The * elongata* mutants identify a functional Elongator complex in plants with a role in cell proliferation during organ growth

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The key enzyme for transcription of protein-encoding genes in eukaryotes is RNA polymerase II (RNAPII). The recruitment of this enzyme during transcription initiation and its passage along the template during transcription elongation is regulated through the association and dissociation of several complexes. Elongator is a histone acetyl transferase complex, consisting of six subunits (ELP1–ELP6), that copurifies with the elongating RNAPII in yeast and humans. We demonstrate that point mutations in three * Arabidopsis thaliana* genes, encoding homologs of the yeast Elongator subunits ELP1, ELP3 (histone acetyl transferase), and ELP4 are responsible for the phenotypes of the * elongata* (elo) mutants, respectively. The elo mutants are characterized by narrow leaves and reduced root growth that results from a decreased cell division rate. Morphological and molecular phenotypes show that the * elongata* (elo) genes function in the same biological process and the epistatic interactions between the elo genes can be explained by the model of complex formation in yeast. Furthermore, the plant Elongator complex is genetically positioned in the process of RNAPII-mediated transcription downstream of Mediator. Our data indicate that the Elongator complex is evolutionarily conserved in structure and function but reveal that the mechanism by which it stimulates cell proliferation is different in yeast and plants.

Current acetylation is the best-characterized histone modification and plays a role in the regulation of transcription (1–3). This reversible modification results from a balance between the activity of histone deacetylases and histone acetyl transferases (HATs). The Elongator complex consists of six subunits, of which one displays HAT activity (4). Elongator copurifies with RNA polymerase II (RNAPII) during transcriptional elongation, presumably by rendering the DNA more accessible for the passage of the polymerase (5, 6). In * Arabidopsis thaliana* (L.) Heynh., 16 histone deacetylases and 12 HATs are encoded by the genome (7), and mutational analysis has already shown that some of these genes are involved in stress responses, growth, and flower development (8–11).

In a large-scale screening of ethane methylsulfonyl-mutagenized * Arabidopsis* for mutants with abnormally shaped leaves, the * elongata* (elo) mutations have been identified (12) and mapped at low resolution by linkage analysis (13). The elo mutants have a narrow leaf phenotype (12), similar to that of the null mutant * drl1*-2. Subsequently, elo4 was shown to be allelic to * drl1* and was redesignated * drl1* (14). * DRL1* is the ortholog of the yeast * KTI12* protein (14), a putative regulator of the Elongator complex (15). Homologs of the six structural components of Elongator are present in the * Arabidopsis* genome (14). We report that the elo mutations are in genes encoding components of the Elongator complex. Phenotypical analyses showed that mutations in the plant Elongator HAT and its structural components interfere principally with leaf shape, and leaf and root growth by affecting cell proliferation.

Materials and Methods

**Plant Material and Growth Conditions.** Mutants elo1, elo2, elo3, elo4/* drl1*-4 (12), * drl1*-2 (code N9360, Nottingham Seed Stock Centre, Nottingham, United Kingdom) (14), and * swp1* (16) have been described previously; elo3-3, elo3-4, and elo3-5 (The Plant Chromatin Database available at www.chromdb.org), elo3-2 (FLAGdb/FST) (17), and SALK insertion lines (Nottingham Seed Stock Centre) are publicly available. Plants were grown as described in ref. 14. For transcript profiling experiments, the medium was 2.15 g/liter Murashige and Skoog medium salts (Duchefa, Haarlem, The Netherlands)/1 g/liter sucrose/0.5 g/liter Mes, pH 6.0/6 g/liter plant tissue culture agar. Seeds were vernalized for 3 days after sowing.

**Morphological and Cellular Analysis.** Morphological, cellular, and statistical analyses were performed on the expanded first two leaves of the * elongata* mutants (35 days after germination (DAG)) and * Ler* (28 DAG) as described in ref. 18. Seeds were germinated in * vitro* on vertical plates; every 2 days, primary root growth was measured. An analysis of variance was performed on each double mutant (DM), its respective parents, and * Ler*, with the two mutated genes as fixed factors and the slope of root growth as a variable (n = 10). A significant interaction between the two loci showed an epistatic effect between the genes.

Leaf primordia of 7-DAG * Ler*, elo1, elo2, elo4/* drl1*-4, and * drl1*-2 plants (n = 7) were analyzed (19). The distance between 10 neighboring nuclei was measured (five times per primordia) to account for errors on the measurements.

**Flow Cytometry Analysis of the Leaves.** The * elo* and * drl* mutants have delayed development after germination, and growth is very heterogeneous among individuals; therefore, the first two leaves of the mutants and * Ler* were harvested according to developmental stages (1.02, 1.03, etc.) (20). In wild-type plants, three successive phases could be determined during leaf development: proliferation (mitotic cell cycles, illustrated by the presence of...
cells in 2C and 4C), expansion [the intersection between the curves of cells in 2C and 4C and the occurrence of higher ploidy levels (8C, 16C)], and maturation (a stable DNA distribution) (21). Flow cytometry analysis was performed as described in ref. 22. For each time point, two biological and three technical replicates were taken.

Identification of elo Mutations. The genomic and cDNA sequences of the ELO2 (locus tag At5g13680), ELO1 (locus tag At3g11220), and ELO3 (locus tag At5g50320) transcription units (including 500 bp upstream and downstream of the coding sequence) were amplified and sequenced in the elo1, elo2, and elo3 mutants, respectively, and compared with those in Ler (Table 2, which is published as supporting information on the PNAS web site).

DM Analysis. Derived cleaved amplified polymorphic sequence primers (23) were designed with the DCAPS finder 2.0 software (24) with one or two mismatches in the wild-type sequence for elo1, elo4/drl-1, and elo2 (Table 3, which is published as supporting information on the PNAS web site).

cDNA-Amplified Fragment-Length Polymorphism (AFLP), Microarrays, and Clustering. Shoot apices of in vitro grown plants (including the shoot apical meristem and first and second leaf primordia) were harvested at stage 1.0 (20). The experimental design comprised three independent RNA extractions.

The cDNA-AFLP analysis (25) using BstYI and MseI restriction enzymes and 73 primer combinations gave 2,609 cDNA-AFLP fragments. One-way analyses of variance with one fixed factor (genotype) and Tukey’s pairwise comparisons between genotypes (at P < 0.001) with the GENSTAT software (VSN International, Hemel Hempstead, United Kingdom). ATH1 Affymetrix chips (VIB Microarray Facility laboratory, Leuven, Belgium) were used according to the manufacturer’s protocol. The raw data (CEL files) were normalized and summarized with the Robust MultiArray average method from the affy package of BioConductor, Project Release 1.4 (26). The DE genes between each mutant and the wild type were identified at P < 0.05 with an empirical Bayes’ t test and Holm’s P correction by using the limma package of BioConductor (27). The expression ratio of DE transcript was calculated as the mean value of the mutant over that of Ler and then log-transformed for complete linkage hierarchical clustering with Euclidean distance by using cluster and treeview software (28). The microarray data are in ArrayExpress (E-MEXP-300).

Results and Discussion

elo Mutations Affect Genes Encoding Components of the Elongator Complex. We compared the physical location of the six genes encoding Elongator homologs in Arabidopsis with the genetic map positions of the elo mutations. The elo1, elo2, and elo3 mutations colocalized with the genes encoding the ELP4, ELP1, and ELP3 homologs, respectively (Fig. 4, which is published as supporting information on the PNAS web site). Each of the elo mutants carried a point mutation in one of these Elongator genes. The sequencing of the ELO2 gene in elo2 plants revealed a single nucleotide change (G→A) that caused a premature stop codon (TGG→TAG) toward the 3‘ end of the third exon (Fig. 1A). In the elo3 mutant, a single nucleotide change (G→A) in the fifth exon of the ELO3 gene changed an aspartic acid residue, which is conserved in all ELO3 homologs, into an asparagine (D→N) (Fig. 1B). A single base pair change (G→A) at the accpetor splice site of the third intron of the ELO1 gene caused missplicing in the elo1 mutant. Three different mRNAs of ELO1 were present in the elo1 plants, resulting from three different splicing events: exon 4 was spliced out, intron 3 was spliced out incorrectly using the first AG in exon 4, or intron 3 was not spliced out (Fig. 1C). The three splicing events had a similar effect on the formation of the putative protein: at the N terminus, 142 amino acids were identical to those of wild type, followed by divergent amino acids and a stop codon. The microarray experiment using mRNA of the shoot apices of the elo mutants showed that the expression of ELO2 in elo2 was only 20.1% that of the wild type; no difference in expression level was seen for the ELO3 gene between the elo3 mutant and Ler. Overexpression of ELO3 and ELO1 cDNAs in elo and elo1 mutants, respectively, restored wild-type leaf and root phenotypes.

Two T-DNA (transferred DNA of Agrobacterium) insertion lines were examined for the ELO1 gene: In lines N503548 and N579793, the T-DNA was inserted into exons 2 and 7, respectively (Table 1) (29). Most SALK lines available for ELO2 carry an insertion in the third exon, of which two lines (N011529 and N004690) were examined. The sequencing of the flanking region confirmed T-DNA insertions as predicted with SIGnAL. PCR amplifications with gene-specific primers were performed to identify homozygous plants whose progenies displayed a narrow leaf phenotype very similar to that of the elo mutants but with incomplete penetrance. These observations indicated that the Col-0 genetic background of the SALK lines suppressed to some extent the elo leaf phenotype. However, a T-DNA allele of the AtELP3 gene (FST/FLAGDB 219E08) and
ELO1 is probably not a recombinase binding signatures are present in the neither the recombinase (accession no. PS00674) nor the ATP- the eukaryotic Rad51 and DMC1 proteins (33). However, because was first identified in bacterial recombinases and is also present in PS00321) at the N-terminal region of the protein. The RecA motif be an inactive ATPase homolog (32). By using the demonstrated the importance of this aspartic acid for the function- plex. The proteins, supporting a putative role for ELO2 in stabilizing the present in other scaffold protein families, such as the 14-3-3 protein to form a “bridge” between the two subcomplexes (4).

Table 1. Alleles of the ELO2, ELO3, and ELO1 genes

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RNAi, RNA interference; EMS, ethane methylsulfonate; MIPS, Munich Information Center for Protein Sequences; ChromDB, Plant Chromatin Database.

three independent RNA interference lines of ELO3 (N3981, N3982, and N3983) in Wassilewskija (Ws-2) ecotype had leaf and root phenotypes (see below) very similar to those of the elo mutants (Table 1).

The Functional Domains of the Elongator Proteins Are Evolutionarily Conserved. An in silico analysis showed that the functional domains of the ELO2, ELO3, and ELO1 proteins were conserved between yeast and Arabidopsis. In yeast, the Elongator complex consists of two distinct subcomplexes. ELPI was previously described as part of the core subcomplex of Elongator where it functions as a scaffold protein to form a “bridge” between the two subcomplexes (4). Alignments of the homologs (from eight species) with the Arabidopsis ELO2 protein revealed conserved domains that were also present in other scaffold protein families, such as the 14-3-3 proteins, supporting a putative role for ELO2 in stabilizing the interaction between different components of the Elongator complex. The elo2 mutation is predicted to form a truncated protein that does not contain a conserved C-terminal domain. The yeast ELPI3 protein was reported to possess HAT activity in vitro (30). By sequence analysis, the ELPI3 HAT was classified in the GCN5-related N-acetyltransferase superfamily (7), and a methyltransferase domain was found as well (31). The presence of a GCN5-related N-acetyltransferase family domain (accession no. PF00583) and a radical S-adenosyl methionine superfamily domain (accession no. PF04055) imply a similar functionality of ELO3 in Arabidopsis. The HAT and methyltransferase domains of ELO3 suggested that this protein might be the key enzyme of the Elongator complex because it would render the chromatin more accessible for the passage of the RNAII transcription machinery by modifying histones. In the elo3 mutants, an aspartic acid residue, which is conserved in all ELO3 homologs (from >32 species) within the HAT domain, is converted to an asparagine. The extreme phenotype of the elo3 mutants, compared with the other elo mutants, demonstrated the importance of this aspartic acid for the functionality of the ELO3 protein. The yeast ELP4, which forms the accessory subcomplex with ELP5 and ELP6, has been reported to be an inactive ATPase homolog (32). By using the Arabidopsis ELO1 protein, we identified a RecA domain (accession no. PS00321) at the N-terminal region of the protein. The RecA motif was first identified in bacterial recombinases and is also present in the eukaryotic Rad51 and DMC1 proteins (33). However, because neither the recombinein (accession no. PS00674) nor the ATP- binding signatures are present in the Arabidopsis ELO1 protein, ELO1 is probably not a recombinase sensu strictu. The sequence similarity between ELO1 and the recombinein superfamily of ATPases indicates that ELO1 might represent a previously uncharacterized type of DNA binding/processing protein. The elo1 mu-
tation is predicted to cause truncation of the moderately conserved C-terminal part of the ELO1 protein.

The expression of the ELO1, ELO2, and ELO3 genes was monitored in different plant organs by RT-PCR: roots, young first leaves, whole seedlings (14 DAG), cotyledons, expanded first leaves, floral buds, and shoot apices (27 DAG). The three ELO genes were expressed in every plant organ examined (data not shown) in the same manner as the DRL1 gene (14). These expression data were confirmed in silico for each organ at different developmental stages by using the Genevestigator database (34).

Reduced Organ Growth Phenotype. Although the elo mutants were originally isolated as leaf mutants (12), they also displayed a pleio-
tropic phenotype: The growth of the primary roots was reduced, and the architecture of the inflorescences was altered and reduced in length compared with wild type. Furthermore, upon germina-
tion, seedling growth was delayed in all elo mutants and germina-
tion was severely retarded in elo3. The phenotypes of the different elo mutants were very similar and resembled that of drl1-2 (Fig. 1D). However, quantification of some of the phenotypes resulted in differences in phenotypic severity between the different elo and drl mutants. Fully expanded first and second leaves collected 28 DAG were analyzed for a number of parameters (18). The lamina, petiole, and total leaf length were increased significantly in elo1, elo2, and elo4/drl1-4 compared with Ler (n ≥ 10), and the transition between lamina and petiole was unclear. The lamina width was smaller in all mutants because of a reduced cell number as measured by the number of palisade cells (Fig. 2B). elo3 displayed the most severe phenotype, with a 52.4% reduction in palisade cell number (Fig. 2A and B). In elo4/drl1-4, the cell number was not significantly reduced (Fig. 2B), confirming that it was a weak allele of DRL1 (14). In transverse sections through first and second fully expanded elo3 leaves, larger and more irregularly shaped palisade cells, more intercellular spaces, and normal vascular bundles with dorsal xylem and ventral phloem tissue were seen, a leaf anatomy reminiscent of that of drl1-2 (Fig. 2A) (14). In conclusion, the narrow leaf phenotype of elo mutants was very similar to that of the drl1-2 mutant (14) and was also associated with a reduction in cell number.

Kinetics of primary root growth was measured in elo1, elo2, elo3, drl1-2, and elo4/drl1-4 homozygotes and compared with the wild-type Ler (n ≥ 10). Root growth was significantly inhibited in all mutants (Fig. 2C). Statistical analysis positioned each of the mu-
tants in a distinct group, except for the drl1-2 and elo2 mutants that formed one group, based on their root growth (Fig. 2C). Other alleles of ELO3 (elo3-2, elo3-3, elo3-4, and elo3-5) also showed retarded root growth compared with that of Ws-2 (n = 10) (Fig. 5A), which is published as supporting information on the PNAS web site). The growth rate of the primary roots was calculated as root length difference between two successive time points divided by the time interval between these time points. The growth rate of the last time points was used to determine cell production in the roots, which was a measure for the number of cells produced per hour and is the ratio of the root growth rate and the length of the mature root cortex cells. The length of the mature cortex cells was significantly shorter in all elo and drl1-2 mutants (data not shown), and cell production was severely decreased in the mutant root (Fig. 2D).

The phenotypic analyses of the Elongator mutants showed that the Elongator genes have a positive effect on lateral leaf and primary root growth, which coincides with their expression in these organs. The similar phenotypes of the different Elongator mutants suggest that the Elongator genes play a role in the same pathway, process, or even in the same complex, as previously shown for mutants with similar developmental phenotypes, such as the clavata mutants (35). Comparable phenotypes were obtained upon mutation of ELO3, the putative HAT, DRL1, a putative regulator, and the structural components ELO2 and ELO1, implying that each component examined was important for the functionality of Elongator in plants.
The elo Mutants Have a Reduced Cell Proliferation Rate, Probably Without Affecting the Core Cell Cycle Machinery. The serious delay in growth after germination and the reduced cell numbers underlying the elo and drl phenotypes suggest a decrease in cell division rate. Cell division activity is also decreased in the yeast Elongator mutants; the cells undergo a G1 delay and are often not able to divide. The reduction of cell division activity in plants, because of cell size being negatively correlated with cell division rate, as previously shown for the yeast Elongator complex was also due to a G1 delay in plants, the mechanism through which this is exerted differs between the two species.

To assess whether the reduced cell division rate upon mutation of the Elongator complex was also due to a G1 delay in plants, the rates of mitosis in mutants and wild-type plants at several time points during development were determined by flow cytometry in the first two leaves. The mitotic index was significantly lower in the mutant primordia 7 DAG (Fig. 2E), implying that the duration of interphase was prolonged over that of mitosis (37). Moreover, the distance between the nuclei, which is a direct measure of cell size (19), was significantly higher in the elo mutants, and the cells were larger (Fig. 2F). This observation is in agreement with a decrease in cell division rate, because cell size is negatively correlated with cell division rate, as observed for the lines overexpressing E2Fa/DPa and Kip-related proteins (22, 38). Previously, an altered cell division rate in the leaf primordia was shown to have a serious impact on leaf morphogenesis (39).

Furthermore, the flow cytometry profiles of the first two leaves also ruled out the possibility that the reduced cell number was due to an early exit from the mitotic cell divisions in the elo mutants, because the curves of cells in 2C and 4C intersected around the same developmental time point as in wild type (Fig. 5B and C). When the growth retardation of the elo and drl mutants is taken into account, the mitotic cell divisions would stop even later than those of the wild type. The differences in endoreduplication between the mutant and wild-type plants were not significant and could have resulted from differences in age of the seedlings at the time of harvesting. The cell expansion that is correlated to endoreduplication goes on longer in the elo mutants, explaining a slightly higher ploidy level in the mutants (Fig. 5B and C). In conclusion, no direct defect was observed on the cell cycle transitions or on the endoreduplication; therefore, the effect of the elo and drl mutations on cell division is probably not related to core cell cycle activity. This hypothesis is supported by the observation that no core cell cycle genes were DE in the microarray experiment using shoot apices of elo mutants.

Molecular Phenotyping. We studied the molecular phenotype to assess whether the same sets of genes were DE in the mutants. To distinguish the DE genes that were not a direct consequence of the elo and drl mutations but were, rather, a secondary effect of the leaf phenotype, another narrow leaf mutant, angusta-4 (ang4) (12), was included as a control. The analysis of 2609 cDNA-AFLP transcripts derived from the seven genotypes (Ler, elo1, elo2, elo3, elo4/drl1-4, drl1-2, and ang4) showed that 763 (29%) transcripts were DE in the mutants and the wild type (at \( P < 0.001 \)). These 763 DE transcripts were used for clustering to observe the distances between the genome expression profiles of mutants. The clustering result showed clearly that ang4 was distant from the elongata genotypes, which, in turn, clustered together, but were divided into two subgroups: drl1-2, elo4/drl1-4 (the two alleles of DRL1), and elo3 in one group, which comprises mutants in a regulator and the key enzyme of the complex, and elo1 and elo2 in a second group, whose respective genes are involved in Elongator architecture (Fig. 2G).

A genome-wide analysis with the Affymetrix ATH1 microarrays on elo2, elo3, drl1-2, and ang4 mutants identified a total number of 2,897 genes that were DE in at least one mutant and
the contrast to growth of the primary root and architecture of the Elongator genes without extensive microscopic analysis in phenotypic trait could not be used to determine epistasis among corresponding wild-type alleles in F2 putative DMs. A PCR analysis to similarly. The analogous molecular processes affected by the experimental evidence for their function in a complex. The differences in the rosette leaf phenotype are subtle and corresponding to function, together in one process. The differences in the rosette leaf phenotype are subtle among the various elo and drl mutants and, therefore, this phenotypic trait could not be used to determine epistasis among the Elongator genes without extensive microscopic analysis in contrast to growth of the primary root and architecture of the inflorescence that allowed a clear distinction between some of the elo and drl mutants. Two cases are exemplified in Fig. 3: elo3 elo1 and elo2 elo1, whose DMs exhibit a root and inflorescence phenotype similar to that of the less dramatic parent, elo1. A pairwise statistical analysis was performed on the root growth and positioned the DMs in the same group as elo1, distant from elo2 and elo3 (Fig. 3D); also, the architecture of the DM inflorescences was less dramatically affected and similar to that of the elo1 mutant (Fig. 3A and B). The other DM combinations were also phenotypically characterized and compared with their respective parents. The elo2 elo3, elo4 elo1, and elo2 drl1-2 DMs had phenotypes similar to those of elo2, elo4/drl1-4, and drl1-2, respectively. Thus, DRL1 was epistatic to ELO2 and ELO1. Because the corresponding proteins have been shown to be part of distinct subcomplexes of Elongator in yeast, this observation is in accordance with a proposed role for DRL1 as a regulator of the holocomplex (15). Furthermore, ELO1 was epistatic over ELO2 and ELO3, indicating the importance of the accessory subcomplex for the function of the core subcomplex. Finally, ELO2 was epistatic to ELO3, suggesting that the ELO2 scaffold protein is important for the maintenance of the integrity of Elongator as a HAT complex. These epistasis data can be correlated with the proposed functions of the proteins in Arabidopsis as well as with the model proposed in yeast for the Elongator complex formation (Fig. 3C) (4).

Genetic Interactions Between SWP1 and DRL1. The strawwelpeter1 (swp1) recessive mutation, which was isolated in a Ws-2 background, also causes narrow leaves with a reduced cell number. The SWP1 gene encodes a component of the Mediator complex that associates with the RNAII transcription initiation complex (17). A SWP1/swp1 × drl1-2/drl1-2 cross was performed, of which the F1 plants were selected on phosphinotricin, the marker present on the T-DNA inducing the swp1 mutation, to select F1 plants that contained the swp1 mutation. All plants that survived this selection displayed a wild-type phenotype. In the F2 population, no novel or additive phenotype could be detected; so, the mutant plants from this population were analyzed molecularly by PCR to identify drl1-2 homozygotes. From these
plants, the F₃ seeds were grown selectively on phosphinothricin, and the 100% resistant lines were identified as swirl1 drl1-2 DMs. All individuals from two DM lines had a phenotype similar to that of the swirl1 mutants: no growth retardation, small serrations in the first leaves, and normal growth of the primary root and inflorescence (Fig. 3E), indicating that swirl1 is epistatic to DRL1. These genetic data position Elongator more downstream in the RNAPII-mediated transcription process than Mediator in the plant system, which is consistent with the role of the yeast elongator complex in transcription elongation.

**Conclusion**

We demonstrated that the Elongator complex is present in plants and that the functional domains of three component proteins are conserved. Several lines of evidence also suggest complex formation in plants, indicating that Elongator is structurally conserved. By positioning the Elongator complex in the process of transcription elongation, downstream of Mediator, we showed that the function of the complex is conserved.

Although the Elongator complex is very well conserved, the phenotypes of the Elongator mutant plants show that its function differs in unicellular and multicellular organisms. In yeast, Elongator mutants are retarded in growth because of a slow adaptation to changing environmental conditions (40). However, in plants, the disruption of the Elongator complex has effects throughout development: germination, vegetative growth, and the reproductive phase were affected in the mutants.

In humans, the Elongator complex also plays a role in development, as a mutation in one of the Elongator components is associated with the neuronal syndrome, familial dysautonomia (41). This neurodevelopmental genetic disorder affects development and maintenance of sensory and autonomic neurons, resulting in a wide range of pathologies primarily due to malperception of stimuli from the environment and during development (42).

In addition, the analysis of the plant Elongator mutants revealed that the Elongator complex has a positive effect on the cell proliferation rate during organ growth, without affecting the core cell cycle machinery. Our data indicate that the mechanism by which the Elongator complex affects the cell division rate is different in yeast and higher plants. The identification of the Elongator complex in plants now offers opportunities to gain more insights into the role of the Elongator complex in multicellular organisms. Because plants are easily amenable to experimentation, it will be possible to determine the environmental and developmental cues that direct Elongator activity and the molecular processes regulated by this complex. The microarray experiments revealed that only a limited number of processes are affected in the elo and drl mutants, i.e., secondary metabolism, photomorphogenesis, and several stress responses, indicating that Elongator selectively influences transcriptional activity. The mechanism behind these selective roles of Elongator will need to be explored further.

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