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...
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 er 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 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
emum product lacks a large portion of the predicted protein. The emum 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 (At5g02950), 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 athoc6 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 athto2 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.
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 (Lobbes 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; Lobbes 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...
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 mutant phenotype conferred by the emu mutation enhances the 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; Raczynska 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.
(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_072001), attho2-2 (SALK_130342), attho2-3 (CS848337), attho2-4 (SALK_129441), atttho1-1 (SALK_051022C), atttho1-2 (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 RCH1B. 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′-CCCGGGAATTT CGTTTCCTCACTC-3′) and 5g09860-RV101 (5′-CCCGGGTTAC GCAAAAAGACTTTAATTG-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 Smal and cloned into the Smal 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 atttho6 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′-CGGGCGACT CATAGTCGGTG-3′) and 5g09860-RV3 (5′-CGGTCAATTCCT CAGACG-3′). After digestion with AfII, the emu allele produces two fragments (0.5 and 0.4 kb), while the wild-type allele is resistant to AfII 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′-TTGACGAAACAAAAAGTTGG AT-3′) and 5g09860-RV4 (5′-AAGTATATACGCCATACACG-3′) produces the full-length product of 0.13 kb, which becomes 0.11 kb after digestion with Mbol. The emu allele is resistant to Mbol. For the T-DNA insertion mutants, the presence of T-DNA insertions was confirmed by PCR using the primer pairs LB-FW (5′-GGC GTTACCCCACTTTACGC-3′) and 1g24706-RV (5′-TCCGGCT TTGTGCCAGTCTC-3′) for attho2-1; 1g24706-FW (5′-TCTTAA
TCCAGTGCCACAGC-3') and RB-RV (5'-AAACGATCCGATCGTTGAC-3') for attho-2; 1g24706-FW2 (5'-GTCATGCTCGAGGATGC-3') and pCSA110LB-RV (5'-TACAGCAAGAACGCGAATTCG-3') for attho2-3; 1g24706-FW2 and LB-FW for attho2-4; and THOC6-FW (5'-GCAAGCTTTAATTTGATCC-3') and LB-FW for atthoxc-1 and atthoxc-2. The respective wild-type alleles were genotyped by PCR using the primer pairs 1g24706-FW2 and 1g24706-RV2 (5'-GCGTCTGTAACCCAATGT-3') for attho2-4; and THOC6-FW and THOC6-RV (5'-GGCCAAAGTTCCAGCTG-3') for atthoxc-1 and atthoxc-2. The se-1 allele was detected by a PCR-based genotyping marker generated based on the information previously described. Total RNA (5 μg) was loaded onto a Leica DMRX E microscope (Leica Microsystems).

**Microscopy**

For examination of embryo phenotypes, tissues were cleared as previously described. 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 summarized in 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 (rsb.info.nih.gov/ij), and normalized miRNA/rRNA values are represented.

**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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