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Overexpression of *SlREV* alters the development of the flower pedicel abscission zone and fruit formation in tomato

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ABSTRACT

Versatile roles of REVOLUTA (REV), a Class III homeodomain-leucine zipper (HD-ZIP III) transcription factor, have been depicted mainly in *Arabidopsis* and *Populus*. In this study, we investigated the functions of its tomato homolog, namely SlREV. Overexpression of a microRNA166-resistant version of *SlREV* (35S::REV^{Ris}) not only resulted in vegetative abnormalities such as curly leaves and fasciated stems, but also caused dramatic reproductive alterations including continuous production of flowers at the pedicel abscission zone (AZ) and ectopic fruit formation on receptacles. Microscopic analysis showed that meristem-like structures continuously emerged from the exodermises of the pedicel AZs and that ectopic carpels formed between the first and second whorl of floral buds in 35S::REV^{Ris} plants. Transcriptional data suggest that *SlREV* may regulate genes related to meristem maintenance and cell differentiation in the development of the flower pedicel abscission zone, and modulate genes in homeodomain and MADS-box families and hormone pathways during fruit formation. Altogether, these results reveal novel roles of *SlREV* in tomato flower development and fruit formation.

1. Introduction

Flowering is an important process that determines fruit and seed production in angiosperms. It consists of two seemingly distinct phases: a transition phase from vegetative to reproductive development and a floral organogenesis phase for pattern formation. Upon floral transition, shoot apical meristem (SAM) switches into inflorescence meristem (IM) to initiate floral meristems (FM) on its flanks afterwards. Specific floral organ primordia subsequently emerge on each position of FM, after which they differentiate into various reproductive structures. Once the primordia of the female reproductive organs (carpels) are formed, floral stem cells are genetically programmed to terminate. This process, called FM determinacy [1], is an important step which can directly affect normal blooming, pollination and fertilization, and fruit development.

The flower development is controlled by a complicated regulation network which involves diverse transcription factor families, such as the MADS-box family [2,3] and the Homeodomain (HD) family [4–7]. REVOLUTA is a transcription factor known to regulate vegetative development in Arabidopsis [8] and Populus [9], but alteration of its expression also caused flower deficiency [10], suggesting its roles in reproductive development. It belongs to the family of homeodomain. The homeodomain is highly conserved among diverse proteins from different kingdoms, participating in a wide variety of developmental processes. For instance, when a HD binding to one or two Meionx domains forms a KNOX or a BELL protein, it can interactively regulate meristem maintenance [11], floral transition and inflorescence determination [12]. When it is attached to a finger domain (forming a PHD) or a zinc finger (forming a ZF-HD), the proteins influence flower pollen maturation [13] and floral development [6], respectively. Mostly, proteins possessing a HD and a leucine zipper domain (HD-ZIP) are involved in multiple activities including response to environmental conditions [14], meristem regulation [15], organ and vascular development, hormones action mediation, trichome branching [16], ethylene biosynthesis and floral organogenesis [7]. Class III homeodomain leucine-zipper (HD-ZIP III) transcription factors as one subfamily of HD-ZIP play prominent roles in embryo [17], root [18], shoot and leaf development [19]. It is well documented that these miRNA165/166-mediated subfamily genes are required for the SAM establishment, vascular development, and polarity formation of lateral organs by promoting the adaxial identity. Among the five single mutant of HD-ZIP III genes (*REV*, *PHABULOSA* [*PHB*],

Abbreviations: AZ, abscission zone; FM, floral meristem; HD-ZIP, homeodomain-leucine zipper protein; REV, REVOLUTA; SAM, shoot apical meristem.

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PHAVOLUTA [*PHV*], *INCURVATA4*/*CORONA* [*ICU4*/*CNA*], and *ATHB8*) in Arabidopsis, only *rev* has readily observable phenotypes [19]. The loss-of function-of *REV* leads to a failure in producing axillary meristems and functional floral meristems [8]. Conversely, the gain-of-function mutants of *rev-d*, *phb-d*, *phv-d*, *avb1*, and *icu4* in Arabidopsis and *rld1* in maize (*Zea mays*), each introducing a single-nucleotide mutation within the miR165/166 binding site, leads to a dominant repression of HD-ZIP III transcripts, and subsequently results in the ectopic expression of gene products [10,20–23]. One of the mutations *avb1* in Arabidopsis causes enlarged meristems, adaxialized leaves, fasciated inflorescence and ectopic carpels [10]. In Populus, overexpressing the miR165/166-resistant form of *popREVOLUTA* caused an abnormal formation of cambia within cortical parenchyma leading to production of secondary vascular tissues in reverse polarity [9]. The same strategy has also been used in rice (*Oryza sativa*): constitutive expression of miR166-resistant *OSHB1m*, *OSHB3m* and *OSHB5m* affects the adaxial-abaxial patterning of leaves and vascular bundle development [24]. However, only a few regulators are found in the regulation network of HD-ZIP III. LITTLE ZIPPER encoding small leucine zipper-containing proteins are suggested to form a negative feedback with Arabidopsis REV (AtREV) [25]. Another study showed that HD-ZIP III are restricted by miR165/166 via competition of ARGONAUTE10 (AGO10) with AGO1 to regulate Arabidopsis SAM initiation [26]. Recently, a genome-wide binding-site analysis of AtREV indicates that several genes like *YUCCA* in auxin biosynthesis and HD-ZIP II transcription factors are directly targeted by AtREV and involved in the shade avoidance response pathway [27]. These findings suggest the versatile roles of REV in plant development, but its regulatory network in flower or fruit development remains elusive.

Tomato (*Solanum lycopersicum*) is a model plant for research on flesh fruit. In this work, we probed the roles of REV in tomato by overexpressing *SlREV* and a miR166-resistant form of *SlREV*, and found that alteration of *SlREV* expression not only caused vegetative abnormalities but also affected the development of the floral pedicel abscission zone (AZ) and fruit formation. Furthermore, we performed transcriptional analysis to screen genes that are potentially regulated by SlREV in the formation of tomato fruit and the development of pedicel AZ. These results provide new insight into the regulation network of SlREV in reproductive development.

2. Materials and methods

2.1. Plant material and growth conditions

Wild-type (WT) tomato (cv. Micro-TOM) and transgenic plants were grown in a vermiculite-perlite mix with nutrition soil under greenhouse conditions with a temperature ranging from 18 °C to 25 °C. For in vitro culture, seeds were surface sterilized by treatment with a 5% sodium hypochlorite solution for 15 min, rinsed three times with sterile distilled water, and sown on 1/2 Murashige and Skoog (MS, Sigma) culture medium containing 0.8% (w/v) phytoagar without sucrose (pH 5.8). Seedlings were transferred into a growth chamber with a 16-h light (25 °C)/8-h dark (18 °C) period (photosynthetic photon flux density: 50–70 $\mu mol\ m^{-2}\ s^{-1}$).

2.2. Plasmid construction and plant transformation

Previously, a tomato homolog of *REV* gene (SGN-U562744 in Tomato Sol Genomic Network database, http://solgenomics.net/) was validated being cleaved by *Sly*-miR166 with 5′ rapid amplification of cDNA ends (5′ RACE) experiment [28]. Based on the sequence of SGN-U562744, a 2523 bp coding fragment was cloned with primers *SlREV*f_F: 5′-CTGGGGTTTGGATTTGAGGGAA-3′ and *SlREV*f_R: 5′-CCCCTCATTCAACTGTGTTCCT-3′. To generate miR166-resistant *SlREV* clone (named $SlREV^{Ris}$), 4 synonymous nucleotide substitutions in the *Sly*-miR166 target site of *SlREV* were introduced by two-step PCR mutagenesis. The *SlREV*f_F primer and $SlREV^{Ris}$_R primer: 5′-GGCAAAAATCCCAACTGAATC**A**GG**T**CC**T**GG**T**TTCATCCCAGGCATCGGG-3′ were used to amplify a partial fragment of 623 bp. This amplicon used as the forward primer was combined with the reverse primer *SlREV*f_R to obtain the full-length of $SlREV^{Ris}$. Then, both *SlREV* and $SlREV^{Ris}$ fragments were cloned into the modified binary vector pLP100 under the CaMV 35S promoter. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation according to Ren et al. [29]. Transformants were first selected on kanamycin (100 mg/L) and then confirmed by both PCR and GUS (β-glucosidase) staining.

2.3. Histological analysis

Histological preparations were performed according to Gabe [30]. Multiple flower buds of 2–3 mm in length at stage 8–9 [31] and 2 mm in length of pedicel AZ were embedded in FAA solution [50% (v/v) ethanol, 5% (v/v) acetic acid and 3.7% (v/v) paraformaldehyde]. After placed under vacuum for 10 min, the samples were incubated at room temperature for 24 h, and then were dehydrated in gradient ethanol and embedded in paraffin (Paraplast plus; Sigma). For light microscopy analysis, 8 mm-thick sections were stained with 0.05% toluidine blue, and observed under a light microscope (OLYMPUS BX-URA2, Japan).

2.4. Digital gene expression profile

Total RNA was isolated from 1 to 2 mm floral buds at stage 6–8 of three individual 35S::REV^{Ris} (T2 generation of Line 1) plants and corresponding WT controls by TRIZOL method. RNA quantity and quality were assayed in the Agilent 2100 Bioanalyzer (Agilent Technologies). Three aliquots of RNA from transgenic or WT plants were pooled, respectively, and sent to Illumina Cluster Station and Illumina HiSeq™ 2000 System (BGI Inc.) for RNA library construction and deep sequencing. Clean tags were obtained after quality filtering, and mapped to the annotated genome sequence of *S. lycopersicum* in Tomato Sol Genomic Network database (http://solgenomics.net/). The number of tags for each gene was normalized to Tags Per Million (TPM) to obtain the gene expression data. Differentially expressed genes between 35S::REV^{Ris} and WT samples were analyzed as described [32]. Genes with a false discovery rate (FDR) ≤ 0.001 and fold change ≥ 2 were considered to be differentially expressed. Gene Ontology analysis was performed as described by Nicholas [33] by using Arabidopsis (*Arabidopsis thaliana*) best BLAST hits as input. The transcriptome data have been deposited in NCBI's Gene Expression Omnibus [34] and are accessible through GEO Series accession number GSE58305 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58305).

2.5. Quantitative reverse transcription-PCR (QRT-PCR)

Total RNA was treated with DNase (Fermentas) prior to RT by using the RevertAid™ first strand cDNA synthesis kit (Fermentas). PCR was performed in a Bio-Rad CFX96 with real-time PCR reagents (SsoFast™ EvaGreen® Supermix, Bio-Rad). PCR conditions were as follows: 5 min at 95 °C, then 42 cycles of 5 s at 95 °C, 5 s at 60 °C and 5 s at 72 °C. The sequences of the specific primers are listed in Supplementary Table S1. *ACTIN* was used as a reference gene. The relative quantification of gene expression level was determined by the comparative C_T method $2^{-\Delta\Delta C_T}$ [35].