were elucidated to uncover their physiological roles in plant growth and development, senescence, response to immunity, and heat-shock tolerance (Clément et al. 2011; Li et al. 2016; Noël et al. 2007; Sung et al. 2001). The two genes, *AtHSP70-1* (*AT5G02500.1*) and *AtHSP70-2* (*AT5G02490*.1), exhibited 80% identity in genomic sequence and 92% identity in amino acid sequence. Moreover, both genes and the targeted gene showed high similarity in the amino acid sequence (80%) at the genomic level. The target gene *SlHSP70-1* (*Solyc06g076020.2.1*) showed 80% identity with gene *AtHSP70-1* (*AT5G02500.1*). This suggests that *SlHSP70-1* may contribute to other physiological events although further investigations are required.

The internode elongation length of the *SlHSP70-OE*

and *Sliaa9* mutants may involve cell elongation and/or division in tomato stem. Cell division and elongation are closely related to phytohormone signals, particularly auxin and gibberellin, in which auxin signals promote the activity of auxin-responsive genes directly or via intermediates through gibberellin biosynthesis (Campanoni and Nick 2005; Ross et al. 2000; van den Heuvel et al. 2001). The functions of *SlIAA9* and *SlDELLA* were revealed not only in the process of fruit formation and development but also in the growth of other organs of the tomato plant (Bassel et al. 2008; Wang et al. 2005). The *SlIAA9* was highly expressed in most organs of the tomato plants (Koenig et al. 2013). The point and antisense mutants showed remarkable simplification in leaf structure and internode elongation in tomato (Wang et al. 2005; Zhang et al. 2007). *SlDELLA* also contributed to morphological changes in leaves and internode elongation (Bassel et al. 2008; Nir et al. 2017). In this study, the expression levels of *SlIAA9* and *SlDELLA* in *SlHSP70-OE* leaves were significantly higher than those in WT leaves (Figure 5A, B). The low expression level of *SlIAA9* in the leaves was observed in WT tomato (Koenig et al. 2013). *SlDELLA* was expressed differently in distinct organs, in which the expression levels in the leaves were extremely lower than those in the flowers and stems. These findings implied that the high expression of the *SlIAA9* and *SlDELLA* in *SlHSP70-OE* leaves seems to maintain a stable leaf morphology in tomato.

High expression of the *SlHSP70-1* gene in the *SlHSP70-OE* and *Sliaa9* mutants in stems was associated with internode elongation in tomato (Figure 5C). These results suggested that the expression of the targeted *SlHSP70-1* probably contributed to healthy stem elongation in tomato. Further investigations are necessary to understand how the *SlHSP70-1* gene affects specific phenotypes of *Sliaa9* and *Sldella* mutants.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

MK supervised the study. MK, NTV, KK, NW carried out the experiments, analyzed and interpreted the results. TA and SH provided *Sliaa9* mutant line and TA and HE provided Agrobacterium strain. KK and AF conducted gene co-expression network analysis. NTV, KK and MK prepared the manuscript. All authors have read and approved the manuscript.

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