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Functional plasticity of miR165/166 in plant development revealed by small tandem target mimic

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ABSTRACT

MicroRNA 165 and 166 (miR165/166) is composed of nine members and targets five members (*PHB*, *PHV*, *REV*, *ATHB8* and *ATHB15*) of the *HD-ZIP III* transcription factor family. Mutants generated by traditional methods could hardly reveal the overall functions of miR165/166 in plant development. In this study, the expressions of all miR165/166 members were simultaneously blocked by over-expressing STTM165/166-31 in *Arabidopsis* and tomato for functional dissection of miR165/166 family. Following a down-regulation of over 90% endogenous miR165/166, the target *HD-ZIP III* genes were correspondingly up-regulated in the STTM transgenic *Arabidopsis* and tomato plants. Notably, the STTM165/166-31 over-expressed *Arabidopsis* and tomato displayed pleiotropic effects on development which were not frequently observed in previously identified genetic mutants of either individual miR165/166 gene or any of the five target genes. Furthermore, the transgenic *Arabidopsis* showed increased IAA content and decreased IAA sensitivity accompanied by enhanced expressions of genes responsible for auxin biosynthesis and signaling, suggesting possible roles of auxin in mediation of miR165/166-regulated processes. Importantly, the transgenic *Arabidopsis* exhibited the improved behavior under salt stress. Overall, such diverse variations in plant development and physiological process revealed by STTM165/166 demonstrate a key role of miR165/166-mediated network in regulating plant development and responses to abiotic stresses.

1. Introduction

MicroRNAs (miRNAs), a major class of 21–24 nt endogenous non-coding RNAs, regulate the expression of protein-coding genes by affecting the mRNA stability or translation of the target genes [1]. A number of miRNAs have been revealed to play important roles in plant growth and development [2–4]. One of such well-defined miRNAs in plants is miR165/166. There are two miR165 genes, miR165a and miR165b, and seven miR166 genes (miR166a-g) in the *Arabidopsis* genome [1]. The mature miR165 and miR166 sequences are almost identical except for C/U difference at position 17. Transgenic *Arabidopsis* over-expressing each of miR165a, miR165b and miR166a displayed similar phenotypic alterations, suggesting functional redundancy of miR165/166 family [5–7].

MiR165/166 has been characterized to down-regulate the expressions of five members of class III homeodomain-leucine zipper (*HD-ZIP III*) transcription factor gene family, namely *PHABULOSA* (*PHB*)/*ATHB14*, *PHAVOLUTA* (*PHV*)/*ATHB9*, *INTERFASCICULAR FIBERLESS*/*REVOLUTA* (*IFL1*/*REV*), *INCURVATA4*/*CORONA*/*ATHB15* and *ATHB8* [8]. Increasing data showed that miR165/166 and their targets *HD-ZIP III* genes regulate a number of plant developmental processes, such as shoot apical meristem (SAM) and lateral meristem formation, leaf adaxial/abaxial polarity, vascular patterning, and floral development [5–7,9,10].

As a highly conserved gene family in land plants, the five *HD-ZIP III* genes also have been identified to possess a complex pattern

Abbreviations: HD-ZIP III, class III homeodomain-leucine zipper; IAA, Indole-3-acetic acid; PHB, PHABULOSA; PHV, PHAVOLUTA; REV, REVOLUTA; SAM, shoot apical meristem; STTM, small tandem target mimic.

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of overlapping functions [11]. Loss-of-function mutants of *phb*, *phv*, *athb8* and *athb15* exhibit indistinguishable phenotypes compared to the wild-type except that loss-of-function mutations in *ifl1*/*rev* result in a clear mutant phenotype [12]. *ifl1*/*rev* mutant was screened from ethylmethanesulfonate-mutagenized populations of *Arabidopsis* and showed altered differentiation of interfascicular fibers and secondary xylem, reduced auxin transport in the inflorescence stems and reduced lateral branches [13]. The *phb phv rev* triple mutant exhibited leaves lacking adaxial characters and SAM [8,14]. However, the gain-of-function mutants in the *phb*, *phv* and *rev* genes generated by disrupting the miR165/166 binding sites displayed leaves with adaxial trait [5,10,14–16].

The redundant functions of miR165/166 genes and their target *HD-ZIP III* genes suggest an extreme complexity of miR165/166-mediated regulatory network and its resultant physiological functions. Thus, loss-of-function alleles or gain-of-function alleles of a single gene either in miR165/166 family or in *HD-ZIP III* members are unlikely to reveal the full spectrum of such gene regulatory network. It is much needed to study how the high inactivation of miR165/166 family members could affect the expressions of all *HD-ZIP III* transcriptional factors or their related gene network, and consequently what phenotypic variations are masked by such redundancy in miR165/166 and their target gene family members.

Small Tandem Target Mimic (STTM) developed in our lab is a highly effective technology that can target and degrade/block simultaneously all members of a small RNA family, and concomitantly up-regulate all target genes by expression of a simple small artificial RNA structure [17]. This approach is especially useful in revealing the overall functions of specific miRNA families that have multiple members and multiple target genes. STTM consists of two non-cleanable small RNA binding sites, which are partially complementary with the target small RNAs, and linked by an empirically tested spacer of different functional lengths (31, 48 and 88 nucleotides). STTM165/166 is such an artificial structure with two miR165/166 binding sites (24 nt each) that contain a "CUA" bulge opposite to the middle of the miRNAs when binding complexes are formed. STTM165/166-31 and STTM165/166-48 represent that the two miR165/166 binding sites were linked with 31-nucleotide and 48-nucleotide spacer, respectively. The length, thermostability, the secondary structure of the spacer and the two miRNA binding sites *versus* one of STTM, in relation to STTM efficacies, have been analyzed in our previous paper. We also reported that the degradation of miRNA by STTM is possibly through the SMALL RNA DEGRADING NUCLEASE (SDN) enzymes [17]. Other unidentified protein co-factors may also be involved in the miRNA reduction by STTM [18]. Both STTM165/166-48 and STTM165/166-31 were effective enough in triggering the degradation of majority miR165/166 and generated dramatic phenotypic changes in development to a minor different extent, but STTM165/166-31 over-expressed *Arabidopsis* produced a decent amount of seeds for further analysis. STTM165/166-31 transgenic *Arabidopsis* and tomato plants were thus used primarily in this study. The efficacy and the phenotype of STTM165/166 in tomato were evaluated for first time as an application of STTM in fruit crops. The STTM165/166-31 over-expressed *Arabidopsis* was further used to investigate roles of miR165/166 in plant development and their gene regulatory network in detail.

Our data demonstrated that over-expression of STTM165/166-31 not only significantly induced the degradation of overall mature miR165/166 in corresponding to an up-regulation of *HD-ZIP III* genes in both *Arabidopsis* and tomato, but also documented varieties of strong and distinct phenotypic effects on plant developments that were not observed in previous genetic studies. Furthermore, the STTM165/166-31 over-expressed *Arabidopsis* showed enhanced expressions of auxin synthetic genes, increased IAA content and decreased IAA sensitivity, suggesting important roles of miR165/166 for auxin signaling. Of interest, the STTM165/166-31 over-expressed *Arabidopsis* exhibited improved behavior under salt stress. Finally, a modified model with new role of miR165/166 in their mediated regulatory network in plant biological processes was proposed. Together, our results suggest that STTM is powerful in revealing full functions of miR165/166 including their new functions in auxin signaling and stress responses.

2. Materials and methods

2.1. Plant materials and growth conditions

All *Arabidopsis* lines used here were derived from the Columbia-0 background (Col). *Arabidopsis* were grown in a controlled environmental room at 22 °C with a relative humidity of 60% under long day conditions (16 h light and 8 h dark) with white light illumination. Tomato seeds of the *Solanum lycopersicum* cv. Ailsa Craig were used and planted under the following conditions: 16 h light/8 h dark cycle and 25 °C.

Arabidopsis seedlings were germinated on MS plates for two days. For IAA response assays, 2-day-old seedlings were transferred onto new agar medium with or without 1 μM IAA and grown vertically for another 3 or 6 days. Lateral root numbers were counted under a dissection microscope. Root elongation was measured using the ImageJ software. For salt treatment, 2-day-old seedlings were moved onto new agar medium with 0, 170, 180, 190 or 200 mmol L^{-1} NaCl. To evaluate the root growth, the plates with seedlings were placed vertically in upside down position for additional 6 d. The seedlings with white color leaves were counted. Seedlings with a white and a green cotyledon were counted as white. Values are mean ± SE (n = 30 seedlings).

2.2. Transformation of Arabidopsis and tomato

Agrobacterium tumefaciens GV3101 containing the STTM165/166-31 vector were used for the *Arabidopsis* transformation by a simplified floral dip method [19] and for tomato cotyledon transformation as previously described by Smith et al. [20]. More than 30 T1 transgenic *Arabidopsis* lines and 19 independent T1 transgenic tomato lines were obtained. The proportion of abnormal phenotypes was about 27% for STTM165/166-31 transgenic *Arabidopsis* and about 21% for transgenic tomato. The homozygous transgenic plants were used for further analysis.

2.3. RNA isolation, Northern blot and real-time PCR analysis

RNA isolation, Northern blot and real-time PCR analysis were performed as described previously [21]. Leaves of 7-week-old *Arabidopsis* and tomato were used for real-time PCR analysis, respectively. The expression level of *actin* in *Arabidopsis* and sly-CAC gene in tomato were used as an internal control for gene expressions, respectively. The primers used for amplification of *HD-ZIP III* mRNAs are located on two sides of the miR165 binding sites to ensure that only the uncleaved mRNAs at the miR165/166 target site could be used as templates for amplification. miR165/166 stem-loop RT primer, miR165/166 specific forward primer and universal reverse primer were used for the quantification of the miR165/166 levels. U6 was used as an internal control for miRNA expressions. Values were normalized to *actin* or *U6* and then to vector control plants. The relative quantity of gene expression was calculated using $2^{-\Delta\Delta CT}$ method [22]. Probes and primers used in this work are listed in Supplemental Table S1. The reverse primer of 165-166-STTMSwa31ntlink-PR was used as probe in Northern blots to detect STTM RNA from the transgenic plants. All gene expression data are from three biological replicates.