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The involvement of InMIR167 in the regulation of expression of its target gene InARF8, and their participation in the vegetative and generative development of *Ipomoea nil* plants

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ABSTRACT

The plant hormone auxin plays a critical role in regulating plant growth and development. Recent advances have been made that having improved our understanding of auxin response pathways, primarily by characterizing the genes encoding auxin response factors (ARFs) in *Arabidopsis*. In addition, the expression of some *ARFs* is regulated by microRNAs (miRNAs). In *Arabidopsis thaliana*, *ARF6* and *ARF8* are targeted by miR167, whereas *ARF10*, *ARF16* and *ARF17* are targeted by miR160. Nevertheless, little is known about any possible interactions between miRNAs and the auxin signaling pathway during plant development. In this study, we isolated the miR167 target gene *InARF8* cDNA from the cotyledons of the short day plant (SDP) *Ipomoea nil* (named also *Pharbitis nil*). Additionally, the In-miR167 precursor was identified from the *I. nil* EST database and analyses of *InARF8* mRNA, In-pre-miR167 and mature miR167 accumulation in the plant's vegetative and generative organs were performed. The identified cDNA of *InARF8* contains a miR167 complementary sequence and shows significant similarity to *ARF8* cDNAs of other plant species. The predicted amino acid sequence of InARF8 includes all of the characteristic domains for ARF family transcription factors (B3 DNA-binding domain, AUX/IAA-CTD and a glutamine-rich region). Quantitative RT-PCR reactions and *in situ* hybridization indicated that *InARF8* was expressed primarily in the shoot apices, leaf primordia and hypocotyls of *I. nil* seedlings, as well as in flower pistils and petals. The *InARF8* transcript level increased consistently during the entire period of pistil development, whereas in the stamens, the greatest transcriptional activity occurred only during the intensive elongation phase. Additionally, an expression analysis of both the precursor In-pre-miR167 molecules identified and mature miRNA was performed. We observed that, in most of the organs examined, the *InARF8* expression pattern was opposite to that of *MIR167*, indicating that the gene's activity was regulated by mRNA cleavage. Our findings suggested that *InARF8* and *InMIR167* participated in the development of young tissues, especially the shoot apices and flower elements. The main function of *MIR167* appears to be to regulate *InARF8* organ localization.

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Introduction

The plant hormone auxin is one of the key growth regulators that play a crucial role in developmental processes throughout the life cycle of a plant, including cell division and elongation, vascular tissue differentiation, phototropic responses, flower development and organ abscission (Eckardt, 2001). The identification of a gene family encoding proteins called auxin response factors (ARFs) in *Arabidopsis* provides a good entry to study auxin regulation at the molecular level (Ulmasov et al., 1999a,b; Liscum and Reed, 2002; Tiwari et al., 2003).

Recent studies have indicated that several *ARF* genes are negatively regulated *via* small RNAs at the post-transcriptional level. For example, *ARF2*, *ARF3*, and *ARF4* are repressed by trans-acting siRNA (Williams et al., 2005). Some others are regulated by microRNAs (miRNAs), which are single-stranded RNA molecules of ~21 nt in length that are known to control the expression of many

Abbreviations: ARF, auxin response factor; CTD, C-terminal dimerization domain; DBD, DNA-binding domain; FISH, fluorescence *in situ* hybridization; IAA, indole-3 acetic acid; miRNA, microRNA; LD, long day; MR, middle region.

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developmentally important transcription factor genes, either by cleavage of mRNAs or by translational repression (Bonnet et al., 2006; Zhang et al., 2007; Voinnet, 2009). So far, three miRNAs have been identified that are related to auxin-regulated plant development, namely miR160, miR164 and miR167, targeting *ARF10*, *16,17* (Mallory et al., 2005; Wang et al., 2005; Liu et al., 2007), *NAC1* (Mallory et al., 2004; Guo et al., 2005) and *ARF6*, *-8* genes (Reinhart et al., 2002; Rhoades et al., 2002; Wu et al., 2006), respectively.

The *Arabidopsis* genome encodes 23 known ARF proteins, which have in the N-terminal region a DBD (DNA-binding domain) B3 domain binding the specific nucleotide sequence AuxRE in target gene promoters. ARF proteins can either activate or repress downstream genes, primarily depending on the amino acid sequence of their MR (middle region): a Q/L/S-rich MR determines the protein as an activator, and a P/S/T-rich one does so as a repressor (Tiwari et al., 2003). The C-terminal region of these proteins (except for ARF3 and ARF17) carries a CTD (C-terminal dimerization domain) responsible for dimerization and interactions with other proteins. Within this domain, two characteristic amino acid motifs III and IV have been identified. These motifs were also identified in the AUX/IAA (indole-3 acetic acid) proteins. ARF and AUX/IAA proteins enable the formation of homo- and heterodimers with the participation of these domains. This is the basis for regulating auxin response gene expression (Ulmasov et al., 1999a,b; Tiwari et al., 2003; Woodward and Bartel, 2005). A sequence complementary to miRNA is present in the CTD-coding region of the *ARF6* and *ARF8* transcripts (Reinhart et al., 2002; Rhoades et al., 2002).

Although a few *ARF* genes have been characterized functionally (Tian et al., 2004; Nagpal et al., 2005; Okushima et al., 2005a,b), the functions of many other *ARFs* remain unknown. Recently, the functions of *ARF6* and *ARF8* have been shown by analyzing *arf6* and *arf8* mutant plants. While the *arf8-1* mutation only affected hypocotyl elongation under light conditions (Tian et al., 2004), the null mutants *arf6-2* and *arf8-3* showed slightly enhanced developmental abnormalities (Nagpal et al., 2005). A double mutation in the *arf6* and *arf8* genes, as well as overexpression of *MIR167* in *Arabidopsis*, produce similar phenotypes manifesting, for example, complete sterility (Reinhart et al., 2002; Rhoades et al., 2002; Ru et al., 2006; Wu et al., 2006).

In this research, we describe, for the first time, the element (*InARF8*) of the auxin signal transduction pathway that is regulated by miRNAs for *Ipomoea nil* (a model plant for studies on generative growth). The miR167 target gene *InARF8* cDNA was isolated from *I. nil* cotyledons. Additionally, the In-miR167 precursor was identified from the *I. nil* EST database. Also, analyses of *InARF8* mRNA, In-pre-miR167 and mature miR167 accumulation in the plant's vegetative and generative organs were performed. Our results highlight a possible general regulation of *ARF* expression by miRNAs, indicating the importance of small RNAs in auxin signaling responses in various plant species.

Materials and methods

Plant materials

Seeds of *Ipomoea nil* Chois cv. *Violet* (Marutane Seed Co., Kyoto, Japan) were treated according to Glazińska et al. (2009).

The apexes, cotyledons, hypocotyls and roots were picked after a short night in 5 d of growth and immediately frozen in liquid nitrogen and stored at −80 °C. Additionally, pistils, stamens, sepals and petals were harvested from fully open flowers. Leaves were harvested from 3-week-old plants.

For developmental analysis of pistils and stamens, flower buds were collected at various stages of growth (stage 1–5), from the appearance of flower buds with visible stamens and pistils to fully open flowers (Fig.). Flower elements, pistils and stamens, were collected from all stages of growth. Thereafter, material was frozen in liquid nitrogen and stored at −80 °C for RNA isolation.

Molecular cloning of InARF8 cDNA

The tissue of *I. nil* (1.0–1.5 g) was homogenized in a sterile chilled mortar with a pestle. Total RNA was isolated with Tri Reagent (Sigma–Aldrich, St. Louis, USA) and genomic DNA was removed with Deoxyribonuclease I (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. All primers used were synthesized by the "oligo.pl" Laboratory of DNA Sequencing and Oligonucleotides Synthesis (Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland). One μg of total RNA primed with anchored oligo(dT)$_{19}$ primers was used for first strand synthesis with the RevertAid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. PCR, using degenerated primers 5′-AGATYCTGTWCGCTGSCCAAWTTCAC-3′ (forward) and 5′-TCAACAAAWACAAGCTSCCAGHCTG-3′ (reverse), was performed in the T3 Thermocycler (Biometra, Göttingen, Germany). Primers were designed from sequences conserved between *ARF8* from *Arabidopsis thaliana*, *Solanum lycopersicum* and *Oryza sativa*. Mixtures and conditions of the PCR reaction were prepared according to Glazińska et al. (2009). A 1350 bp amplified cDNA fragment was isolated from an agarose gel with the GeneMATRIX Agarose Out DNA Purification Kit (EurX, Gdansk, Poland), cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced by "oligo.pl". A full-length cDNA coding for *InARF8* was isolated using the BD SMART RACE cDNA Amplification Kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). mRNA used in 5′-and 3′-RACE-PCR was isolated with Dynabeads Oligo (dT)$_{25}$ (Dynal Biotech, Oslo, Sweden). Gene-specific primers for 5′-RACE (5′-CCCCATCATATCCTGGGAAAGAGGCATC-3′) and 3′-RACE (5′-GCACTTAGCGGCTGCAATGTCATCTGTG-3′) were selected from a cDNA sequence of the identified clone using Primer3 (http://frodo.wi.mit.edu/primer3/primer3-code.html). PCR reactions were performed using the Advantage 2 PCR Enzyme System (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). The RACE products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced by oligo.pl.

The identified cDNAs of the *InARF8* gene were analyzed using ClustalW (http://www.ebi.ac.uk/clustalw), BLAST 2.2.25 (http://www.ncbi.nlm.nih.gov/BLAST) ExPASY (http://www.expasy.org) and Search miRBase:Sequences (http://microrna.sanger.ac.uk/sequences/). The phylogenetic analysis was made using the Phylogeny.fr web service (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi). Sequence alignments were carried out using the MUSCLE software and then the Gblocks program was used to eliminate poorly aligned positions and divergent regions. The phylogenetic analysis was performed using the PhyML program. The phylogenetic tree was constructed using the TreeDyn program.

Identification of In-pre-miRNA167 sequence

BLAST (basic local alignment search tool) (Altschul et al., 1990) was used to search for the fragment pri-miR167 including the pre-miRNA sequence in the EST sequences database of *I. nil*. The MiR167 sequence was obtained from the miRBase database (ftp://mirbase.org/pub/mirbase/12.0/) (Griffiths-Jones et al., 2008). Because sequences of miR167 show conservation across different kingdoms (Axtell and Bartel, 2005; Zhang et al., 2006), we use the miRNAs from *A. thaliana*. ESTs that potentially contain miRNA sequences were obtained after the BLAST search. The RNAstructure software (http://rna.urmc.rochester.edu/

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