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Arabidopsis VIP6/ELF8, the homolog of CTR9 component of the transcriptional complex PAF1, is essential for plant development

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Abstract *The VERNALIZATION INDEPENDENCE 6* (*VIP6*)/*EARLY FLOWERING 8* (*ELF8*) gene of *Arabidopsis thaliana* encodes a protein homologous to CYCLIN THREE REQUIRING 9 (CTR9) component of the yeast transcriptional complex, RNA polymerase II-associated factor 1 (PAF1). It has been demonstrated that mutation alleles in the *VIP6*/*ELF8* show early flowering phenotype, as well as other pleiotropic developmental defects. Here, we provide evidence that seeds with *vip6*/*elf8* homozygous mutations were rarely obtained in all three independent lines of T-DNA insertion. Although viable seeds with homozygous mutant alleles were rarely obtained, they showed pleiotropic phenotype like mutants of other PAF1-related genes, and were sterile. These results suggest that the *VIP6*/*ELF8* gene is essential for plant development.

Key words: CTR9, ELF8, embryonic lethality, PAF1 complex, VIP6.

Gene regulation in eukaryotes is affected by chromatin structure. Changes in chromatin structure are regulated by multiple ways of histone modifications, such as acetylation, methylation, and ubiquitination. It is believed that the trimethylated histone H3 at lysine 4 (H3K4), 36 (H3K36) and 79 (H3K79) are hallmarks of active chromatins (Shilatifard 2006). In yeast, trimethylation of H3K4 in the 5′ region of the target gene is catalyzed by the SET1 histone methyltransferase, and the recruitment of SET1 protein requires the RNA polymerase II-associated factor 1 (PAF1) complex together with serine-5 phosphorylation of the C-terminal domain of RNA polymerase II (Hampsey and Reinberg 2003). The yeast PAF1 complex consists of five components, PAF1, LEO1, RTF1, CTR9 and CDC73 (Mueller and Jaehning 2002; Krogan et al. 2003; Squazzo et al. 2002), and the PAF1 complex is required for efficient transcription elongation by RNA polymerase II (Marton and Desiderio 2008). Moreover, the PAF1 complex may regulate gene expression through a post-transcriptional mechanism, such as 3′-processing of pre-mRNAs (Mueller et al. 2004; Penheiter et al. 2005; Sheldon et al. 2005). In human, hSki8 was identified as another component of the human PAF1 complex (Zhu et al. 2005). Human hSki8 subunit is the homolog of yeast SKI8/REC103 (Gardiner et al. 1997) that is a component of yeast superkiller (SKI) complex, which is required for 3′-to-5′ degradation of cytoplasmic mRNAs (Masison et al. 1995; Anderson and Parker 1998). Human hSKI complex localizes to both nucleus and cytoplasm and interacts with human PAF1 complex, and associates with transcriptionally active genes depending on the presence of human PAF1 complex (Zhu et al. 2005). Thus, in addition to coordinating events during transcription, hPAF1 complex also coordinates events in RNA quality control.

In *Arabidopsis thaliana*, growing evidences indicate that the major floral repressor FLOWERING LOCUS C (FLC), a MADS-box transcription factor, is under the control of multiple chromatin modifiers. It is evident that H3K4 trimethylation plays an important role in the activation of *FLC* transcription (Dennis and Peacock 2007; He and Amasino 2005; Noh and Noh 2006; Reyes 2006; Schmitz and Amasino 2007). Recently, it was reported that methylation of H3K36 is also involved in the activation of *FLC*, because the loss-of-function

Abbreviations: Cln, cyclin; CTR9, Cln three requiring 9; ELF, EARLY FLOWERING; EFS, EARLY FLOWERING IN SHORT DAYS; FLC, FLOWERING LOCUS C; FLM, FLOWERING LOCUS M; H3K4, histone H3 lysine 8; H3K36, histone H3 lysine 36; H3K79, histone H3 lysine 79; MAF, MADS AFFECTING FLOWERING; NES, nuclear export signal; PAF1, RNA polymerase II-associated factor 1; SKI, superkiller; SDG8, SET DOMAIN GROUP 8; TPR, tetratricopeptide-repeat; VIP, VERNALIZATION INDEPENDENCE

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mutants of *EARLY FLOWERING IN SHORT DAYS* (*EFS*)/*SET DOMAIN GROUP 8* (*SDG8*) gene that encodes a homolog of H3K36-specific histone methyltransferase, SET2, show a dramatically reduced level of H3K36 di-methylation and an early-flowering phenotype (Kim et al. 2005; Zhao et al. 2005).

EARLY FLOWERING 7 (ELF7, also known as VERNALIZATION INDEPENDENCE, VIP2) is a homolog of yeast PAF1 and is required for the elevated expression of *FLC* in the autonomous-pathway late-flowering mutants and in a winter-annual accession (He et al. 2004; Oh et al. 2004). In addition, *Arabidopsis* homologs of LEO1 (VIP4), RTF1 (VIP5), and CTR9 (ELF8, also known as VIP6) are also required for the transcriptional activation of *FLC*, and mutations in these genes result in early flowering phenotype. *VIP6*/*ELF8* interacts with VIP4 (He et al. 2004; Oh et al. 2004) and also with a WD-repeat protein VIP3 (Oh et al. 2004) that is the *Arabidopsis* homolog of SKI8 (Jolivet et al. 2006), indicating that, like human PAF1 complex, *Arabidopsis* PAF1 complex contains a SKI8 ortholog. On the other hand, there is no evidence that *Arabidopsis* PAF1 complex contains a CDC73 homolog. Thus, there may be slight difference in the composition of PAF1 complex among different organisms. It is believed that the *Arabidopsis* PAF1-like complex is required for the general activation of *FLC* transcription (Dennis and Peacock 2007; He and Amasino 2005; Noh and Noh 2006; Reyes 2006; Schmitz and Amasino 2007). In addition to suppressing *FLC* expression, mutations in the PAF1-like genes also suppress the expression of *FLC*-related MADS-box genes, *MADS AFFECTING FLOWERING* (*MAF*) *1* to *MAF5* (He et al. 2004; Oh et al. 2004; Xu et al. 2008). Consistent with this, trimethylation of H3K4 in *FLC* as well as in *FLOWERING LOCUS M* (*FLM*)/*MAF1* chromatin was reduced in *elf7* and *elf8* mutants (He et al. 2004). Similarly, decreased levels of trimethyl-H3K4 in *FLC* and *FLM*/*MAF1* chromatins were observed in *vip4* mutants (Xu et al. 2008).

Arabidopsis PAF1-like complex mutants, *vip2*/*elf7*, *vip3*, *vip4*, *vip5* and *vip6*/*elf8*, show similar phenotypes of pleiotropic developmental defects that are not observed in *flc* null alleles (He et al. 2004; Oh et al. 2004; Xu et al. 2008; Jolivet et al. 2006; Zhang and van Nocker 2002; Zhang et al. 2003). Those phenotypes include reduced plant size, reduced apical dominance, reduced fertility and specific morphological defects in flowers, but any mutations in genes encoding components of PAF1 complex never resulted in lethality. Contrary to reports in the literature, we present evidence that seeds with *vip6*/*elf8* homozygous mutations were hardly obtained in all three independent lines of T-DNA insertion.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Columbia (Col) was used as wild type. The T-DNA-tagged *Arabidopsis* lines (Alonso et al. 2003), SALK_065364, SALK_090130, SALK_090131 and SALK_090141 were obtained from the Arabidopsis Biological Resource Center. Seeds were sterilized in 70% ethanol for 1 to 2 min, 1% sodium hypochlorite plus 0.02% Triton X-100 for 10 min, and thoroughly rinsed in sterile water for 5 times. The sterilized seeds were sown on plates that contained 0.8% agar (BA-10, Ina Food Industry Co., Ltd., Nagano), 1×Murashige and Skoog Plant Salt Mixture (Nihon Pharmaceutical Co. Ltd., Tokyo), 1×Gamborg's Vitamin Solution (Sigma), 1% sucrose, and 0.5 g l^{-1} 2-morpholinoethanesulfonic acid, pH adjusted to 5.7 with KOH. After being stored in a refrigerator at 4°C for 2 days, they were germinated in a growth chamber (MLR-350, Sanyo) under white fluorescent light (FL40SSW/37, Toshiba) at 30 to 44 μmol m^{-2} s^{-1} in long days (16 h light/8 h dark) at 22°C, 60% relative humidity. When antibiotic selection of transformants was needed, 25 μg ml^{-1} kanamycin (for SALK lines) or 15 μg ml^{-1} hygromycin B (for Kazusa line KG6249) was included in the agar plates. Seven to 10 days after germination, seedlings were individually transplanted to cubes of rockwool (Nittobo, Tokyo), put on mixture of perlite-vermiculite (1 : 1) in plastic pots, and further grown on shelves under white fluorescent light (FL40SW, Toshiba) at 32 to 44 μmol m^{-2} s^{-1} in long days (16 h light/8 h dark) in a air-conditioned room at 22°C, 60% relative humidity. Pots were put on plastic trays and irrigated weekly with MGRL medium (Fujiwara et al. 1992).

Identification of KG6249

Additional mutant line KG6249 was identified from the collection of T-DNA insertion mutants of Kazusa DNA Research Institute (Kato et al. 2007), using polymerase chain reaction (PCR) with appropriate primers coupled with DNA hybridization with a labeled probe prepared from the cDNA clone (RAFL09-37-I15, AY070455) provided by RIKEN Bioresource Center. KG6249 was confirmed to carry a T-DNA insertion in the immediate downstream of 5′-end of the 2nd intron of the gene (Figure 1, DDBJ Genome Survey Sequence AB429509).

Genotyping analysis

The following primer combinations were used for amplification and sequencing of the *Arabidopsis* genomic DNA next to the T-DNA insertions:

KG6249: RB with 06R (5′-TTCCCTTAATTCTCCGCTC-ATGATC-3′)/KG6249-A1 (5′-CAGGAACATTATCAGGGGC-3′); wild-type allele with KG6249-S1 (5′-ATCTCTGGCT-CATCATCGC-3′)/KG6249-A1. SALK_090130: LB with SIG-LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′)/SIG090-S1 (5′-GCCCCTGATAATGTTCCTG-3′); wild-type allele with SIG090-S1/SIG090-A2 (5′-CCCCCTAAAACAAGACCA-3′). SALK_065364: LB with SIG-LBb1/SIG065-S1 (5′-TGCC-TTTTCTATGGGTCC-3′); wild-type allele with SIG065S1/SIG065-A1 (5′-CCCATACATCAGGCATCTG-3′).

Figure 1. Structure of the *VIP6*/*ELF8* gene (At2g06210). The horizontal line indicates introns, and exons are shown as open (untranslated region) or closed (translated region) boxes. The positions of T-DNA insertions are indicated. Gene-specific primers used for genotyping are indicated by filled arrowheads, and primers used for RT-PCR are shown by open arrowheads.

RT-PCR

Total RNA was isolated using Concert Plant RNA Reagent (Invitrogen) according to the manufacturer's protocol. The *VIP6*/*ELF8* cDNA was amplified using ReverTra Dash kit (Toyobo) with the primer pair AtIABP4mRNA-S1 (5′-TCCATAGGCTGATTGAAAGTGGTC-3′) and AtIABP4mRNA-A1 (5′-CCAGTTCCCCAGAGAAAGAATA-GC-3′). Primer combination for Actin cDNA was Actin1-F (5′-TCCATCTTGGCATCTCTCAG-3′) and Actin1-R (5′-GTAC-CCGCATCAGGCATCTG-3′).

Microscopy

Fruits of different developmental stages were dissected with needles and observed with a binocular microscope. Developing ovules and seeds were cleared and observed by a Nomarski microscope as described (Aida et al. 1997).

Accession numbers

Sequence data from this article can be found in DDBJ/GenBank/EMBL data libraries under accession numbers AB429509 (genomic sequence of KG6249 flanking T-DNA insertion) and BR000721 (*VIP6*/*ELF8* mRNA).

Results

Identification of mutant alleles in Arabidopsis At2g06210 gene (VIP6/ELF8)

Previously, we isolated a rice (Oryza sativa) cDNA (AB057651, Os07g0476200/LOC_Os07g29360, OsCTR9) encoding a putative nuclear protein with tetratricopeptide-repeats (TPRs) that shares sequence homology with $p150^{TSP}$ of mouse (also known as SH2BP1 or mCTR9; Malek et al. 1996) whose physiological function was unknown (Shiraya et al., manuscript in preparation). To understand function of CTR9 homologs in plants, we decided to investigate T-DNA insertion mutants of its homologous gene (At2g06210) in *Arabidopsis*, and obtained mutant alleles, SALK_065364, SALK_090130, SALK_090131 and SALK_090141 from a collection developed at the Salk Institute Genomic Analysis Laboratory (SIGnAL; Alonso et al. 2003), and KG6249 from Kazusa DNA Research Institute (Kato et al. 2007). Later, there appeared reports demonstrating that the gene At2g06210 encodes the *Arabidopsis* homolog of CTR9 (ELF8/VIP6; He et al. 2004; Oh et al. 2004). Oh et al. (2004) used *vip6-1* allele that had been generated by fast-neutron mutagenesis and lacked the entire region of At2g06210, and T-DNA insertional mutants *vip6-2* and *vip6-3*, as well as two alleles SALK_065364 and SALK_090130, which were the same alleles that we used in this study. In addition, the mutant alleles reported as *elf8-1*, *elf8-2* and *elf8-3* correspond to SALK_090130, SALK_065364, and SALK_103938, respectively (He et al. 2004; YueHui He, personal communication). The corresponding disrupted positions in the *VIP6*/*ELF8* gene are presented (Figure 1). There are no reports on point-mutated alleles in the At2g06210 gene.

In addition, we consider that the gene model in the public databases (encoding a protein of 1064 amino acids; He et al. 2004) is incorrect and instead the model encoding a protein of 1091 amino acids (Oh et al. 2004) is correct. The misinterpretation seems to have been resulted from an error in the *Arabidopsis* genome sequence (NC_003071), because the sequences of a cDNA (AY070455) and an expressed sequence tag (EH819393) as well as the Genome Survey Sequences BH241404, BH862564/ED598602 (flanking genome sequences of SALK_090131), BH862563/ED598601 (flanking genome sequences of SALK_090130) and BH241331 support the presence of an additional 'C' at position 712 in mRNA (DDBJ Third Party Annotation BR000721). The 27-amino acid sequence from amino acid position 199 to 225 had been inadequately excluded by misinterpreting the corresponding 81-bp nucleotide sequence at the 3′-end of the 6th exon as an intron. The inclusion of this region gives better sequence alignment with other homologs of CTR9, and this region includes a conserved amino acid sequence that has been identified as the functional nuclear export signal (NES) in OsCTR9 (Shiraya et al., manuscript in preparation).

Seedlings with homozygous vip6/elf8 mutation alleles were hardly obtained

Heterozygous plants were identified by PCR screening for mutant alleles KG6249, SALK_090130, SALK090131 and SALK_065364, but not for SALK_090141 allele. These heterozygous plants showed no obvious growth phenotype, and indeed they expressed the *VIP6*/*ELF8* transcript as well as wild type plants (Figure 4B). Mutant alleles, KG6249, SALK_090130 and SALK_065364, were analyzed further. However, we were unable, despite extensive screening, to identify homozygous mutants among the progeny of each heterozygous plant (Figure 2). We first checked the germination rates of seeds collected from self-pollinated heterozygous plants, but there was no difference between mutant alleles and wild type (Table 1), indicating that it is not the problem of seed germination. However, when immature fruits on heterozygous plants were opened and examined under a microscope, approximately 36 to 40% of the ovules remained undeveloped (Table 2, Figure 3A) whereas such undeveloped ovules were only 10% in control wild-type plants (Table 2, Figure 3B). The undeveloped ovules were randomly distributed along the fruit and no increase in size was observed as the fruits matured (Figure 3A). The segregation ratio of developed seeds to undeveloped ovules were found to be in the range of 1.47 : 1 to 1.76 : 1, showing distortion from segregation ratio of 3 : 1 as expected for a recessive mutation in an essential nuclear gene (Table 2). These results suggested that a disruption of the *VIP6*/*ELF8* gene is not only causing embryonic lethality but also partial defect in gametophytic transmission of mutant alleles.

To figure out what occurs during the process, flower organs of various stages of development were sampled from heterozygous *vip6*/*elf8* plants, and cleared whole-mounts were observed using Nomarski optics (Figure 3). At the early stages just before and after pollination, forms and the sizes of ovules in *vip6*/*elf8* plants did not differ from those of wild type plants (Figure 3C, D). The embryos corresponding to the undeveloped ovules seemed to be arrested at an early, pre-globular, stage of development although they were not clearly visible (Figure 3G, H). We could not determine, at this stage, whether the first asymmetric division, or even the fertilization, of the mutant zygotes occurred or not. On the contrary, normal-appearing green seeds contained embryos at the normal heart stage (Figure 3F). Numbers of degenerating ovules increased as fruit development proceeded (Figure 3I, J; Table 3), indicating that aborted seeds or unfertilized ovules are directed to degeneration process. By staining with aniline blue, pollen tube growth into such degenerating ovules was observed (data not shown), indicating that some defects other than pollen tube growth is causing sterility. These observations thus suggest that a defect in *VIP6*/*ELF8* is causing embryonic lethality and some defect in fertilization.

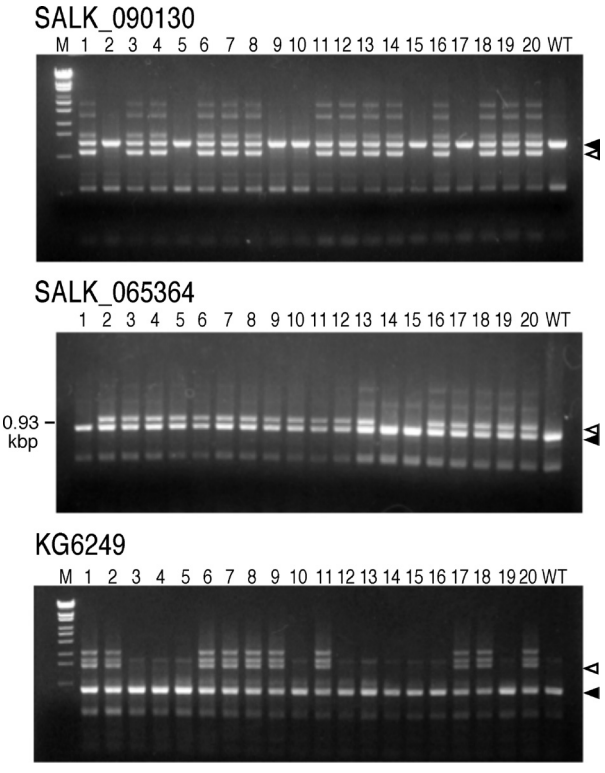

Figure 2. Analysis of T-DNA insertion mutants of the *VIP6*/*ELF8* gene. Twenty progenies from each heterozygous plant were analyzed by multiplex genomic PCR with a mixture of a T-DNA specific primer and a pair of gene-specific primers. Open arrowheads indicate products from mutant alleles, and closed arrowheads indicate those from wild-type (WT) allele. M: λ/*Sty*I marker.

Table 1. Germination rate of seeds collected from *vip6*/*elf8* heterozygous mutants 5 days after sowing

	Germination (%)	n
SALK_090130	88.4	498
SALK_065364	89.8	139
KG6249	90.1	142
Wild type	88.1	134

Table 2. Seed development in fruits of *vip6*/*elf8* heterozygous mutants

	Developed seeds	Undeveloped ovules	Ratio	Total
SALK_090130	452 (59.6%)	307 (40.4%)	1.47 : 1	759 (100%)
SALK_065364	702 (63.7%)	400 (36.2%)	1.76 : 1	1102 (100%)
KG6249	708 (62.2%)	430 (37.8%)	1.65 : 1	1138 (100%)
Wild type	804 (90.0%)	89 (10.0%)	Not applicable	893

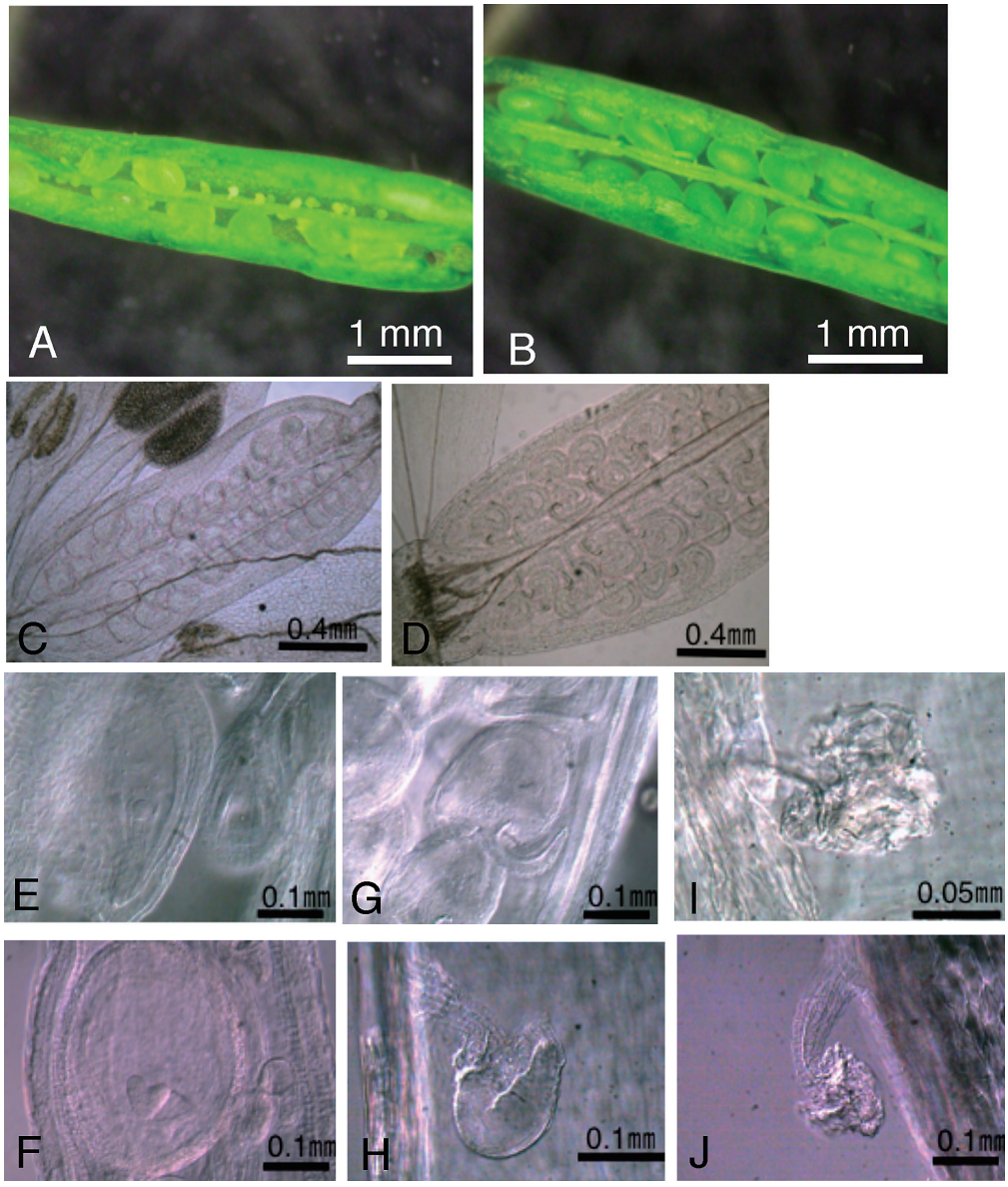

Figure 3. Phenotype of heterozygous *vip6*/*elf8* mutants. (A) Open young fruit of heterozygous SALK_090130. (B) Open young fruit of wild-type plants. (C to J) Images of heterozygous SALK_090130 plants observed by Nomarski optics after tissue clearing. Ovules before (C) and 1 day after (D) pollination. A two-cell stage embryo (E) and a heart stage embryo (F) in normal seeds. (G) and (H) are those of aborted seeds, and (I) and (J) are degenerating ovules, each at similar time-points as (E) and (F), respectively. Same phenotype was observed in heterozygous SALK_065364 and KG6249 (data not shown).

Table 3. Developmental state of ovules or seeds after self pollination in *vip6*/*elf8* heterozygous mutant of SALK_090130 allele

Days after pollination	Stage	Normal-appearing ovules or seeds (%)	Aborted seeds (%)	Degenerating ovules (%)	n
0	(fertilization)	100	0	0	228
1	1 or 2 cell	91.6	8.4	0	311
2	8 or 16 cell	55.8	37.0	7.1	154
3	Globular	56.6	26.2	17.1	198
4	Globular	56.8	30.8	12.3	227
5	Heart	53.7	30.5	15.7	229
6	Heart	61.6	26.2	11.6	112
7	Heart	51.4	24.2	24.2	107
8	Mature	59.6	12.8	23.6	156
9	Mature	54.2	12.5	28.3	208

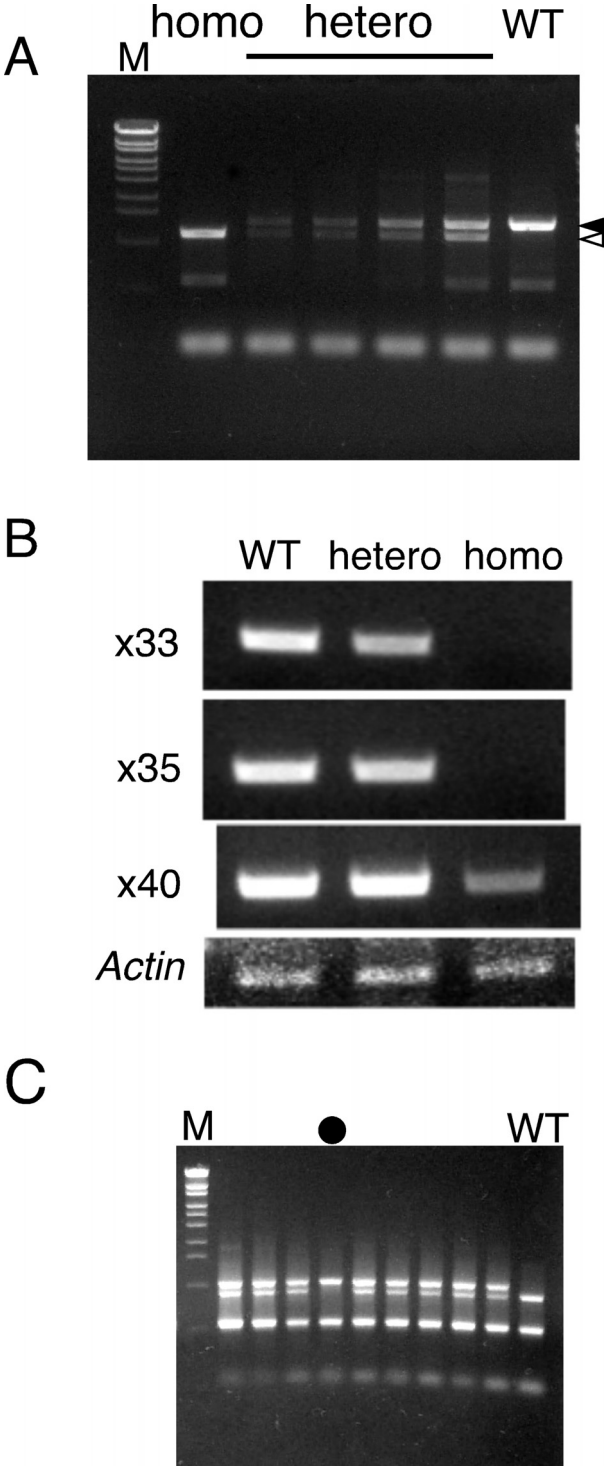

Figure 4. Identification of homozygous *vip6*/*elf8* mutants. (A) Genotyping analysis of SALK_090130 allele by multiplex PCR as described in Figure 2. From left to right: a homozygous (homo) mutant, four heterozygous (hetero) mutants and a wild-type (WT) plant. Open arrowheads indicate products from mutant alleles, and closed arrowheads indicate those from wild-type (WT) allele. (B) Analysis of *VIP6*/*ELF8* expression by RT-PCR in wild-type (WT), heterozygous (hetero) and homozygous (homo) SALK_090130 mutant plants. Numbers of PCR cycles are indicated on the left. *Actin* is used as control. (C) Genotyping analysis of SALK_065364 allele by multiplex PCR as described in Figure 2. One homozygous mutant (closed circle) was identified. WT: wild type. Others are heterozygous mutants. M: λ/*Sty*I marker.

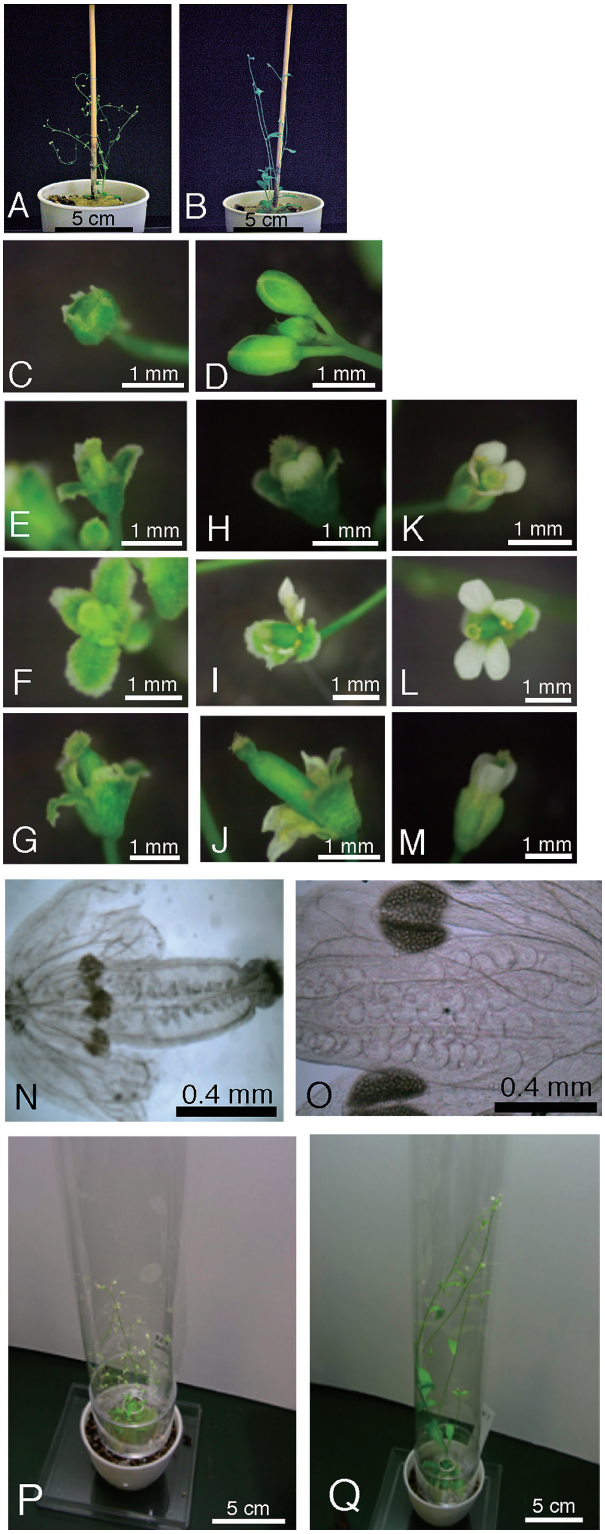

Figure 5. Phenotype of homozygous *vip6*/*elf8* mutants. (A) A homozygous mutant of SALK_090130. (B) A wild-type plant. Flower buds just before flowering in the mutant (C) and in wild-type (D) plants. (E to M) Morphology of flowers just before (E, H, K) or after (F, I, L) flowering, and at later stages (G, J, M). Severe (E to G) and moderate (H to J) phenotypes of a homozygous SALK_090130 mutant plant are shown, along with those of wild-type plants (K to M). Nomarski images observed after clearing of flowers of homozygous SALK_090130 mutant (N) and wild-type (O) plant before dehiscence of anthers are shown. (P) Putative homozygous mutant of KG6249. (Q) A wild-type plant.

Homozygous vip6/elf8 plants rarely occur and show pleiotropic developmental phenotype

As our results that the homozygous mutations in the *VIP6*/*ELF8* gene lead to embryonic lethality contradicted the previous reports (He et al. 2004; Oh et al. 2004), we further searched for homozygous plants and found that, although quite rarely, homozygous *vip6*/*elf8* plants do exist. So far we have definitely identified one homozygous mutant plant with similar developmental phenotype each from SALK_090130 (Figure 4A, Figure 5A) and SALK_065364 (Figure 4C). In the KG6249 line, one putative homozygous mutant plant was also found, but supporting data other than photograph (Figure 5P) is absent. In addition, we observed before another putative homozygous plant of SALK_065364, predicted from the presence of T-DNA insertion in the gene and the absence of the *VIP6*/*ELF8* transcript (data not shown). In common, they were dwarf, had thin stems and small leaves and showed reduced apical dominance, as compared with wild type plants. Though they got old earlier, they did not produce any seeds. Although we could examine in detail only one homozygous mutant of SALK_090130 (Figure 5C to O), morphologically abnormal flowers were often observed, similar to those reported in PAF1-related *vip* and *elf* mutants (He et al. 2004; Oh et al. 2004; Xu et al. 2008; Jolivet et al. 2006; Zhang and van Nocker 2002; Zhang et al. 2003), with sepals with white margins, undeveloped petals, short stamen filaments, green anthers without any pollens, and small ovules. RT-PCR experiments indicate that the viable homozygous mutant plant expressed extremely low amount of *VIP6*/*ELF8* transcript (Figure 4B). These results suggest that *vip6*/*elf8* homozygous plants are largely embryonic lethal without expression of *VIP6*/*ELF8*, but in extremely rare case, they are viable with leaky expression of *VIP6*/*ELF8*, although they are sterile.

Discussion

Pleiotropic phenotypes observed in mutants of Arabidopsis PAF1 complex components

The pleiotropic phenotypes observed for *vip6*/*elf8* homozygous mutants in this study were similar to those previously reported for mutants of other *Arabidopsis* PAF1 complex components. The pleiotropic phenotypes, other than early flowering, observed in *elf7*, *elf8* and *vip4* mutations were specific to the Col background, and it was suggested that the additional phenotypes of these mutations in Col are the result of a single recessive locus in the Col genetic background (He et al. 2004). On the other hand, the *vip3* mutation conferred essentially identical pleiotropy when introgressed into the Lansberg erecta (L*er*) background (Zhang et al. 2003). At this point, we have no evidence for the identity of the effect of the genetic background on the pleiotropic phenotype that is common in mutations in PAF1-related genes.

Lethality of homozygous mutants in the VIP6/ELF8 gene

It has been reported that although individual components of the PAF1 complex are not essential in yeast, many combinations of mutations are lethal (Chang et al. 1999), indicating that the complex as a whole play an essential role. Loss of single genes encoding the yeast PAF1 complex components results in pleiotropic phenotypic changes (Betz et al. 2002; Chang et al. 1999; Mueller and Jaehning 2002). Among them, most severe phenotypes of the various known PAF1 complex mutants were those of single deletions of *PAF1* and *CTR9*, and they showed identical phenotypes including increased sensitivity to higher and lower temperature, many compounds; significantly slower growth rate than wild type at normal growth condition, and reduced viability (Betz et al. 2002). Combining *PAF1* deletion with deletion of *CTR9* does not result in any phenotypic enhancement (Koch et al. 1999). It is noteworthy that yeast Ctr9 had been identified, in association with Paf1, as a factor required for full expression of the G_1 cyclin *CLN2* (Koch et al. 1999), and for proper chromosomal segregation (Foreman and Davis 1996), and in both cases *ctr9* mutants were temperature-sensitive.

Our results suggest that homozygous *vip6*/*elf8* mutant plants cannot be obtained because of embryonic lethality and/or defects in fertilization process. Since we observed similar results with three independent alleles that have T-DNA insertion at different sites, and there are no genes whose defect may lead to lethality in the neighborhood of At2g06210, we consider that the mutant phenotype is caused by mutations in the At2g06210 gene. As we used the same alleles of the 'SALK lines' that have been used in the previous studies (He et al. 2004; Oh et al. 2004) and we grew plants under conditions that are generally used for *Arabidopsis*, at present we cannot understand the reason for the conflicting results. One possible reason for this inconsistency may be slight differences in growth conditions such as temperature and/or other unknown stresses. For *vip3* mutants, it has been reported that flowers were typically male sterile, and self-pollination was rare (Jolivet et al. 2006; Zhang et al. 2003), and that when plants were grown at a lower temperature (18°C), these floral defects were attenuated, and plants were typically fertile (Zhang et al. 2003). Indeed, for *vip6* mutants, similar temperature-dependence of fertility was noted (Steven van Nocker, personal communication).

It is noteworthy that the yeast PAF1 complex is not only required for efficient transcription elongation by RNA polymerase II (Marton and Desiderio 2008), but also have a role in 3′-formation of RNAs (Mueller et al. 2004; Penheiter et al. 2005; Sheldon et al. 2005).

Significant role of mRNA 3′-end formation not only in flowering-time control but also in other developmental modulation in plants has been described. FY is the mRNA 3′-end processing factor related to Pfs2p of yeast, and was found to interact with FCA which is a nuclear RNA binding protein controlling flowering time (Simpson et al. 2003). FCA/FY interaction is required to regulate *FCA* expression through pre-mRNA processing, which results in the down-regulation of the floral repressor *FLC*. Interestingly, null alleles of *FY* gene in autonomous flowering pathway are also embryonic lethal (Henderson et al. 2005) and pleiotropic functions for FY in development had been previously suggested by the genetic analysis of the autonomous promotion pathway mutants (Koornneef et al. 1998). Therefore, the pleiotropic phenotype and the embryonic lethality caused by deletion of a PAF1 complex component VIP6/ELF8 may be ascribed to defect in 3′-processing of RNAs.

Recent reports indicate the essential roles of the PAF1 complex in growth and development: deletion of expression of the *HPRT2* gene that encodes parafibromin, the human ortholog of CDC73, leads to embryonic lethality (Wang et al. 2008), and zebrafish embryos deficient in RTF1 or CTR9 show abnormal development (Akanuma et al. 2007). Further studies are needed to clarify the role of the PAF1 complex in *Arabidopsis* and other plants.

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