Arabidopsis CLP1-SIMILAR PROTEIN3, an Ortholog of Human Polyadenylation Factor CLP1, Functions in Gametophyte, Embryo, and Postembryonic Development[1][C][W][OA]

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Polyadenylation factor CLP1 is essential for mRNA 3′-end processing in yeast and mammals. The Arabidopsis (*Arabidopsis thaliana*) CLP1-SIMILAR PROTEIN3 (CLPS3) is an ortholog of human hCLP1. CLPS3 was previously found to be a subunit in the affinity-purified PCFS4-TAP (tandem affinity purification) complex involved in the alternative polyadenylation of *FCA* and flowering time control in Arabidopsis. In this article, we further explored the components in the affinity-purified CLPS3-TAP complex, from which Arabidopsis cleavage and polyadenylation specificity factor (CPSF) subunits AtCPSF100 and AtCPSF160 were found. This result implies that CLPS3 may bridge CPSF to the PCFS4 complex. Characterization of the *CLPS3* mutant revealed that *CLPS3* was essential for embryo development and important for female gametophyte transmission. Overexpression of CLPS3-TAP fusion caused a range of postembryonic development abnormalities, including early flowering time, altered phyllotaxy, and abnormal numbers and shapes of flower organs. These phenotypes are associated with the altered gene expression levels of *FCA*, *WUS*, and *CUC1*. The decreased ratio of *FCA-β* to *FCA-γ* in the overexpression plants suggests that CLPS3 favored the usage of *FCA* regular poly(A) site over the alternative site. These observations indicate that Arabidopsis CLPS3 might be involved in the processing of pre-mRNAs encoded by a distinct subset of genes that are important in plant development.

Posttranscriptional mRNA 3′-end processing is an integral part of eukaryotic mRNA biogenesis. Based on in vitro biochemical assays, the essential components for 3′-end processing are largely defined in yeast and human (Zhao et al., 1999). In yeast, four components, cleavage factor I (CF I), cleavage factor II (CF II), cleavage/polyadenylation factor (CPF), and poly(A) polymerase (PAP), are necessary for mRNA 3′-end processing (Zhao et al., 1999). CF I, comprising five polypeptide subunits, including yClp1p and yPcf11p, is required for both specific 3′-end cleavage and addition of a poly(A) track (Gross and Moore, 2001). yClp1p and yPcf11p directly interact with each other (Gross and Moore, 2001). In humans, in vitro assays defined six components that are essential for cleavage and polyadenylation of pre-mRNA; these have been designated as the mammalian CF I and CF II (CF I_M and CF II_M), cleavage/polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CstF), PAP, and poly(A) binding protein II (PAB II; Zhao et al., 1999). Most polypeptide subunits in the human mRNA 3′-end processing components were found to be orthologs to the subunits of yeast polyadenylation complex and vice versa (Keller and Minvielle-Sebastia, 1997). The human orthologs of yeast yPcf11p and yClp1p, hPCf11 and hCLP1, directly interact with each other as do their yeast counterparts and constitute human CF II_M (de Vries et al., 2000). Furthermore, hCLP1 interacts with CF I_M and CPSF, bridging three components together (de Vries et al., 2000). In contrast to yeast, however, neither human hCLP1 nor hPCF11 is required for addition of the poly(A) tail (de Vries et al., 2000).

Although the protein components for plant polyadenylation are largely uncharacterized at the biochemical level, the completed sequences of several plant genomes and large amount of plant cDNA data in the public databases allow the identification of the plant orthologs of yeast and human polyadenylation factors. Indeed, most yeast or human polyadenylation factors have their counterparts in Arabidopsis (*Arabidopsis thaliana*; Hunt, 2008). A systematic assay of pairwise

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interactions of Arabidopsis polyadenylation factors revealed an interaction topology showing features both similar to that of yeast or human and unique for Arabidopsis (Hunt et al., 2008). This interaction network revealed three hubs centering on AtCPSF100, AtCLPS3, and AtFIPS (Hunt et al., 2008). The AtCPSF100 and AtCLPS3 hubs are similar to the complexes in human and the AtFIPS hub is unique to plants (Hunt et al., 2008). The AtCPSF100 hub has been largely confirmed by coimmunoprecipitation assays (Herr et al., 2006; Xu et al., 2006). The same is true for the AtFIPS hub (Forbes et al., 2006). The Arabidopsis PCFS4 and CLP1-SIMILAR PROTEIN3 (CLPS3) have been identified based on sequence and phylogenetic analysis as orthologs of human hPCF11 and hCLP1 (Xing et al., 2008). PCFS4 and CLPS3 are located in CLPS3 hub and directly interact with each other in vivo, as do their orthologs in yeast and mammals (Xing et al., 2008).

Whereas the homology and the similar interaction topology of polyadenylation factors from plants, fungi, and mammals clearly suggest a conserved mRNA polyadenylation mechanism in eukaryotes, unique features (in addition to the unique AtFIPS hub) and greater complexity for plants may exist. First, for most polyadenylation factor subunits, there is only one corresponding gene in the yeast and human genome. In Arabidopsis, however, some polyadenylation factors are encoded by gene families (Hunt et al., 2008). For example, there are two CLP1 and four PCF11 paralogs in Arabidopsis (Xing et al., 2008). Second, instead of being essential, as in yeast and human, some Arabidopsis polyadenylation factors are not essential and instead affect only specific biological functions. For instance, mutants of Arabidopsis deficient in PCFS4 showed only a delayed flowering time and slightly altered leaf shape (Xing et al., 2008). In the case of CPSF30, mutants were viable and showed a slightly smaller plant size and an enhanced resistance to oxidative stress (Delaney et al., 2006; Zhang et al., 2008). Although the nonessential nature of PCFS4 might be explained by the possible functional redundancy or subfunctionalization of related paralogs, there is no simple explanation for the nonessential nature of CPSF30 because there are no obvious paralogs in Arabidopsis (Hunt, 2008).

How do polyadenylation factors function in specific biological processes? Two models, which are not necessarily exclusive, have been proposed (Hunt, 2008). First, there might be multiple polyadenylation complexes (MPCs) in plants, each of them being responsible for the 3′-end processing of a subset of genes. The MPC model was supported by the differential tissue-specific expression of genes encoding some polyadenylation factor subunits (Hunt, 2008). The second model proposes that the subset of genes responsible for a specific biological process might be regulated by alternative polyadenylation. In this model, the core polyadenylation machinery (CPM) is recruited by a specific RNA-binding protein to the alternative polyadenylation sites of the subset of genes (Hunt, 2008). The CPM model is best illustrated by the alternative polyadenylation of *FCA* pre-mRNA. FCA is a RNA-binding protein and required for the alternative polyadenylation of its own pre-mRNA. The polyadenylation at the regular 3′-untranslated region of *FCA* results in the *FCA-γ* transcripts, whereas *FCA-β* is derived from the polyadenylation occurring within the third intron (Macknight et al., 1997; Quesada et al., 2003). *FCA-γ* encodes the functional FCA protein that is required for the polyadenylation within the third intron, as well as inhibition of FLC activity (Macknight et al., 1997; Quesada et al., 2003). In addition to FCA, the polyadenylation factors FY and PCFS4 are also required for polyadenylation in the third intron (Simpson et al., 2003; Xing et al., 2008). By affinity purification and yeast two-hybrid (Y2H) assays, it was proved that PCFS4, FY, CLPS3 and other unknown proteins formed a complex in vivo (Simpson et al., 2003; Xing et al., 2008). This complex is presumably recruited to the intron 3 polyadenylation site through an interaction between FY and FCA. It is not clear how AtCLPS3 affects the alternative polyadenylation of *FCA*.

In this study, we explored the proteins that are associated with affinity-purified CLPS3 and found the Arabidopsis CPSF subunits, thus linking the *FCA* alternative polyadenylation to the core polyadenylation machinery. We also characterized loss- and gain-of-function mutants of *CLPS3* and found that *CLPS3* functions in gametophyte, embryo, and postembryonic development, including flowering time. Further, the early flowering time of CLPS3 overexpression was associated with altered polyadenylation of *FCA* transcripts, suggesting a role for CLPS3 in *FCA* mRNA 3′-end processing. The altered expression of *WUS* and *CUC1*, which are involved in shoot apical meristem (SAM) maintenance and differentiation, and the range of developmental abnormalities of plants that overexpress CLPS3, suggest that CLPS3-mediated regulation of 3′-end processing might target more genes in addition to *FCA*.

RESULTS

Arabidopsis Orthologs of Human hCLP1 and Yeast yClp1

Both human hCLP1 and yeast yClp1p are essential for mRNA 3′-end processing and the CLP1 polypeptide sequences are conserved across metazoan and fungi (de Vries et al., 2000; Gross and Moore, 2001; Noble et al., 2007). A recognizable feature of CLP1 proteins is the Walk A motif, suggesting that CLP1 is a GTP/ATP-binding protein (de Vries et al., 2000; Gross and Moore, 2001; Noble et al., 2007). A recent study also revealed that hCLP1 is a RNA kinase and functions in tRNA splicing (Weitzer and Martinez, 2007). To see whether plants contain orthologs of CLP1 protein, the nonredundant database of GenBank was searched with TBLASTN using a yClp1 sequence as a

query. Two predicted polypeptides each from rice (*Oryza sativa*) and Arabidopsis genomes were identified. The Arabidopsis polypeptides, designated as CLPS3 (At3g04680) and CLPS5 (At5g39930), were highly similar to yClp1p, in which CLPS3 shows 50% similarity and CLPS5 shows 47% similarity across the whole sequence. Alignment of the Arabidopsis and rice polypeptide sequences with sequences from metazoan and fungi indicated that the Walker A motif was also conserved in plants (Supplemental Fig. S1).

To see whether the two Arabidopsis proteins are indeed the orthologs of yeast yClp1, the aligned sequences with ClustalW were analyzed with parsimony and bootstrap programs within the PAUP 4.0 package. The sequences from metazoan clustered together and the sequences from plants formed a group closer to fungi and algae than to metazoans, suggesting that two Arabidopsis polypeptides are orthologs of human and yeast CLP proteins (Fig. 1). An interesting observation was that both Arabidopsis and rice had two CLP1 orthologs that clustered within species, suggesting that the gene duplication occurred twice independently after the divergence of the rice and Arabidopsis lineages (Fig. 1). Because knockout T-DNA insertion mutants of *CLPS5* showed no altered phenotype and CLPS5 did not interact with PCFS4 (Hunt et al., 2008; D.H. Xing, unpublished data), the following studies focused only on CLPS3.

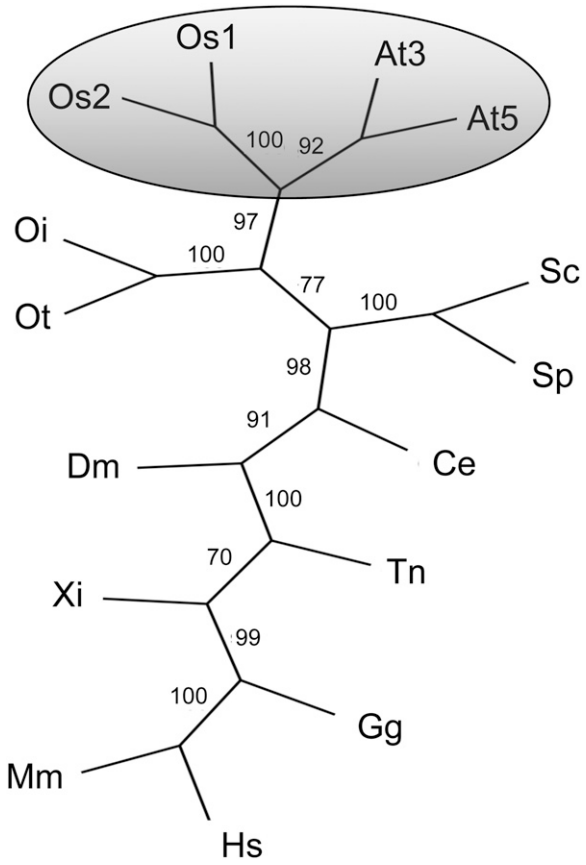

Figure 1. Phylogenetic relationship of yeast Clp1p homologs. The phylogenic tree was generated by parsimony and bootstrap programs within the PAUP 4.0 package based on the aligned amino acid sequences (Supplemental Fig. S1). The orthologs from plants are circled. Sc, *Saccharomyces cerevisiae*, NP_014893; Sp, *Schizosaccharomyces pombe*, NP_593741; Hs, *Homo sapiens*, NP_006822; Mm, *Mus musculus*, NP_598601; Gg, *Gallus gallus*, NP_001012292; Xl, *Xenopus laevis*, NP_001084787; Tn, *Tetraodon nigroviridis*, CAG04774; Ce, *Caenorhabditis elegans*, NP_001040858; Dm, *Drosophila melanogaster*, NP_610876; At3, At5, *Arabidopsis thaliana*, NP_187119, NP_198809; Os1, Os2, *Oryza sativa*, NP_001046299, EAZ22244; Ot, *Ostreococcus tauri*, CAL52668; Ol, *Ostreococcus lucimarinus*, XP_001416530.

CLPS3 Formed a Complex with Other Polyadenylation Factors

Previous studies had shown that CLPS3 directly interacts with PCFS4 and forms a complex in vivo with FY and the unknown proteins (Xing et al., 2008). To further explore the complex, cultured Arabidopsis suspension cells were transformed with *CLPS3-TAP* (tandem affinity purification) fusion or the empty *TAP* vector. Whole cell extracts from transgenic cells were prepared and subjected to affinity purification. The affinity-purified complex was probed with antibodies against a variety of polyadenylation factors. As shown in Figure 2, in addition to FY, Arabidopsis CPSF100 and CPSF160 were detected in the purified CLPS3:TAP complex, but not in the purified TAP control. However, we could not detect Arabidopsis CPSF73-I, CPSF73-II, and CPSF30 in the complex with their corresponding antibodies (data not shown).

CLPS3 Is a Nuclear Protein and Universally Expressed throughout the Arabidopsis Life Cycle

To define the subcellular localization of CLPS3, stable transgenic plants containing a *CLPS3-GFP* gene driven by the 35S promoter were generated. Confocal microscope inspection of guard cells of the cotyledon revealed that cells containing *CLPS3-GFP* fusion showed a strong GFP signal in the nucleus, but, if any, a very weak signal in the cytoplasm, in contrast to the strong GFP signals in both the nucleus and cytoplasm for the cells containing *GFP* alone (Fig. 3A). This result suggests that CLPS3 is largely a nuclear protein, consistent with its putative function as a polyadenylation factor.

To explore the temporal and spatial expression patterns of the *CLPS3* gene, the genomic sequence 950 bp upstream and 50 bp downstream of the *CLPS3* translation start site was fused to the GUS coding sequence and the chimeric gene was transformed into plants. GUS activity was examined in the transgenic plants throughout their life cycles. As shown in Figure 3B, GUS activity could be detected from the early to late stage of developing embryos, from 2- to 12-d-old seedlings, in roots, stems, leaves, and inflorescences, and through reproductive development (in flowers

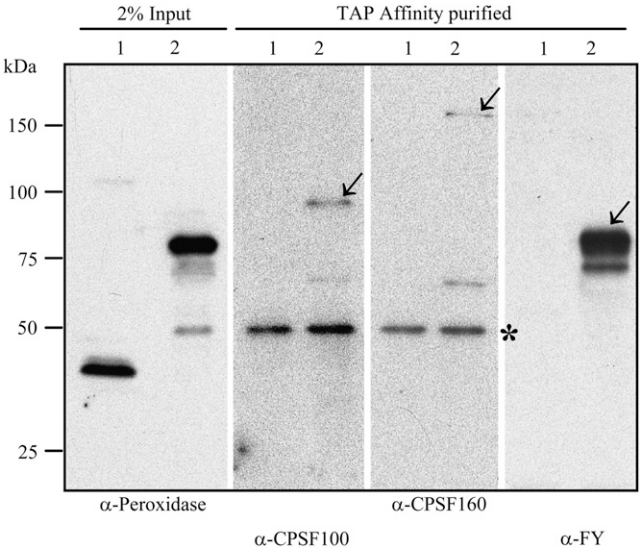

Figure 2. Protein components detected in the affinity-purified CLPS3:TAP complex. The affinity-purified protein complex from cultured suspension cells expressing 35S:CLPS3:TAP (lanes 2) or 35S:TAP (lanes 1 as a negative control) was resolved on 12% SDS-PAGE gel, transferred to membrane, and probed with designated antibodies separately. Two percent total protein was resolved and probed with peroxidase-conjugated antiperoxidase (α-Peroxidase) to show equal loading for the purified proteins. Arrows point to CPSF100, CPSF160, and FY, respectively. *, Nonspecific cross-reacting polypeptide. The low-M_r band in the CPSF100 and CPSF160 images is likely from degradation.

and flower organs and in siliques). The intensity of GUS staining was significantly stronger in the rapidly growing tissues, such as root tips, shoot tips, and pollens, likely reflecting the cell density or intensive metabolism of these tissues. These results suggest that the CLPS3 promoter is active throughout the whole life cycle and in most of tissues and organs.

CLPS3 Knockout Mutant Is Lethal and Affects Female Gametophyte Transmission

To better understand the biological functions of *CLPS3*, we characterized a T-DNA insertion mutant of *CLPS3*. A line with the T-DNA insertion at the last intron of *CLPS3* gene, designated as *clps3-1*, was obtained from the Arabidopsis Biological Resource Center (ABRC; Alonso et al., 2003). Germinating the seeds on Murashige and Skoog medium indicated that the transformants lost their kanamycin resistance. Therefore, mutant screening was performed by PCR genotyping (Fig. 4A). After extensive screening (n = 60 seedlings), only hemizygotes with T-DNA insertion (t/+) were identified, suggesting that the homozygote *clps3-1* plants (t/t) are not viable. Close inspection of the dissected siliques set on hemizygous plants H (t/+) revealed aborted seeds that were not observed in the siliques from wild-type plants (Fig. 4B).The ratio of normal seeds to aborted seeds was close to 3:1 (n = 130; χ^2 P = 0.80), suggesting that aborted seeds were likely homozygous for the T-DNA insertion. If so, the ratio of wild-type W (+/+) to hemizygote H (t/+) in the progeny from hemizygote plants H (t/+) should be 1:2. Genotyping the progeny, however, indicated that the ratio is closer to 2:3 (Table I), suggesting that the transmission of a mutated gametophyte was likely reduced.

To test which gametophyte (male or female) is responsible for the reduced transmission, reciprocal crosses of the hemizygote H (t/+) to wild-type plants W (+/+) were performed and the progeny from the cross was genotyped. When the hemizygote H (t/+) was the paternal parent, the ratio of hemizygote wild-type W (+/+) to H (t/+) in the progeny population was 1:1, indicating that the transmission of mutated male gametophyte was equally efficient as that of the wild type (Table I). On the other hand, when the hemizygote H (t/+) was the maternal parent, the ratio was 2:1 instead of 1:1, suggesting that the transmission of a mutated female gametophyte was only about 50% as efficient as that of the wild type (Table I). Thus, the T-DNA insertion mutation negatively affects female gametophyte transmission.

Lethality of *clps3-1* Is Due to the Failure of Embryo Development

To see what might be the cause of seed abortion, we examined the seed development under confocal microscopy. The embryo development of presumed *clps3-1* (t/t) homozygotes failed at a very early stage because no developing embryo was observed at all in aborted ovules (n = 73; Fig. 4C). Thus, the seed abortion of the *clps3-1* homozygote is due to the failure of its embryo development.

Genotyping and phenotyping the progeny from hemizygote selfing or from crosses between the hemizygote and wild-type plants revealed that the seed abortion phenotype cosegregated with the hemizygote (n = 237), suggesting that the seed abortion was caused either by the mutation of *CLPS3* itself or a mutation closely linked to *CLPS3*. To rule out the latter possibility, a complementation test was performed. For this, the *CLPS3-TAP* construct was used to transform the H (t/+) hemizygote plants. T0 seedlings were screened with Basta and resistant plants were recovered. These plants were further genotyped by PCR (Fig. 4A), using primer pairs that allowed differentiation of the wild-type *CLPS3* allele (higher M_r band), the T-DNA insertion allele (no amplification), and the *CLPS3-TAP* transgene allele (lower M_r band; Fig. 4D). Three of seven transgenic plants were found to be hemizygotes H (t/+). We further genotyped 36 progeny from one of three hemizygous transformants. We found that eight of 27 plants containing *CLPS3-TAP* transgene were *clps3-1* (t/t) homozygotes and no seed abortion was observed in those plants (Fig. 4B). Western-blotting assay with the antibody against the TAP tag indicated that the *CLPS3-TAP* transgene was expressed (Fig. 4D). These results indicate that the *CLPS3-TAP* transgene successfully rescued *clps3-1* (t/t) homozygous mu-

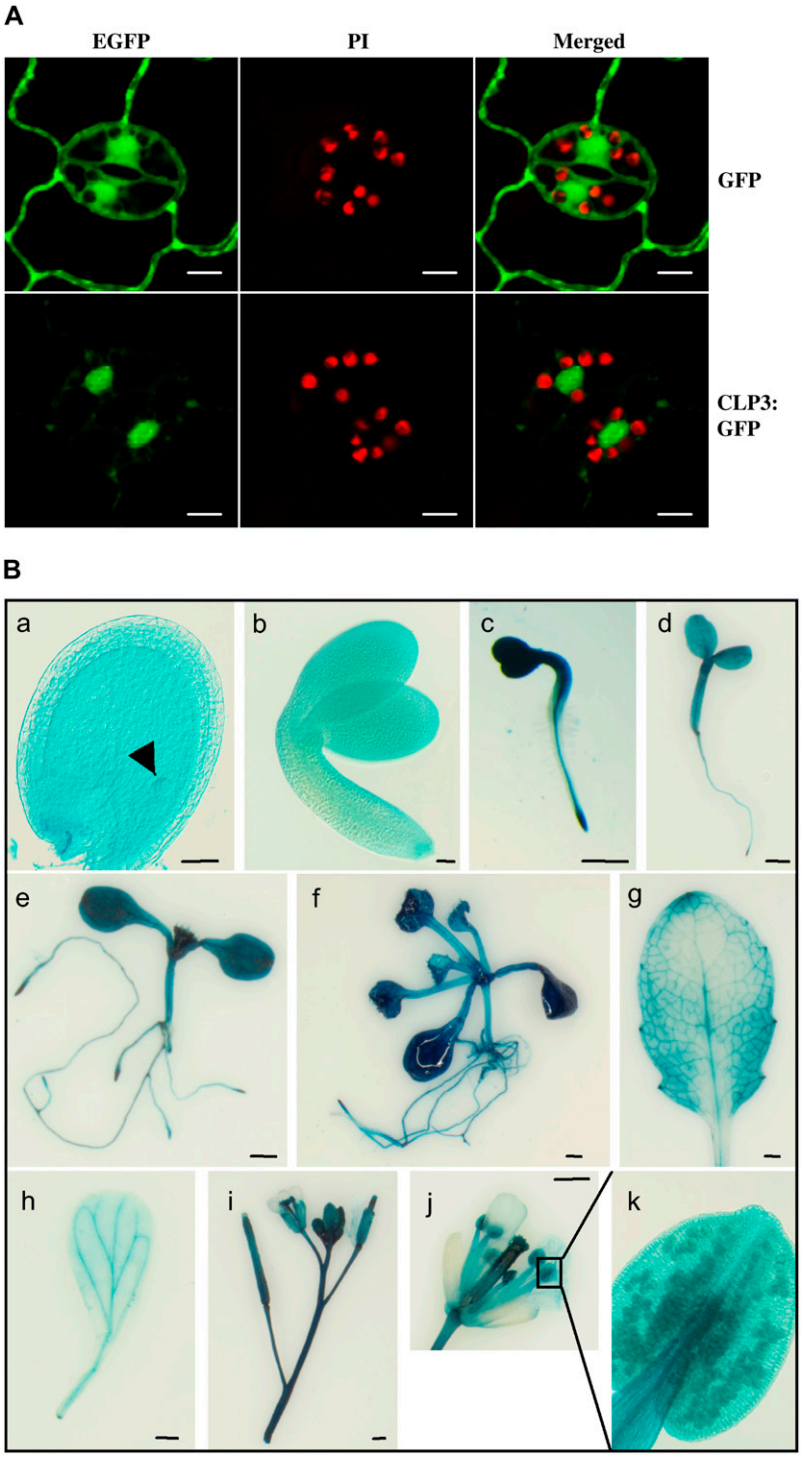

Figure 3. Subcellular localization and expression domain of CLPS3. A, GFP fluorescence and fluorescence from chloroplasts were examined with enhanced GFP and PI filter in the guard cells of wild-type plants (Col), transgenic plants containing *GFP* (GFP), and *CLPS3*:*GFP* (CLPS3:GFP) protein fusion, respectively. The green fluorescence and PI pictures were merged to show the accurate localization of GFP fluorescence (Merged). Bar = 10 μm. B, Expression profiles of Arabidopsis *CLPS3* examined by GUS activity in transgenic plants containing $CLPS3_{pro}$:*GUS* fusion in a variety of developmental stages and organs. a and b, Early stage (globular; a) and late stage (b) of embryo development; c to f, seedlings at 2, 4, 7, and 14 d postgermination; g and h, fully expanded rosette leaf and cauline leaf; i, inflorescence; j, open flower; k, anther. The arrow in a points to the embryo. Bar = 25 μm (a and b); bar = 1 mm (c–j).

tants. Thus, the T-DNA mutation of *CLPS3* was responsible for embryo lethality.

CLPS3 Overexpression Results in Altered Postembryonic Development

While the homozygote T-DNA insertion mutant *clps3-1* (t/t) was rescued by the *CLPS3-TAP* transgene, the phenotype of the rescued plants was different from the wild type. In particular, a range of developmental abnormalities was observed (Fig. 5). To see whether the CLPS3:TAP fusion was overexpressed in the plants, western-blot analysis with α-CLPS3 antibody was performed. A strong band with the M_r corresponding to CLPS3:TAP was detected in samples from *clps3-1* (t/t) *CLPS3-TAP* plants, but no signal was seen

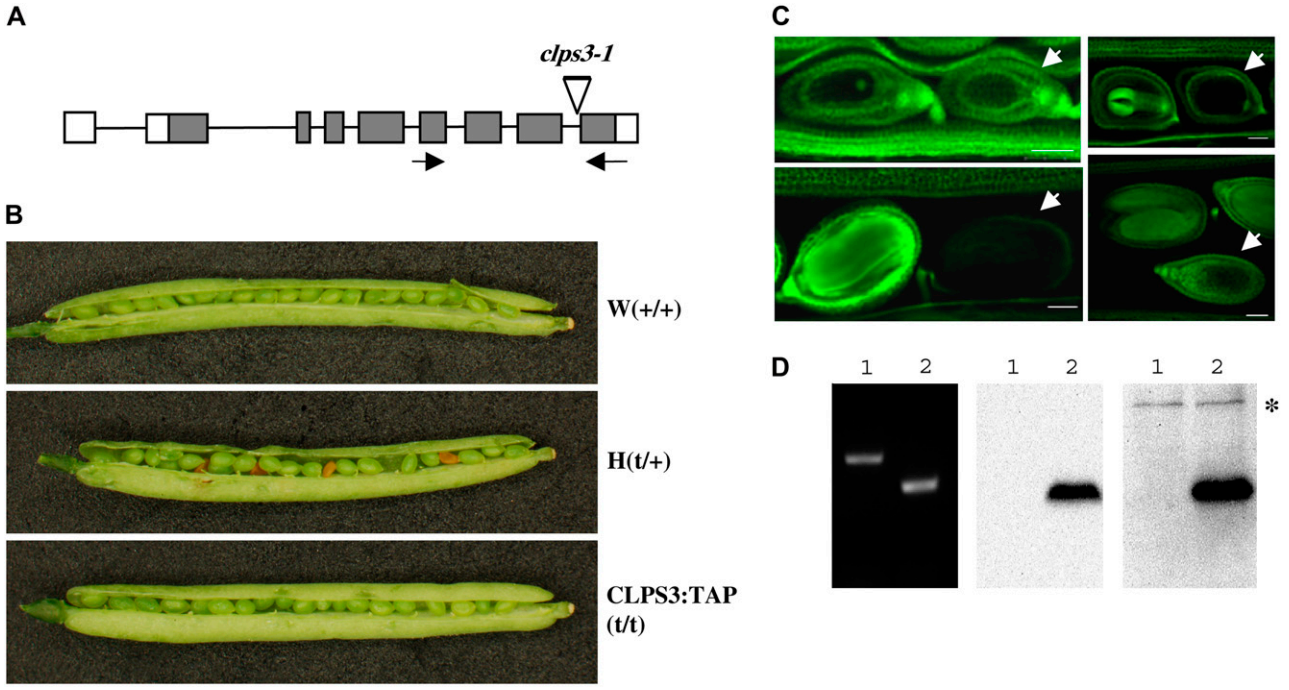

Figure 4. Mutation of *CLPS3* causes aborted embryo development. A, Schematic gene structure of *CLPS3* and the position of T-DNA insertion. The boxes and lines represent exons and introns, respectively. Gray boxes denote the coding regions. Inverted triangle points to the T-DNA insertion position. Arrows denote the annealing positions for a primer pair (p421/p499) used for PCR genotyping the *CLPS3* allele. B, Seed segregation and abortion in siliques from wild-type plants W (+/+) (top), T-DNA insertion hemizygote plants H (t/+) (middle), and T-DNA insertion homozygote plants expressing *35S:CLPS3:TAP* transgene, CLPS3:TAP (t/t) (bottom). C, Fluorescence microscopic examination of the embryo development. Siliques from T-DNA insertion hemizygote plants at a variety of postpollination stages were collected, fixed, cleared, and examined under the confocal microscope with the fluorescein isothiocyanate filter. Embryos in globular (top left), torpedo (top right), early mature (bottom right), and late mature (bottom left) stages are shown. White arrows point to the empty ovules presumably due to the developmental failure of embryos that are homozygotes for the T-DNA insertion. D, The existence and expression of CLPS3:TAP fusion in the transgenic plants were confirmed by PCR-based genotyping (using primers in A; left), western blotting with peroxidase-conjugated antiperoxidase (middle), and western blotting with antibodies against CLPS3 (right). Lanes 1, Wild-type plant; lanes 2, CLPS3:TAP (t/t) transgenic plants. *, Nonspecific cross-reaction.

in samples from wild-type plants (Fig. 4D). This indicates that CLPS3:TAP was indeed overexpressed in the rescued plants and the endogenous CLPS3 level was too low to be detected with α-CLPS3. The overexpression of CLPS3:TAP cosegregated with the developmental abnormality ($n = 36$). This abnormality was not likely due to the expression of the TAP tag because the transgenic plants overexpressing the TAP tag alone were identical to wild-type plants (data not shown). Therefore, the developmental abnormality was caused by overexpression of CLPS3:TAP.

One of the first abnormalities in the postembryonic development was the arrest of the SAM, which occurred after the cotyledon expanded or after bolting (Fig. 5A, a–d). Five of 36 T1 transgenic plants showed the premature meristem arrest. Four of 36 T1 plants showed branched main stems and/or several main stems at the base of rosette leaves, suggesting weaker apical dominance. Both rosette and cauline leaves had variable extents of leaf edge serration (Fig. 5A, e). Several cauline leaves could be also formed at one node (Fig. 5A, d). This leaf abnormality was observed in all 36 T1 plants. The most obvious abnormalities were an altered phyllotaxy (Fig. 5A, f and g) and flowering time, with the overexpression plant flowering earlier than the wild-type plants in both long-day and short-day growth conditions (Fig. 5, C and D). The altered phyllotaxy occurred in all 31 T1 plants that grew to the reproductive stage. Flower development also showed a variety of abnormalities, including altered shape and number of flower organs (Fig. 5B; Supplemental Table S1). The numbers of sepals, petals, and stamens range from 2 to 5, 3 to 5, and 3 to 7, respectively (Supplemental Table S1). The carpels,

Table I. *Inheritance of T-DNA insertion allele at CLPS3 locus*

M, Maternal parent; F, paternal parent; *N*, sample size; W (+/+), wild type; H (t/+), T-DNA insertion hemizygote; W:H, theoretical ratio for wild type to hemizygote; *P*, *P* value from χ^2 test.

Cross (M × F)	*N*	W (+/+)	H (t/+)	W:H	*P*
H (t/+) × H (t/+)	67	25	42	2:3	0.63
W (+/+) × H (t/+)	66	31	35	1:1	0.62
H (t/+) × W (+/+)	67	45	22	1:1	0.005

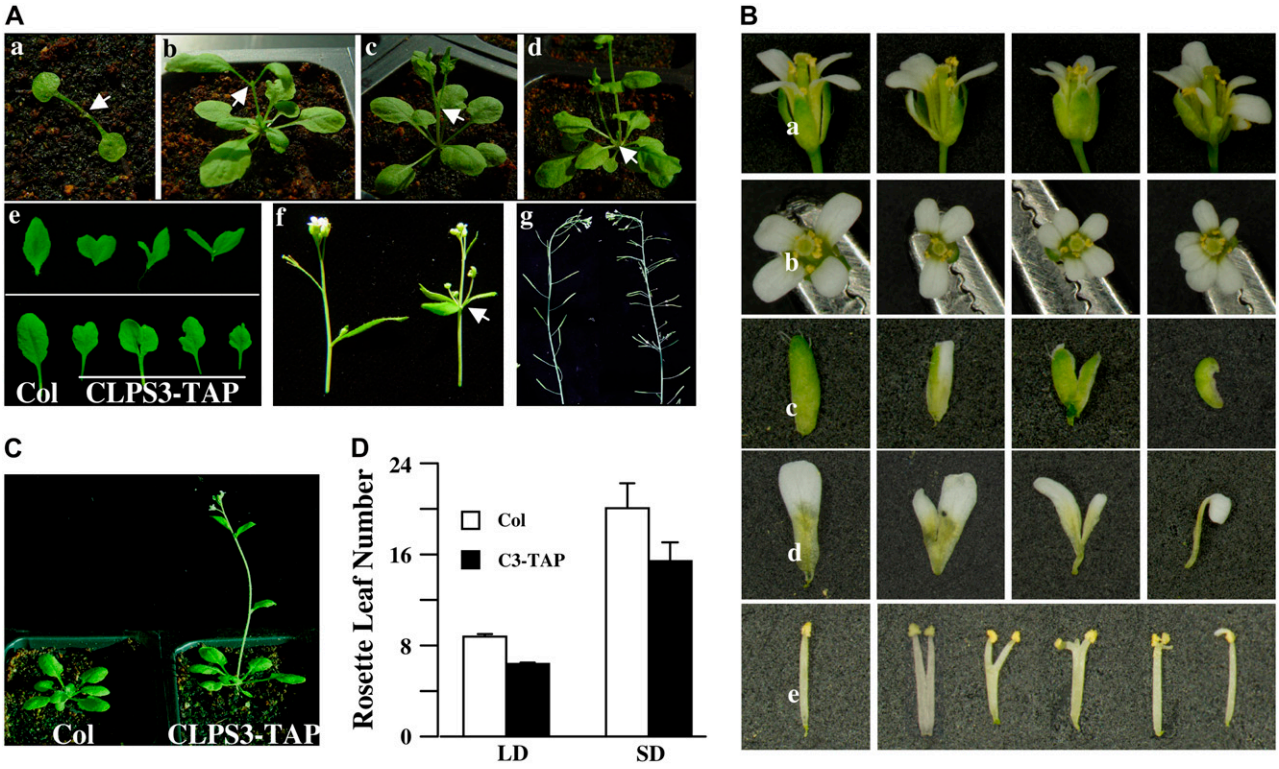

Figure 5. Altered morphology and development of the transgenic plants overexpressing CLPS3:TAP fusion protein. A, Loss of SAM after cotyledon expansion (a) or after bolting (b), branched (c), or multiple (d) main stems, altered shape for both rosette (e; bottom) and cauline (e; top) leaves, altered cauline leaf number at a single node (f), and altered phyllotaxy (g). f and g, Wild-type Col-0 at left and the CLPS3:TAP overexpression plant at right. Seedlings (a–d) are 35 days old. B, Altered flower morphology. a, Side view of the flowers; b, top view of the flowers with a variety of number of petals; c to f, altered shape of sepals, petals, and stamens. Each far-left image was from wild-type Col-0 and the other images from CLPS3:TAP overexpression plants. C, Photograph of 25-d-old CLPS3:TAP overexpression plant and wild-type Col-0 plant showing the earlier flowering phenotype. D, The reduced rosette leaf number of CLPS3:TAP overexpression plants growing in long-day (LD) and short-day (SD) conditions. [See online article for color version of this figure.]

however, were normal (Supplemental Table S1). The altered shape of sepals, petals, and stamens showed a similar pattern to that observed in the abnormalities of leaves, which was likely caused by bifurcation or fasciation. Petal-like anthers were also observed (Fig. 5B, e). In spite of multiple developmental alterations, the CLPS3:TAP overexpression plants showed no difference from wild type in both root development and seed set (data not shown).

The Early Flowering Time of CLPS3:TAP Overexpression Is Associated with Altered *FCA* Expression Pattern

Previous studies indicated that *PCFS4* is involved in the alternative polyadenylation of *FCA* pre-mRNA and the *pcfs4* mutants show a delay in flowering time (Xing et al., 2008). Because CLPS3 directly interacts with PCFS4 (Xing et al., 2008) and overexpression of CLPS3:TAP fusion led to early flowering, it was of interest to see whether the *FCA* pre-mRNA processing was also affected in the CLPS3:TAP overexpression plants. To do so, semiquantitative reverse transcription (RT)-PCR with total RNA from 14-d-old seedlings was conducted. The results indicate that the ratio of *FCA-β* and *FCA-β* was significantly decreased in CLPS3:TAP overexpression plants, suggesting that CLPS3 favors the usage of the *FCA* normal, instead of the intron 3, poly(A) site (Fig. 6, A and B).

Because the *PCFS4*-regulated flowering time was partially mediated by *FLC* and the overexpression of PCFS4-interacting protein CLPS3 causes early flowering (Fig. 6, C and D; Xing et al., 2008), we also asked whether *FLC* expression was altered in CLPS3:TAP overexpression plants. Indeed, the abundance of the *FLC* messenger was reduced in CLPS3:TAP overexpression plants when comparing to wild-type plants (Fig. 6A).

WUS and *CUC1* Expression Was Increased in CLPS3:TAP Overexpression Plants

The weaker apical dominance, altered leaf shape, phyllotaxy, and flower organs in CLPS3:TAP overexpression plants (Fig. 5) suggest that the maintenance

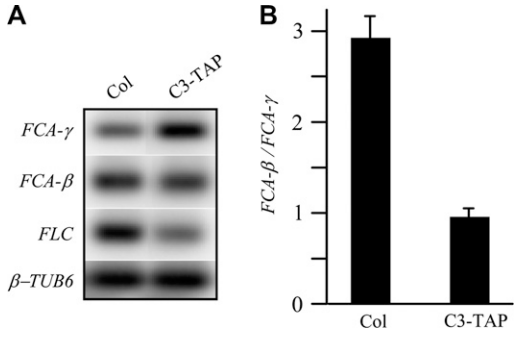

Figure 6. Expression of *FCA* and *FLC* in CLPS3:TAP overexpression plants. Semiquantitative RT-PCR was performed with DNase-treated total RNA extracted from 14-d-old seedlings. The expression of *β-TUB6* was used as an internal control. A, *FCA-γ*, *FCA-β*, and *FLC* were amplified, respectively, with transcript-specific primer pairs. Shown is a representative of three independent experimental results. B, The band intensities in A were quantified with ImageQuant and the ratios of *FCA-β* to *FCA-γ* abundance normalized with *β-TUB6* are presented. Ratios are expressed as means of three independent experiments.

and differentiation of the SAM might be disrupted in the transgenic plants. To explore the molecular mechanism of the developmental alterations, we measured the expression level of a set of genes that are involved in SAM maintenance and differentiation, including *WUS*, *STM*, *CLV1*, *CLV2*, *CLV3*, *CUC1*, *AS1*, *AS2*, *Knat1*, *Knat2*, *CycD*, *PIN1*, and *BLR* (Castellano and Sablowski, 2005; Fleming, 2005). These were done in the shoots of 10-d-old seedlings. The abundance of *WUS* and *CUC1*, but not *β-TUB6*, was significantly increased in the CLPS3:TAP overexpression plants when compared to the wild-type or transgenic plants overexpressing TAP alone (Fig. 7). However, the expression of other genes listed above was not different from controls (data not shown). This result suggests that the developmental abnormality of CLPS3:TAP overexpression plants might be accounted for by the altered expression of *WUS* and *CUC1*.

DISCUSSION

CLP1 is a predicted ATP/GTP-binding protein required for mRNA 3′-end processing in both yeast and human (de Vries et al., 2000; Gross and Moore, 2001). As a subunit of CF II_M in human, CLP1 also bridges the CF II_M, CF I_M, and CPSF complexes (de Vries et al., 2000; Gross and Moore, 2001). In addition to its role in mRNA 3′-end processing, CLP1 functions also in tRNA splicing as a RNA kinase (Weitzer and Martinez, 2007). To study the functions of plant orthologs of CLP1, we affinity purified the Arabidopsis CLPS3:TAP complex from transgenic suspension cell cultures and found that some of the CPSF subunits (CPSF100 and CPSF160; Fig. 2) reside in the complex. In previous studies, both in vitro pull-down and affinity assays with CPSF100:Flag fusion revealed that CPSF73-I is an integral part of the Arabidopsis CPSF complex (Herr et al., 2006; Xu et al., 2006). However, we could not detect the CPSF73-I in the purified CLPS3:TAP complex; either there are multiple CPSF complexes in planta with some lacking CPSF73-I or the interaction of CPSF73-I with other CPSF members is weak so that it was lost under our purification conditions. Inconsistent with the CPSF100:Flag purification, we also could not detect another CPSF subunit, CPSF30, in the purified CLPS3:TAP complex. Although the CPSF30 was found to interact with CPSF100 by Y2H and in vitro pull-down assays (Xu et al., 2006), it is likely that CPSF30 is not an essential part of the CPSF complex given that the CPSF30 null mutant did not show lethal phenotype (Delaney et al., 2006; Zhang et al., 2008). Previous Y2H studies indicated that CLPS3 did not directly interact with any of CPSF73-1, CPSF100, or CPSF160. Thus, how CLPS3 interacts with CPSF complex remains elusive (Hunt et al., 2008). It is possible that an unknown subunit in the complex might bridge CLPS3 and other CPSF members together.

Both affinity purifications with CLPS3-TAP in this study and PCFS4-TAP in a previous study from cultured suspension cells revealed that FY was in the complex (Fig. 2; Xing et al., 2008). Further analysis

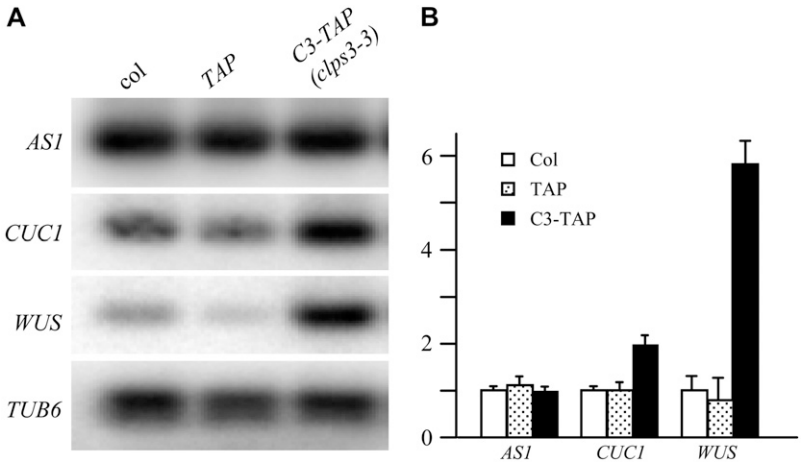

Figure 7. mRNA abundance of *AS1*, *CUC1*, *WUS*, and *β-TUB6* in Col-0, transgenic plants containing TAP alone (TAP), and CLPS3:TAP (C3-TAP) overexpression plants. A, Semiquantitative RT-PCR was performed with DNase-treated total RNA extracted from shoots of 10-d-old seedlings. No amplifications were observed for the genes investigated in the non-RT control (data not shown). B, Band intensities in A were quantified with ImageQuant and normalized with *β-TUB6*. The abundance of the gene transcripts was presented relative to that in Col-0, which was arbitrarily set as 1. Data were means of three independent experimental results.

with Y2H assay, however, indicated that while CLPS3 and PCF4 directly interacted with each other, FY does not directly interact with either of them (Xing et al., 2008). FY had likely been copurified with CLPS3:TAP and PCFS4:TAP by interacting with CPSF100, as suggested by the CPSF100-Flag purification and Y2H assay (Herr et al., 2006; Hunt et al., 2008). The current data support a model in which CLPS3 plays a similar role to that of hCLP1 in humans, to bridge different polyadenylation complexes together (de Vries et al., 2000). The combination of Y2H assays, in vitro pull-down assays, and affinity purification followed by mass spectrometry assay of multiple Arabidopsis polyadenylation factors might reveal a more completed interaction topology of Arabidopsis polyadenylation machinery.

Whereas Arabidopsis CLPS3 forms a complex with other polyadenylation factors in planta, nothing is known about its biological functions. Characterization of the *clps3-1* mutant in this study revealed, not surprisingly, that *CLPS3* is essential for embryo development (Fig. 4). Intriguingly, however, it is only partially required for female gametophyte development and not at all for male gametophyte development (Table I), suggesting that CLPS3 is not essential for gametophyte development. The characterization of the CLPS3:TAP overexpression plants suggests that CLPS3 functions in Arabidopsis postembryonic development. The effects of overexpressing CLPS3 on the Arabidopsis development are unlikely due to the disruption of the general or core polyadenylation machinery because only specific biological functions and the expression of a specific set of genes were affected in the mutant (Figs. 5–7). For instance, while apical dominance, leaf shape, phyllotaxy, and flower development were affected by the overexpression, no alteration was observed for the root and seed development (Fig. 5; data not shown). Similarly, only the expression of two of 13 genes (*WUS* and *CUC1*) examined in the SAM maintenance and differentiation was affected by the overexpression (Fig. 7; data not shown). These observations suggest that CLPS3 is unlikely to be a subunit of the core polyadenylation machinery.

CLPS3 is not the first to be identified as an Arabidopsis polyadenylation factor differentially affecting the female gametophyte transmission. Mutants of Arabidopsis CPSF73-II, the paralog of Arabidopsis CPSF73-I, also showed reduced female gametophyte transmission, but normal male gametophyte transmission (Xu et al., 2004). In contrast, CPSF73-1 overexpression was associated with male sterility (Xu et al., 2006). In mouse, testis-specific isoforms of polyadenylation factor CstF64 and poly(A) polymerase were proposed to play specialized roles in mRNA 3′-end processing during male gametogenesis (Wallace et al., 1999). A recent study revealed that the *Drosophila* sex-lethal (SXL) protein regulated alternative polyadenylation of the transcript from enhancer of rudimentary [e(r)] gene in the female germline (Gawande et al., 2006). SXL, a RNA-binding protein, switches poly(A) site choices by competitively, with CstF64, binding to the 3′-untranslated region of the e(r) transcript (Gawande et al., 2006). These observations suggest that the germline of high eukaryotes might possess special polyadenylation apparatus. It will be interesting to see whether Arabidopsis female gametophyte possesses a specific polyadenylation apparatus that contains CLPS3 and CPSF73-II subunits. That both CLPS3 and CPSF73-II have paralogs (CLPS5 and CPSF73-I, respectively) in the Arabidopsis genome provides the potential for such functional specialization.

Because CLPS3 interacts with PCFS4 in vivo (Xing et al., 2008), it is reasonable to expect that CLPS3 and PCFS4 function in the same biological process. Supporting this hypothesis, the early flowering phenotype of CLPS3:TAP overexpression is consistent with the delayed flowering of *pcfs4* loss-of-function mutants (Fig. 5; Xing et al., 2008). It will be interesting to see the flowering phenotype of CLPS3:TAP overexpression in the *pcfs4* mutant background. Further evidence that CLPS3 and PCFS4 function together is provided by the observation that both of them regulate the alternative polyadenylation of *FCA* (Fig. 6; Xing et al., 2008). It seems, however, that CLPS3 showed an antagonistic effect to PCFS4 on *FCA* alternative polyadenylation in which CLPS3 favors the usage of the regular *FCA* poly (A) site while PCFS4 enhances the usage of the intron 3 poly(A) site (Fig. 6; Xing et al., 2008). Detailed molecular events on the *FCA* alternative polyadenylation remain to be resolved.

The altered postembryonic development of CLPS3:TAP overexpression plants, specifically the weak apical dominance, the altered phyllotaxy, and flower organs, led us to speculate that some molecular events involved in SAM maintenance and differentiation might be disrupted by the overexpression. Indeed, the abundance of *WUS* and *CUC1* was significantly increased in the shoots of transgenic plants (Fig. 7). *WUS* was known to form a feedback regulation loop with *CLV3* to maintain the size of the SAM (Brand et al., 2000; Schoof et al., 2000). However, we were unable to detect a change of *CLV3* abundance in the overexpression plants. Given that the expression domain of *CLV3* is limited to a few cells within the SAM, it is likely that the change of its abundance was too diluted to be detected when the whole shoot is sampled (Fletcher et al., 1999). Alternatively, the expression domain, but not the overall expression level of CLV3, may be affected by the CLPS3:TAP overexpression in which situation the altered expression could not be detected by RT-PCR analysis. In situ hybridization analysis of CLV3 expression might give the answers to this question. *CUC1* functions in organ boundary definition and mutation of *CUC1* leads to the fused cup-shaped cotyledons (Takada et al., 2001). The altered phyllotaxy, fused leaf, sepal, petal, and anther might be attributed to the disrupted expression of *CUC1* in the CLPS3:TAP overexpression plants.

In conclusion, the protein interaction assay and the characteristics of gain- and loss-of-function of CLPS3 lines strongly suggest that CLPS3 is a polyadenylation factor involving in multiple biological functions. Future studies will focus on how CLPS3 and other polyadenylation factors regulate specific biological functions at molecular and biochemical levels.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) genotypes used in this study were in Columbia-0 (Col-0; CS6000) background. The T-DNA insertion mutant (SALK_025156) at the CLPS3 locus (At3g04680), designated as *clps3-1*, was obtained from the ABRC (Alonso et al., 2003). Seeds were sown in plastic pots (50 × 60 mm) with soil (Sun Gro 360; Sun Gro Horticulture), stratified at 4°C for 2 d, and moved to a growth chamber. The growth conditions were set as the following: temperature, 22°C; light intensity, 100 mmol m^{-2} s^{-1}; photoperiods, 16 h light/8 h dark for long day and 8 h light/16 h dark for short day.

Phylogenetic Analysis

The amino acid sequences of CLP1 homolog proteins from a variety of eukaryotes were downloaded from National Center for Biotechnology Information and analyzed as previously described (Xing et al., 2008).

Plasmid Construction and Transformation

All gene fusions in this study were constructed essentially as described previously (Xing et al., 2008). All clones were confirmed by DNA sequencing. Gene fusion constructs in appropriate T-DNA plasmids were first introduced into *Agrobacterium* strain GV3101 and then transferred into Arabidopsis plants (Clough and Bent, 1998) or cultured suspension cells (Menges and Murray, 2004). The transformants were screened by appropriate antibiotics or herbicide.

For the complementation test, the *CLPS3* CDS amplified from plasmid U17288 (from ABRC) with primers p462/p499 were first cloned into pDONR201, resulting in pD201-CLPS3 and then cloned into pCTAPi plasmid by LR reaction (according to the Gateway vector system from Invitrogen), resulting in pCLPS3-TAP. For *CLPS3-GFP* fusion, the *CLPS3* CDS from pD201-CLPS3 was cloned into pMDC83 by LR reaction, resulting in CLPS3-pMDC83.

For CLPS3 promoter activity assays, the genomic sequence 950 bp upstream and 50 bp downstream of the CLPS3 start codon was amplified with a primer pair p571/p572. The PCR product was first cloned into pTOPO-SD plasmid (Invitrogen) and then introduced into pMDC162 plasmid (Curtis and Grossniklaus, 2003) by LR reaction, resulting in CLPS3-pMDC162, using a protocol as described (Xu and Li, 2008). These related binary vectors were then transformed to Arabidopsis.

Protein Complex Isolation and Western-Blot Analyses

The TAP of the tagged CLPS3 was performed essentially as described (Rohila et al., 2004). Briefly, the protein extract from 10 g of cultured suspension cells was incubated with IgG Sepharose beads (GE Healthcare Bioscience AB) at 4°C for 4 h, washed four times with IPP150 buffer. The binding protein was released with AcTEV protease for 2 h at 16°C. After brief centrifugation, the supernatant was used for immunoblot analysis. Western-blot analysis was performed as described (Xu et al., 2006). The α-FY antibody was a gift from Caroline Dean (John Innes Center). Antibodies against CPSF30, CPSF73-1, CPSF73-2, CPSF100, CPSF160, and CLPS3 were described previously (Xu et al., 2006; Xing et al., 2008). Detection of the TAP tag protein A part was done by using the peroxidase-conjugated antiperoxidase antibody (Sigma).

CLPS3 Subcellular Localization and GUS Activity Assay

The transgenic plants containing the CLPS3-pMDC83 plasmid were germinated in Murashige and Skoog medium for 3 to 5 d. The GFP signals in guard cells of cotyledons were examined under a confocal microscope with an enhanced GFP filter. The GUS activity assay was as described (Xing et al., 2008).

Genotyping and Phenotype Scoring

The screening of the *clps3-1* mutant and genotyping of the segregation population from the T-DNA hemizygote H (t/+) or from the cross between H (t/+) and Col-0 were performed by PCR using two gene-specific primers, p421and p499 or p421, and T-DNA left-border primer p500 (all primer sequences are listed in Supplemental Table S2).

For the seed phenotype, siliques in a series of developmental stages were opened with a syringe and the developing seeds were scored under a dissecting microscope. For scoring flowering time, the seeds of different lines harvested at the same time were germinated and the rosette leaf number was counted when the first flower opens.

For embryo development observations, siliques were fixed in fix solution (4% glutaraldehyde, 12.5 mM cacodylate, pH 6.9) for 24 h, rinsed with distilled water, dehydrated in ethanol series, cleared in clear solution ethanol:benzylbenzoate:benzylalcohol (3:2:1 [v/v]), and kept in storage solution benzylbenzoate:benzylalcohol (2:1 [v/v]) at 4°C in dark. The cleared siliques were mounted with immersion oil on glass slide and covered with coverslips for confocal microscope observation. The fluorescein isothiocyanate filter was used to capture the natural fluorescence from the ovules and embryos.

Gene Expression Analysis

The semiquantitative PCR and GUS staining assays were performed essentially as described previously (Xing et al., 2008). Primer pair p657/p658 was used for the amplification of *β-TUB6*; p687/p689 for *FCA-γ*; P687/688 for *FCA-β*; p662/p663 for *FLC*; p780/p781 for *AS1*; p778/p779 for *CUC1*, and p760/p761 for *WUS*. PCR was performed for 20 cycles with annealing temperature at 55°C. The amplified product was transferred onto membrane, hybridized with a corresponding p32-labeled probe, and detected with a phosphor imager.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NP_187119 (AtCPLS3).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence alignment of yeast Clp1p homologs.

Supplemental Table S1. Statistics of altered flower structure in CLPS3:TAP overexpression plants.

Supplemental Table S2. Oligonucleotides used in this study.

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