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Over-expression of sly-miR156a in tomato results in multiple vegetative and reproductive trait alterations and partial phenocopy of the *sft* mutant

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ABSTRACT

Plant microRNAs (miRNAs) are vital components of the translation control system that regulates plant development and reproduction. The biological function of sly-miR156 was investigated by over-expression in tomato plants. Transgenic tomato plants exhibited a drastically altered phenotype, with reduced height, smaller but more numerous leaves, and smaller fruit. The inflorescence structure of sly-miR156 over-expressing plants phenocopied the *sft* mutant. The putative targets of sly-miR156 were identified by data base search and included six SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-box transcription factor genes. Their expression patterns were then determined in 35S-miR156a and wild type tomato plants. These target genes, as well as the tomato *FLOWERING LOCUS T (FT)* ortholog *SFT*, were significantly down-regulated in sly-miR156 over-expressing plants. These studies reveal novel phenotypes regulated by miR156.

1. Introduction

The microRNAs (miRNAs) are a class of 20–22 nucleotide endogenous RNAs that regulate gene expression by digestion or translational repression of target mRNAs [1]. Plant miRNAs are critical for developmental regulation [1]. The SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-box genes encode plant-specific transcription factors that participate in the regulation of multiple developmental processes. Expression of these DNA binding proteins is regulated by the microRNA miR156. Indeed, 10 out of 16 *Arabidopsis* SBP-box genes and 11 out of 19 rice SBP-box genes are the targets of miR156 [2,3]. Among these SBP-box target genes, *AtSPL9* and *AtSPL15* regulate the plastochron length, organ size, and shoot maturation in *Arabidopsis* [4,5]. The gene *AtSPL15* regulates cell number and cell size in *Arabidopsis* [6], while *AtSPL3, AtSPL4, and AtSPL5* temporally regulate vegetable phase changes and flowering [2,7]. The trichome distribution is also controlled by miR156-targeted *AtSPL* genes in *Arabidopsis* [8].

The sequential actions of miR156 and miR172 control developmental timing and flowering in *Arabidopsis* [9,10]. Over-expression of miR156 in rice resulted in severe dwarfism and delayed flowering [3]. In maize, a mutant with increased miR156 expression exhibited severe morphological alterations, including enhanced leaf and tiller formation and deformed inflorescence architecture [11]. In contrast, over-expression of *OsSPL14* in rice led to a superior plant architecture and higher grain productivity [12,13]. Another miR156-targeted SBP-box gene, *Cnr*, has been shown to control fruit ripening in tomato [14].

Tomato is an important vegetable crop that is cultivated worldwide. It is also a robust model plant for research on fresh fruit development. Many tomato miRNAs have been identified by computational homology search and deep sequencing methods [15–18]. However, the biological functions of most miRNAs in tomato are still poorly characterized. In this study, the function of miR156 in tomato plant development was investigated by over-expression of sly-miR156a.

2. Materials and methods

2.1. Construction of plant expression vector and tomato transformation

A 213 bp DNA fragment harbouring the pre-sly-miR156a hairpin structure was amplified by PCR using tomato genomic DNA

Abbreviations: SBP, SQUAMOSA PROMOTER BINDING PROTEIN; FT, *FLOWERING LOCUS* T; SFT, *SINGLE-FLOWER TRUSS*; SP, *SELF-PRUNING*

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as the template and primers sly-miR156a-Fw (5′-AAAATCTCTAATT TAGTTGTTTGTTTTTTG-3′) and sly-miR156a-Rv (5′-CCTTCACCTCTT TCGTAAAAATATTTAAATCT-3′). The DNA fragment was inserted into the pMD18-T vector (Takara, Japan) for sequencing, and then sub-cloned into the plant binary vector pBI121 to produce the pBI-slymiR156a plasmid driven by the CaMV 35S promoter. The plasmid was transformed in tomato (*Solanum lycopersicum* cv. Ailsa Craig) using *Agrobacterium tumefaciens* strain C58.

2.2. RNA isolation and Northern blot analysis

Total RNA was extracted from plant tissues using TRIzol reagent (Invitrogen, USA) and separated on a 15% denaturing polyacrylamide/TBE/8 M urea gel. The TBE contained 8.9 mM Tris, 8.9 mM boric acid, and 20 mM EDTA. About 30 μg of RNA was separated per gel. Separated bands were transferred to Hybond-N^+ membrane (Amersham Biosciences, USA). The DNA oligonucleotide miR156R (5′-GTGCTCTCTATCTTCTGTCAA-3′) is the exact reverse-complementary sequence of sly-miR156a. It was end-labelled with [γ-^{32}P] ATP and T_4 polynucleotide kinase (New England Biolabs, USA) to generate a highly specific tagged hybrid probe to detect sly-miR156 expression. Hybridization was performed overnight at 37 °C in 5 mL of hybridization buffer containing 7% SDS in 0.20 M sodium phosphate (pH 7.0). Probe-treated membranes were then washed twice at hybridization temperature with 3× SSC containing 0.1% SDS for 10 min, and exposed to Kodak MS film for 7–10 days.

2.3. Real-time RT-PCR

Total RNA (3 μg) was pre-treated with DNase I (Promega) and reverse transcribed using ReverTra Ace (TOYOBO, Japan). The resulting cDNA was diluted to 100 ng/μL with RNase-free water, and 5 μL was used as the template in a 20 μL PCR reaction. Real-time PCR was performed after a pre-incubation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s, in the LightCycler 480 system (Roche, Switzerland) using SYBR Green I Master (Roche, Switzerland). The sequences of the specific primers are listed in Supplementary Table S1. The β-actin transcripts were used as internal controls. Compared Ct method was used for the data analysis.

3. Results

3.1. Over-expression of sly-miR156 in tomato

To analyse the function of miR156 in tomato, a 213 bp DNA fragment harbouring the hairpin structure of pre-Sly-miR156a (Fig. S1) was over-expressed in tomato. A total of 120 independent kanamycin-resistant plants were obtained. The transgenic plants were validated for presence of the transgene by PCR and Southern blotting (Fig. S2A and B). Expression of pre-miR156a was quantified by RT-PCR (Fig. S2C). Three independent transgenic lines (line 53, 56, and 83), containing a single copy of the transgene expressed at high levels, were chosen for further analysis (Fig. S2). The accumulation of mature miR156 in different plant tissues was analyzed by Northern blotting. In wild type plants, miR156 was abundant in bud and leaves, moderately expressed in flower, fruit and root, and barely detectable in stem (Fig. 1). In contrast, transgenic plants showed abundant miR156 accumulation in all these tissues (Fig. 1), indicating successful miR156 over-expression.

3.2. Phenotypes of transgenic plants

The transgenic plants displayed multiple morphological changes. Plants were dwarfs (Fig. 2A) and the leaves were much smaller than wild types (Fig. 2B and H). The fully opened complex leaves and leaflets of transgenic plants were about 50% smaller than wild type leaves (Fig. 2C and D). However, the leaf number (node number) was significantly increased and the internodes were drastically reduced (Fig. 2B, E and F). The lateral shoots developed very early, even in the axil of cotyledons in young seedlings (Fig. 2E). Subsequently, the lateral shoots developed vigorously, and almost every leaf axil formed a lateral shoot (Fig. 2G), giving the plant a "bushy" phenotype (Fig. 2N). The inflorescences of wild type tomato plant were examined (Fig. 2I, left); there were very few definitive vegetative inflorescence shoots generated (≈5% of the total). In transgenic plants, however, more than 50% of the inflorescences generated additional vegetative inflorescence shoots (Fig. 2I, right, and Fig. 2M). This phenotype is similar to the *sft* mutant [19]. The wild type fruits formed clusters (Fig. 2J, left), but the transgenic fruits scattered on the branch (Fig. 2J, right). The transgenic plants produced both smaller and fewer fruits than the wild type plants (Fig. 2N).The average fruit weight of transgenic plants was only 39–45% of the wild type weight (Fig. 2 N and O). In addition, the fruit yield per plant was only 20–30% of the wild type plants (Fig. 2P) indicating a drastic reduction in fruit production. When grown under the same conditions, dense aerial roots developed on the stems of transgenic plants, while none appeared on the stems of wild type plants (Fig. 2L). By dissecting the stems, we observed that the pith was undetectable in the stem of the transgenic plants (Fig. 2K).

3.3. Expression patterns of target genes

The target genes of miR156 identified by searched against the SGN unigene database using the psRNATarget web server (http://bioinfo3.noble.org/psRNATarget/) and tomato miRNA target prediction tool (http://ted.bti.cornell.edu/cgi-bin/TFGD/sRNA/target.cgi). After manually excluding the redundant sequences, 9 unigenes were chosen as candidate targets of sly-miR156 (Supplementary Table S2). Among them, six belonged to the SBP-box gene family; one was a putative pyruvate kinase gene, and two had no annotation (Supplementary Table S2). The phylogenic tree indicated that these six SBP-box genes were the homologues of *AtSPL2, AtSPL3, AtSPL6 and AtSPL15*, which are the targets of miR156 in *Arabidopsis* (Supplementary Fig. S3).

The tissue expression patterns of these putative target genes in transgenic and wild type tomato plants were determined by real-time RT-PCR (Fig. 3). All six SBP-Box target genes, as well as an unidentified protein gene (SGN-U345132), were significantly less abundant in transgenic plant tissues compared to wild type plants. The putative pyruvate kinase gene SGN-U313540 was also down-regulated in some transgenic tissues, but not to the same extent as the SBP-box genes, indicating this gene may be a target of sly-miR156, but is also possible under other regulatory mechanisms. However, the accumulation of SGN-U321529 was not significant different from wild type plants. It was reported

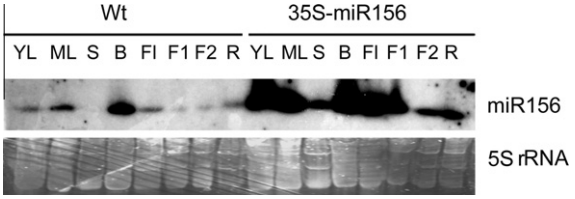

Fig. 1. Northern blot analysis of the expression of miR156 in 35S-miR156a and wild type tissues. YL, young leaf (⩽1 cm length); ML, mature leaf (6th full-opened leaf from shoot apex); S, stem; B, bud; Fl, flower; F1, fruit of 1 cm in diameter; F2, fruit of 4 cm in diameter; R, root. The EtBr staining of rRNA was used as a loading control.

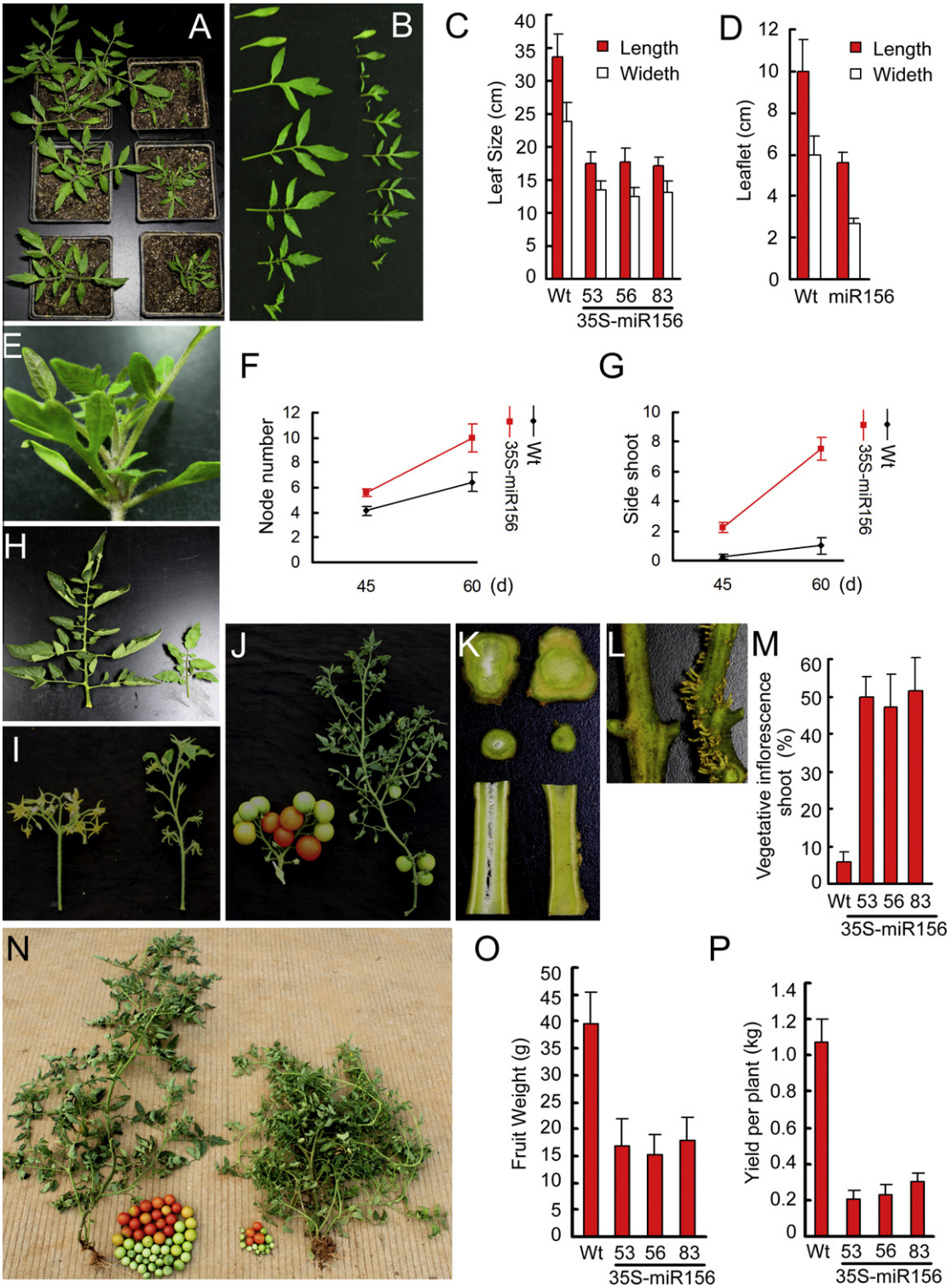

Fig. 2. Phenotypes of 35S-miR156a tomato. (A) Seedling of wild type (left panel) and 35S-miR156a tomato (right panel). (B) Leaves of wild type (left panel) and 35S-miR156a (right panel) tomato seedling. (C) The leaf size (width and length) and (D) the leaflet size of wild type and 35S-miR156a tomato. Wt, wild type; 53, 56 and 83 are three 35S-miR156a lines. The error bar indicates the SE, $n = 15$. (E) The side shoots developed at the axil of cotyledons of 35S-miR156a plant. (F) The node number and (G) the side shoot number of wild type and 35S-miR156a plant at 45 and 60 days after sowing. The error bar indicates the SE, $n = 15$. (H) Mature leaf, (I) inflorescence, (J) fruit bunches, (K) stem pith, and (L) stem air root of wild type (left panel) and 35S-miR156a (right panel) tomato plants. (M) The percentage of vegetative inflorescence shoots formed. Wt, wild type; 53, 56 and 83 are three 35S-miR156a lines. The error bar indicates the SE, $n = 15$. (N) The whole plant of wild type (left panel) and 35S-miR156a (right panel) tomato. (O) The fruit weight and (P) the fruit yield of the wild type and 35S-miR156a tomato. Wt, wild type; 53, 56 and 83 are three 35S-miR156a lines. The error bar indicates the SE, $n = 15$.

that the expression of some target mRNAs and miRNAs are not negatively correlated [20], thus we can not rule out this gene as a target of sly-miR156 base on only the no change at transcript level.

3.4. SFT gene was down-regulated in transgenic tomato

The 35S-miR156 plant phenocopied the inflorescence structure of the *sft* (*SINGLE-FLOWER TRUSS*) mutant [19]. Thus, the

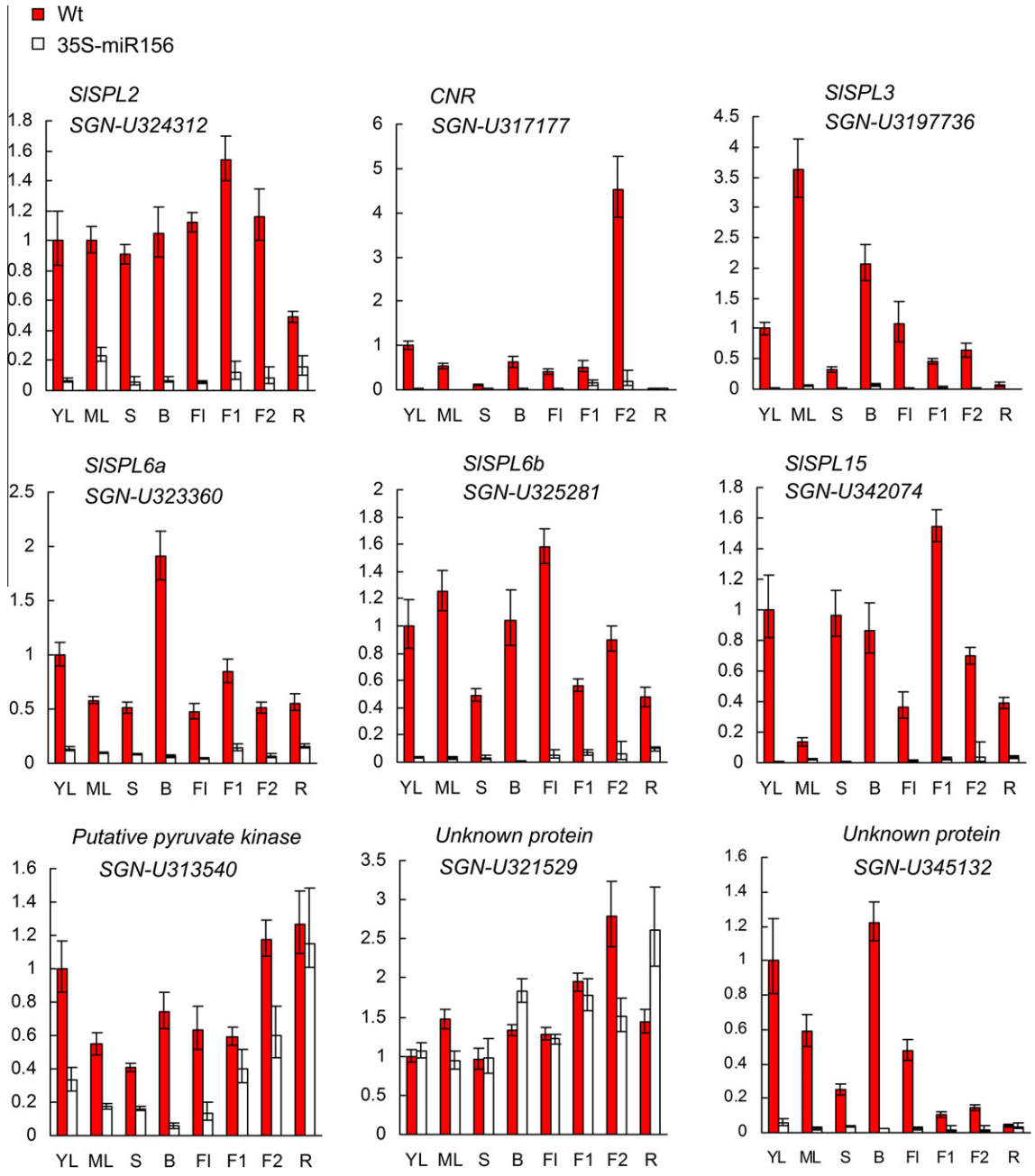

Fig. 3. Real-time RT-PCR analysis of the expression of the target genes of miR156 in wild type and 35S-miR156a tomato tissues. YL, young leaf (⩽1 cm length); ML, mature leaf (6th full-opened leaf from shoot apex); S, stem; B, bud; Fl, flower; F1, fruit of 1 cm in diameter; F2, fruit of 4 cm in diameter; R, root. The error bar indicates the SE of three biological replications (T_2 homozygote progenies of L53, 56 and 83 and three independent wild type plants) and three technological replications.

expression of *SFT* and the related *SELF-PRUNING* (*SP*) gene was monitored in the 35S-miR156 and wild type plant tissues. As shown in Fig. 4, the *SFT* transcripts were significantly lower in 35S-miR156 plants relative to wild types controls. The expression of the *SP* gene was slightly lower in leaf but higher in bud of the 35S-miR156 plants compared to wild types (Fig. 4). Tomato sympodial shoot development is regulated by the SFT/SP balances [21], so the aberrant vegetative inflorescence shoots of 35S-miR156 plants may be attributed to the decreased SFT/SP ratio.

4. Discussion

Transgenic plants constitutively over-expressing miR156 have been constructed in *Arabidopsis* [7], rice [3], and maize [11]. In all these species, and in our miR156 over-expressing tomato plants, similar phenotypes such as dwarfism, a "bush-like" structure, more abundant leaves, shorter plastochron, and later flowering were displayed, suggesting an evolutionarily conserved function of miR156 and SBP-box target genes in plant development. The miR156 over-expressing maize produced more prop roots and our miR156 over-expressing tomato produced numerous adventitious roots, indicating that miR156 plays a common role in adventitious root development. However, some new roles for sly-miR156 were revealed by analysis of 35S-miR156 tomato plants, including control of stem pith, fruit size, and inflorescence structure.

An epigenetic mutation in a tomato SBP-box gene (*Colorless non-ripening, Cnr*) resulted in colourless non-ripening fruits [14]. Cleavage of the *CNR* by miR156 was also demonstrated by 5′-RACE

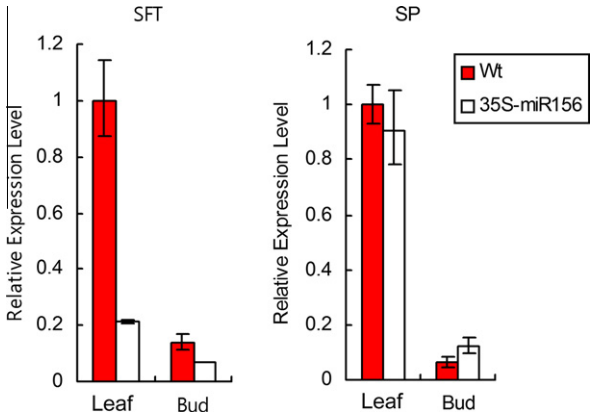

Fig. 4. Real-time RT-PCR analysis of the expression of *SFT* and *SP* gene in wild type and 35S-miR156a tomato. The 6th full-opened leaves from the shoot apex were used in this analysis. The error bar indicates the SE of three independent replications.

analysis [17]. In our 35S-miR156 tomato plants, we noticed that the fruit red colour was slightly lighter than the wild type (data not shown). However, the 35S-miR156 tomato fruits did ripen completely (just later). This could be due to the fact that the over-expression of miR156 down-regulated, but did not eliminate, the expression of *CNR* genes.

In *Arabidopsis*, the FT/FD- and miR156-regulated SPLs pathways converge on an overlapping set of targets at the shoot apex to regulate flowering [10]. The expression of *SFT* may be affected by miR156/SBP-box genes through negative feedback loops; however, the detailed interactions between these two pathways are still unclear. The *sft* mutant tomato produced large fruits and showed a heterosis in fruit yield [22]. In contrast, the 35S-miR156 transgenic tomato plant produced smaller fruits and showed a dramatic decrease in fruit yield, implying *SFT* is only one member of a set of genes affected by miR156/SBP-box genes.

Studies in rice have shown that expression of an *OsSPL14* allele, a mutant gene not regulated by miR156, exhibited higher grain productivity [12,13]. Our 35S-miR156 tomato showed not only a reduced fruit number, but also a decreased fruit weight, implying that miR156 plays an important role in fresh fruit development. Thus, the release one of the SBP-box genes from miR156 control could lead to a higher fruit yield. The expression of miR156 prolonged the vegetative growth and enhanced vitality, traits that are favourable for plant survival under some environmental conditions. Efficient food production, however, depends on shorter growth periods (faster maturation) and higher fruit yields per plant. Thus, tomato plant varieties with reduced miR156 expression could be developed with higher fruit yield and shorter ripening time, at least under appropriate growing conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.12.036.

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