Characterization of *EMU*, the *Arabidopsis* homolog of the yeast THO complex member *HPR1*

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ABSTRACT

Diverse and precise control is essential for eukaryotic gene expression. This is accomplished through the recruitment of a myriad of proteins to a nascent messenger RNA (mRNA) to mediate modifications, such as capping, splicing, 3'-end processing, and export. Despite being important for every cell, however, the mechanism by which the formation of diverse messenger ribonucleoprotein (mRNP) particles contributes to maintaining intricate systems in the multicellular organism remains incompletely defined. We identified and characterized a mutant gene named *erecta mRNA under-expressed (emu)* that leads to the defective mRNA accumulation of *ERECTA*, a developmental regulator in the model plant *Arabidopsis thaliana*. *EMU* encodes a protein homologous to a component of the THO complex that is required for the generation of functional mRNPs. Further analysis suggested that *EMU* is genetically associated with *SERRATE*, *HYPONASTIC LEAVES1*, and *ARGONAUTE1*, which are required for proper RNA maturation or action. Furthermore, mutations in another THO-related gene led to embryonic lethality. These findings support the presence and importance of the THO-related complex in plants as well as yeast and vertebrates.

Keywords: gene regulation; plant; THO complex

INTRODUCTION

The precise regulation of gene expression is essential throughout the life of an organism. During the transcription of primary messenger RNAs (mRNAs), concomitant processing steps take place, including 5'-capping, intron splicing, and 3'-polyadenylation, after which transport-competent mature mRNAs are exported to cytoplasmic translational machinery. A myriad of different proteins bind to the mRNA as it progresses to form mature messenger ribonucleoprotein (mRNP) particles in the nucleus, and these particles are involved in particular steps of the gene expression pathway. Individual mRNP complex components can be thought of as adaptors that allow mRNAs to interface with the numerous intracellular machineries mediating their processing, subcellular localization, translation, and decay (Hieronymus and Silver 2004; Moore 2005; Luna et al. 2008). Despite

being important for every cell, however, the mechanism by which the formation of diverse mRNPs contributes to maintaining intricate systems in the multicellular organism remains incompletely defined.

MicroRNAs (miRNAs) were discovered through studies of developmental timing and morphogenesis. They are endogenous small regulatory RNAs that are loaded onto effector RNP complexes, mediating various functions at the transcriptional and/or post-transcriptional level—such as mRNA cleavage, translational repression, and regulation of chromatin structure. miRNAs show distinct regulatory functions with respect to their targets, which contain a sequence motif partially complementary to the miRNA; some miRNAs determine spatial accumulation, others play a buffering role that ensures the robustness of their expression level and pattern, and still others establish the temporal expression of target genes (Bartel 2004; Carthew and Sontheimer 2009; Voinnet 2009). Except for a few miRNAs that seem to be products of RNA polymerase III (Borchert et al. 2006), most genes encoding miRNAs are first transcribed by RNA polymerase II to produce primary miRNAs (Cai et al. 2004; Lee et al. 2004; Xie et al. 2005). Accordingly, they are subject to an intricate series of transcriptional regulation, as is the case with protein-coding mRNAs (Pawlicki and Steitz 2010). A link between mRNA and

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miRNA biogenesis pathways has been recently indicated in *Arabidopsis*, and this has been extended to RNA silencing in animals (Gregory et al. 2008; Kim et al. 2008; Laubinger et al. 2008; Gruber et al. 2009; Sabin et al. 2009; Büssing et al. 2010). Connecting these two processes permits more robust and accurate regulation of gene expression, and further inquiry into the crosstalk is expected to reveal additional roles.

Here we identified and characterized *Arabidopsis* genes that encode proteins homologous to the components of the THO complex that is required for generation of functional mRNPs. Our findings support the presence and importance of the THO-related complex in plants as well as yeast and vertebrates.

RESULTS

erecta mRNA under-expressed (*emu*) phenotypes

The recessive *emu* mutant was originally identified as a mutation that decreases the transcript accumulation of *ERECTA* (*ER*), which encodes the founding member of the ER family of receptor kinases that function as key regulators of the morphogenesis of above-ground organs in *Arabidopsis* (Fig. 1A; Torii et al. 1996; van Zanten et al. 2009). Apart from the effect on *ER* expression, the *emu* mutation confers pleiotropic phenotypes. For example, the number of cotyledons varies in *emu* (Fig. 1B–D). Whereas wild-type seedlings invariably produced two cotyledons ($n = 725$), 1.3% of *emu* seedlings had one or three cotyledons ($n = 1287$). During *Arabidopsis* embryogenesis, cotyledons grow out of the apical region of embryos at the heart stage (Barton and Poethig 1993). In wild-type embryos, two protrusions were clearly visible at the peripheral zone of the apical region, where cells in the outermost layer divided only anticlinally, developing into the future epidermis (Fig. 1E). In 2.8% of *emu* embryos, abnormal cell division was observed in the outermost cell layer of the apical region ($n = 319$) (Fig. 1F,G). Such defects in cell division were not observed in wild-type embryos and were previously described in other genetic backgrounds that alter the cotyledon number (Saiga et al. 2008; Ploense et al. 2009). Furthermore, the leaves of *emu* plants exhibited a more serrated margin than wild-type plants during later developmental stages (Fig. 1H,I). These phenotypes are not observed in *er* mutants, and *emu* plants did not display *er* phenotypes (Torii et al. 1996). On the other hand, *emu er* double mutants exhibited a novel phenotype, which neither *emu* nor *er* confers. In these double mutants, elongation of the primary inflorescence ceased prematurely, altering plant architecture (Fig. 1J–M). This is reminiscent of *erecta-like1* (*erl1*) *erl2* plants, suggesting close interactions among *EMU* and *ERL* genes, which are functionally orthologous to *ER* (Shpak et al. 2004). These data suggest general roles for *EMU* in both embryonic and post-embryonic development, rather than a specific role as a regulator of

FIGURE 1. *emu* confers pleiotropic phenotypes. (A) RT-PCR analysis of *ER* expression in *emu*. RNA samples were prepared from shoots grown for 10 d after germination. The β -tubulin gene (*TUB2*) was amplified as a control. (B–D) Wild-type (WT) (B) and *emu* (C,D) seedlings. Plants were grown for 3 d after germination. (E–G) Wild-type (E) and *emu* (F,G) embryos. The same *emu* embryo was visualized at different foci to show three cotyledons (F,G). An asterisk indicates abnormal cell division. (H,I) Leaf series of wild-type (H) and *emu* (I) plants. For each genotype, the left two leaves are cotyledons, and the right-most leaf is the first cauline leaf. Plants were grown for 26 d after germination. (J–M) Wild-type (J), *emu* (K), *er-105* (L), and *emu er-105* (M) plants grown for 46 d after germination. *emu er-105* double mutants exhibited a novel phenotype, which neither *emu* nor *er* confers. Arrows indicate the primary inflorescences. Photographs were taken at the same magnification. Scale bars: 1 mm in B–D; 20 μm in E–G; 1 cm in H,I.

ER expression. The F1 offspring from *emu* and wild-type plants exhibited wild-type appearances, and 23% of F2 offspring displayed *emu* phenotypes ($n = 187$), indicating that *emu* is a single nuclear recessive mutation.

EMU encodes a putative THO complex component, Hpr1

To understand the molecular basis of *emu* phenotypes, the *EMU* gene was isolated using a map-based strategy. Sequence analysis revealed a single base substitution at the At5g09860 locus, and constitutive expression of the At5g09860 cDNA in *emu* restored the wild-type appearance (Fig. 2). Thus, we concluded that the identified mutation accounts for the *emu* phenotype. This C-to-T substitution in the third exon produces a stop codon at position 37, and the resulting

FIGURE 2. Map-based cloning of *EMU*. (A) Genetic mapping of *EMU*. The markers used for mapping are shown as well as the number of recombinants between *emu* and these markers. (B) Gene structure of *EMU/At5g09860*, with exons represented as closed boxes. The site of the lesion and nucleotide change in the *emu* allele is indicated. (C–E) Complementation of *emu*. Constitutive expression of wild-type *At5g09860* cDNA in the *emu* mutant restored the wild-type appearance (D). Plants were grown for 17 d after germination. (F) *EMU* expression was analyzed by RT-PCR. Total RNA was extracted from seedlings grown for 7 d after germination. *EF1αA4* was amplified as a control. Scale bars: 1 cm.

emu product lacks a large portion of the predicted protein. The *emu* mutation reduced the accumulation of the *EMU* transcript, which is presumably induced by nonsense-mediated decay (Fig. 2F).

BLAST searches of nonredundant databases within GenBank revealed that the predicted *EMU* product is similar in amino acid sequence to the Hpr1/p84/pThoc1 protein in *Drosophila melanogaster*, *Homo sapiens*, and *Mus musculus* (Supplemental Fig. S1; Durfee et al. 1994; Rehwinkel et al. 2004; Wang et al. 2006). Although the similarity in primary sequence is low, the predicted *EMU* is also similar to the yeast Hpr1 protein, the founding member of these evolutionally conserved proteins, and stretches of conserved regions are recognizable (Supplemental Fig. S2; Aguilera and Klein 1990). Similar proteins are encoded in the genomes of various plant species, suggesting their functional importance in plants (Supplemental Figs. S1, S3).

THO complex components are conserved in *Arabidopsis*

The yeast Hpr1 forms a multimeric protein complex named the THO complex with the proteins Tho2, Mft1, and Thp2 (Chávez et al. 2000). The human and fruit fly THO complexes were recently characterized, and both were shown to contain homologs of yeast Hpr1 and Tho2 (Sträßer et al. 2002; Rehwinkel et al. 2004; Masuda et al. 2005). However, whether a similar complex functions in plants has not yet been established. Using BLAST searches, we identified *Arabidopsis* genes homologous to the fruit fly THO genes—

THO2 and *THOC6*—named *AtTHO2* (At1g24706) and *AtTHOC6* (At2g19430), respectively (Rehwinkel et al. 2004). In the yeast, human, and fruit fly genomes, a single gene encodes each component of the THO complex. However, in the *Arabidopsis* genome, there are two genes homologous to the fruit fly *THOC5* and *THOC7*, respectively, and these genes will be referred to as *AtTHOC5A* (At5g42920), *AtTHOC5B* (At1g45233), *AtTHOC7A* (At3g02950), and *AtTHOC7B* (At5g16790). No obvious homologs of the yeast *MFT1* and *THP2* genes were identified in the *Arabidopsis* genome. Next, we analyzed expression patterns of these genes using publicly available microarray data. No probe set is available for *AtTHOC5B*; however, expression data for other genes were obtained. *EMU*, *AtTHO2*, *AtTHOC5A*, *AtTHOC6*, *AtTHOC7A*, and *AtTHOC7B* are expressed throughout the plant in a similar manner (Supplemental Fig. S4). Interestingly, the expression of *AtTHOC7B* is highly specific to seed. This may suggest a functional divergence between *AtTHOC7A* and *AtTHOC7B*.

AtTHO2 is required for embryo development

To investigate the biological roles of the *Arabidopsis* homologs of the THO components, we searched for T-DNA insertion mutants of *AtTHO2* and *AtTHOC6*, both of which were predicted to be present as a single-copy gene in the genome. Four and two T-DNA insertion mutants were identified for *AtTHO2* and *AtTHOC6*, respectively (Supplemental Fig. S5A,B). Under our growth conditions, the *atthoc6* mutants did not display discernible morphological phenotypes. Given that this gene is absent in yeast, the lack of a mutant phenotype may imply that *AtTHOC6* is not an essential subunit of the putative *Arabidopsis* THO complex. Alternatively, *AtTHOC6* may exhibit another function in *Arabidopsis*. Lee et al. (2008) suggested that a subset of WD40 proteins that contain a damaged DNA binding 1 (DDB1)-binding WD40 (DWD) motif, including *AtTHOC6*, may act as a substrate receptor for E3 ubiquitin ligase.

Attempts to obtain seedlings homozygous for T-DNA insertions in *AtTHO2* by PCR-based genotyping did not identify homozygous plants, and pale-green ovules were segregated in fruits produced from plants heterozygous for the T-DNA insertion (Supplemental Fig. S5C,D). Therefore, it is likely that *attho2* mutants result in lethality during embryo development. To address this possibility, we examined mature embryos produced from plants heterozygous for the T-DNA insertion. Approximately 25% of the embryos exhibited abnormal morphology (Fig. 3A,B). Consistent with our observation, *AtTHO2* is listed in the “pending additions” page in the SeedGenes database at www.seedgenes.org, of which its goal is to establish a catalog of essential genes in *Arabidopsis*, and is called *EMB2793* (Meinke et al. 2008). Abnormal embryos can be recognized from as early as the heart stage by their retarded development and irregular divisions compared with the corresponding wild-type embryo

FIGURE 3. *attho2* mutations lead to embryonic lethality. (A,B) Mature embryos extracted from a plant heterozygous for the *attho2-1* mutant allele, exhibiting either the wild-type (A) or mutant (B) phenotype. (C) A wild-type late-heart stage embryo. (D) An embryo segregating among wild-type late-heart stage embryos. (E,F) Embryos segregating among wild-type heart stage embryos. Asterisks denote aberrant cell divisions. Scale bars: 100 μ m in A,B; 20 μ m in C–F.

(Fig. 3C–F). These results indicate that *AtTHO2* is indispensable for embryogenesis, suggesting a fundamental role for this gene in *Arabidopsis* development.

Splicing defects in *emu*

Similar to *emu*, a weak loss-of-function allele of *SERRATE* (*SE*), *se-1*, causes a serrated-leaf phenotype and alters the cotyledon number (Clarke et al. 1999; Prigge and Wagner 2001). Moreover, a null *se* allele results in embryonic lethality, which is reminiscent of mutations in *AtTHO2* (Lobbess et al. 2006). Therefore, we generated *emu se-1* double-mutant plants to examine the interaction between these two genes. The leaves of *emu se-1* plants exhibited an upward-curling phenotype as seen in stronger *se-2* and *se-3* alleles (Fig. 4A–D; Grigg et al. 2005). In addition, the double mutant was sterile, although *emu* and *se-1* alone have little impact on seed production (Fig. 4I–L). These results suggest a strong genetic interaction between *emu* and *se-1*.

This strong genetic interaction raised the possibility that EMU and SE may have similar functions. *SE* encodes a zinc finger protein that has been implicated in mRNA splicing (Prigge and Wagner 2001; Laubinger et al. 2008). Interestingly, some functions of the THO complex are dependent on splicing, and studies in animals have indicated that the THO complex interacts with the spliceosome (Rappsilber et al. 2002; Zhou et al. 2002; Masuda et al. 2005; Cheng et al. 2006). To determine whether *EMU* as well as *SE* are involved in mRNA splicing, we characterized the splicing

patterns in *emu* mutant plants by examining transcripts of genes encoding serine/arginine-rich (SR) proteins, which belong to a conserved family of splicing regulators in eukaryotes and are extensively alternatively spliced (Reddy 2007; Long and Caceres 2009). The splicing patterns of SR genes were analyzed by RT-PCR using primers described previously (Palusa et al. 2007). As a result, the alternative splicing patterns of *RS31*, *RS40/SR35*, and *SR34b* were found to be altered in *emu* mutant plants, and the expression of *EMU* cDNA restored the wild-type alternative splicing pattern (Fig. 5). This suggests that *EMU* is involved at least in the regulation of alternative splicing of mRNA.

Genetic interactions between *emu* and miRNA mutants

It was demonstrated that SE also functions in miRNA processing through interacting with *HYL1*, which acts solely in miRNA biogenesis (Lu and Fedoroff 2000; Han et al. 2004; Vazquez et al. 2004; Grigg et al. 2005; Lobbess et al. 2006; Yang et al. 2006; Fang and Spector 2007; Dong et al. 2008; Laubinger et al. 2008). Although there is no evidence that the THO complex plays a direct role in miRNA processing

FIGURE 4. Double mutants between *emu* and miRNA mutants exhibit novel phenotypes. (A–H) Plants grown for 21 d after germination. (I–P) Primary inflorescences of plants grown for 51 d after germination. (A,I) Wild type; (B,J) *emu*; (C,K) *se-1*; (D,L) *emu se-1*; (E,M) *hyl1-2*; (F,N) *emu hyl1-2*; (G,O) *ago1-27*; and (H,P) *emu ago1-27*. Scale bars: 1 cm.

FIGURE 5. Alternative splicing patterns of SR genes are altered in *emu*. RT-PCR analysis of the expression of SR genes in seedlings grown for 7 d after germination. (A) Alternative splicing patterns of some SR genes are altered in the *emu* mutant. (B) Expression of *EMU* cDNA restored the wild-type expression pattern of *RS31*. Either CF701-60 (60), CF701-66 (66), or CF701-64 (64) contained a single copy of the 35S::*EMU* transgene as a homozygote and was used for analysis.

in metazoa, the observed genetic interaction between *emu* and *se-1* prompted us to further examine a potential role for *EMU* in miRNA biogenesis. Therefore, we characterized *emu hyl1-2* double-mutant plants. *emu hyl1-2* plants resembled *emu se-1* plants, conferring upward-curling leaves and sterility (Fig. 4E,F,M,N). Similarly, the *emu* mutation enhanced the phenotypes conferred by mutations in other genes involved in miRNA biogenesis, *HUA ENHANCER 1* and *HASTY* (data not shown; Chen et al. 2002; Bollman et al. 2003; Park et al. 2005; Yu et al. 2005). To directly assess the potential role for *EMU* in miRNA biogenesis, miRNA levels were examined. In *emu* plants, various miRNAs accumulated less than in wild-type plants (Fig. 6). Considering that the mutant phenotype conferred by the *emu* mutation is much weaker than the embryonic lethal phenotype seen in the *attho2* mutants, these mild reductions might be due to a modest effect of *emu* on the putative *Arabidopsis* THO complex. To elucidate whether the slight decrease of miRNA accumulation impacts on miRNA-mediated gene regulation in *emu* plants, we generated double-mutant plants between *emu* and plants containing a hypomorphic allele of *ARGONAUTE1* (*AGO1*), *ago1-27*, because miRNAs interact with AGO1 proteins to regulate gene expression (for review, see Vaucheret 2008). Interestingly, the *emu* mutation enhanced the slightly altered phyllotaxy of *ago1-27* inflorescences, wherein the internode length is more random than that in wild-type plants, resulting in multiple fruits emanating from the same node (Fig. 4O,P; Morel et al. 2002; Smith et al. 2009). Taken together, these data suggest that *EMU* may be involved in assuring proper miRNA action.

DISCUSSION

We identified *Arabidopsis* genes encoding proteins homologous to the THO complex components. Although further studies are required to validate the presence of the THO complex in *Arabidopsis*, this study supports the existence of the THO complex in plants as well as in yeast and metazoa

and underscores the importance of THO-related proteins in the context of plant development.

The discovery that *EMU* encodes an *Arabidopsis* homolog of the yeast Hpr1 is particularly important because the in vivo functions of the THO components in multicellular organisms remain elusive. Recently, a role for mouse Thoc1/Hpr1/p84 during embryogenesis was revealed using mice containing the mutant alleles (Wang et al. 2006, 2007), and researchers have now started to analyze THO components in a physiological context in multicellular organisms. In the present study we showed that mutations in *EMU* and *AtTHO2* compromise *Arabidopsis* development. Particularly, *attho2* mutations lead to embryonic lethality, highlighting the essential role of this factor in *Arabidopsis* development.

Our genetic and molecular analyses shed some light on the putative role of *EMU*. As expected from the functions of its counterparts in yeast and animals, *EMU* appears to be required to guarantee accuracy and proper levels of RNA biosynthesis. The observed genetic interactions between *emu* and *se-1* may provide a clue to the mechanism of action of *EMU*. Interestingly, mutations in the *Arabidopsis* genes that encode the two cap-binding complex (CBC) subunits—CAP-BINDING PROTEIN20 (CBP20) and ABSCISIC ACID HYPERSENSITIVE1 (ABH1)/CBP80—cause a serrated leaf phenotype and mRNA splicing defects as observed in *emu* and *se-1* mutants (Hugouvieux et al. 2001; Papp et al. 2004; Kuhn et al. 2007; Laubinger et al. 2008; Raczyńska et al. 2010). It is also noteworthy that the *abh1-8* mutation enhanced the phyllotaxy defect of *ago1-38* mutant inflorescences as seen in *emu ago1-27* plants (Gregory et al. 2008). Furthermore, *emu* was sensitive to the exogenous application of abscisic acid (Supplemental Fig. S6), as are *cbp* and *se-1*

FIGURE 6. miRNA accumulation is reduced in *emu*. (A) RNA samples were prepared from inflorescence apices. (B) RNA samples were prepared from rosette leaves (left) and inflorescences (right) from plants grown for 27 d after germination. Ethidium bromide-stained gels on which rRNA and tRNA bands are visible are shown as a loading control. The intensity of the hybridization signal relative to WT is indicated and was calculated after normalization to rRNA.

(Hugouvieux et al. 2001; Bezerra et al. 2004; Papp et al. 2004). These observations raise the possibility that EMU is involved in connecting SE and CBC, which are involved in miRNA biogenesis but do not appear to interact directly with each other (Gregory et al. 2008; Kim et al. 2008; Laubinger et al. 2008). Although we were unable to detect the two hybrid interactions among ABH1, EMU, SE, and AtTHO2 observed in yeast (C. Furumizu and Y. Komeda, unpubl.), this hypothesis remains to be tested *in planta*. In addition, our speculation is supported by the finding that the human transcription/export (TREX) complex, which is comprised of the core THO complex, is recruited to a region near the 5' end of the mRNA and that TREX recruitment requires the 5' cap (Sträßer et al. 2002; Cheng et al. 2006).

The TREX complex has a conserved role in coupling transcription to mRNA export in yeast and metazoa and consists of two export factors, Yra1/ALY/REF and Sub2/UAP56, in conjunction with the THO complex (Sträßer et al. 2002; Masuda et al. 2005). The *Arabidopsis* genome contains at least one (At5g59950) and two (At5g11170 and At5g11200) genes, which are homologous to Yra1/ALY/REF and Sub2/UAP56, respectively. This suggests the possibility that a complex similar to the TREX complex could exist in *Arabidopsis* as well. In addition to utilizing the genetic resources provided by this and previous studies, future experimental validation and biological characterization of these predicted complexes will allow us to further explore the vast world of RNA and may contribute to a better understanding of the molecular mechanisms, whereby the intricate systems are built up in plants.

MATERIALS AND METHODS

Plant materials and growth conditions

All plants were in the Columbia (Col) background, and Col was used as the wild type. The seeds of *ago1-27* were kindly provided by Hervé Vaucheret. The *attho2-1* (SALK_072011), *attho2-2* (SALK_130342), *attho2-3* (CS848337), *attho2-4* (SALK_129441), *atthoc6-1* (SALK_051022C), *atthoc6-2* (SALK_004825), *hyl1-2* (SALK_064863), and *se-1* (CS3257) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al. 2003). Plants were grown on rock-wool bricks supplemented with vermiculite under cool white fluorescent light in long day conditions (16 h light/8 h dark) at an average temperature of 22°C. For RNA extraction from young seedlings, seeds were germinated on nutrient agar plates containing half-strength Murashige and Skoog Plant Salt Mixture (Wako Pure Chemical Industries), 1% sucrose, and 0.8% Bacto Agar (Difco). The pH of the medium was adjusted to 5.7 with KOH.

Mutant isolation

A genetic screen was conducted as previously described (Furumizu and Komeda 2008). The *emu* mutant was backcrossed six times to wild-type Col, and the individual plants that showed the mutant

phenotypes in the absence of the *ERECTA::GUS* transgene (Yokoyama et al. 1998) were chosen for further analyses.

Map-based cloning

The *emu* mutant was crossed to *Ler*, and F2 seedlings with the *emu* phenotype were examined for recombination between the mutation and PCR-based polymorphic markers. The *emu* mutation was initially mapped to chromosome 5 between markers NGA249 and RCI1B. Further analysis of 678 *emu*-like F2 plants allowed us to locate *EMU* to a region of 138 kb located between markers MASC00149 and SGCSNP13619. Sequencing of this interval identified a C-to-T substitution in the third exon of At5g09860. The entire coding region of At5g09860 in *emu*, in the parental line harboring the *ERECTA::GUS* transgene, in Col, and in *Ler*, was sequenced to confirm that this C-to-T substitution occurred only in the *emu* mutant. The initial mapping used cleaved-amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers from The Arabidopsis Information Resource (TAIR, www.arabidopsis.org). The derived cleaved amplified polymorphic sequence (dCAPS) markers used for fine mapping of the *emu* mutation were generated based on information available from TAIR (Neff et al. 1998). Details regarding primer sequences and polymorphisms are available upon request.

Complementation of *emu*

The *EMU* cDNA was amplified by PCR from the Col cDNA template using primers 5g09860-FW101 (5'-CCCGGGAAATTGT TCTTCCTCCACTC-3') and 5g09860-RV101 (5'-CCCGGGTTAC GCAAAGAAGCTTAATTG-3'), and was subcloned into pGEM-T (Promega) to yield pCF700. pCF700 was sequenced to exclude amplification errors. The *EMU* cDNA was excised from pCF700 by digestion with *Sma*I and cloned into the *Sma*I site of pCF196 (Furumizu and Komeda 2008) to yield pCF701. This complementation construct was introduced into the *Agrobacterium tumefaciens* strain C58C1 by electroporation and was used to transform *emu* plants by the floral dip method using the surfactant Silwet L-77 (Clough and Bent 1998). Transformants were selected on nutrient agar plates containing 50 µg/mL kanamycin.

PCR-based genotyping

PCR-based genotyping markers were designed for *emu*, *attho2*, and *atthoc6* alleles. Two markers were designed for the *emu* allele. In the absence of the complementation construct, the *emu* allele was identified by a CAPS marker. For this, a 0.9-kb genomic fragment was amplified using the primer pair 5g09860-FW (5'-CCGGCGACT CATAGTCCGTG-3') and 5g09860-RV3 (5'-CGGTCAAATCCTC CAGCAG-3'). After digestion with *Afl*III, the *emu* allele produces two fragments (0.5 and 0.4 kb), while the wild-type allele is resistant to *Afl*III digestion. In the presence of the complementation construct, the *emu* allele was identified by a dCAPS marker. In the wild type, the primer pair 12D1M-F2 (5'-TTGACAGAAACAAACAAAGTTGG AT-3') and 5g09860-RV4 (5'-AAGTATATACGCATACCACG-3') produces the full-length product of 0.13 kb, which becomes 0.11 kb after digestion with *Mbo*I. The *emu* allele is resistant to *Mbo*I. For the T-DNA insertion mutants, the presence of T-DNA insertions was confirmed by PCR using the primer pairs LB-FW (5'-GGC GTTACCCAAGCTTAATCGCC-3') and 1g24706-RV (5'-TCCGCT TTGTGCCAGTCTC-3') for *attho2-1*; 1g24706-FW (5'-TCTTAA

TCCAGTGGCACACG-3') and RB-RV (5'-AAACGATCCAGAT CCGGTGC-3') for *attho2-2*; 1g24706-FW2 (5'-GTCAGTGAAC AGTTCGACAG-3') and pCSA110LB-RV (5'-TACAGCAAGAAC GGAATGCG-3') for *attho2-3*; 1g24706-FW2 and LB-FW for *attho2-4*; and THOC6-FW (5'-GCAAGCTAGTAATTTGATCG-3') and LB-FW for *atthoc6-1* and *atthoc6-2*. The respective wild-type alleles were genotyped by PCR using the primer pairs 1g24706-FW and 1g24706-RV for *attho2-1* and *attho2-2*; 1g24706-FW2 and 1g24706-RV2 (5'-TACAACGCTGCAGGAAGTCC-3') for *attho2-3*; 1g24706-FW2 and 1g24706-RV4 (5'-CGTCTGTAAACCAATGT GG-3') for *attho2-4*; and THOC6-FW and THOC6-RV (5'-GCG CAAGAATCACCAAGCTG-3') for *atthoc6-1* and *atthoc6-2*. The *se-1* allele was detected by a PCR-based genotyping marker generated based on the information previously described (Prigge and Wagner 2001). PCR using the primer pair SE-FW3 (5'-AGGTAGCGGG AGAGGTGGAC-3') and SE-RV3 (5'-ACGTTACCTGCGCAGC CGTC-3') produces PCR products of 82 and 75 base pairs for the wild-type and *se-1* alleles, respectively. PCR amplification was performed under standard conditions.

Microscopy

For examination of embryo phenotypes, tissues were cleared as previously described (Yadegari et al. 1994). Samples were visualized using Nomarsky differential interference contrast optics on a Leica DMRX E microscope (Leica Microsystems).

RNA analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

For RT-PCR analysis, total RNA was treated with RNase-free DNase I (Takara Bio). First-strand cDNA was synthesized from 2 µg of RNA with an oligo dT-adaptor primer using AMV Reverse Transcriptase XL (Takara Bio) according to the manufacturer's instructions. One-fortieth of the reverse transcriptase reaction was subjected to PCR amplification. The gene-specific primers used were 5g09860-FW4 (5'-AGCATATACTGGAGCGCG-3') and 5g09860-RV2 (5'-AGCTAGCAGATTTGCACTC-3') for *EMU*. Primers for SR genes, *EF1αA4*, and *TUB2* were previously described (Lee et al. 2000; Nesi et al. 2000; Palusa et al. 2007). The PCR program started at 94°C for 2 min, followed by the optimum number of cycles of 30 sec at 94°C, 30 sec at 56°C, and 1 min at 68°C. PCR products were subjected to agarose gel electrophoresis and were visualized by staining with ethidium bromide. RT-PCRs were performed three times on two independent RNA batches, and the results were comparable in all experiments.

For miRNA gel-blot analysis, total RNA (5 µg) was loaded onto a warmed 15% polyacrylamide, 7 M urea gel and run at 100 V in 0.5X TBE buffer for 2.5–3 h. The gel was divided in two; the upper gel was stained with ethidium bromide to show 5S RNA and tRNAs, and the lower gel was used for RNA blot. Small RNAs were electroblotted to a positively charged Hybond-N+ membrane (Amersham Biosciences) at constant current (4 mA/cm²) for 45 min in 0.5X TBE buffer. After transfer, the membrane was rinsed in 20X SSC, the RNA was crosslinked to the membrane with 1200 × 100 µJ of energy using a Stratilinker UV crosslinker (Stratagene), and the membrane was baked at 80°C for 30 min. The membrane was hybridized with DNA oligonucleotide probes specific for miR156 (5'-GTGCTCACTCTCTTCTGTCA-3'), miR159 (5'-TAGAGCT

CCCTCAATCCAAA-3'), miR165 (5'-GGGGATGAAGCCTGG TCCGA-3'), miR166 (5'-GGGGAATGAAGCCTGGTCCGA-3'), and miR172 (5'-ATGCAGCATCATCAAGATTCT-3'). The probe was end-labeled with [γ -³²P]-ATP using T4 Polynucleotide Kinase (Takara Bio). Unincorporated nucleotides were removed using G-25 spin columns (Amersham Biosciences) as per the manufacturer's instructions. Membranes were prehybridized for at least 3 h and hybridized overnight at 42°C using ULTRAhyb-Oligo Hybridization Buffer (Ambion). Membranes were washed four times for 30 min each with 2X SSC, 0.5% SDS at 42°C. The membranes were visualized using exposure to X-ray films for several days at –80°C with an intensifying screen. The intensities of the bands were quantified from the original figures using the ImageJ 1.43 program (<http://rsb.info.nih.gov/ij/>), and normalized miRNA/rRNA values are represented. RNA gel-blot analyses were performed at least twice on at least two independent RNA batches, and the results were comparable in all experiments.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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NOTE ADDED IN PROOF

Recently, Yelina et al. (2010) published a similar study on the putative *Arabidopsis* THO/TREX complex.

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