Loss-of-function of gynoecium-expressed phospholipase triggers haploid induction

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Abstract

Pollen-specific phospholipase A-mediated in vivo haploid induction system was functionally well conserved in numerous monocots. However, no functional orthologous gene has been identified in dicots plants. Here, we show that loss-of-function of gynoecium-expressed phospholipase AII (pPLAIIγ) triggers haploid plants in Arabidopsis, at an average rate of 1.46%. Reciprocal crosses demonstrate that haploid plants are triggered from the female side and not from the pollen, and the haploid plants carry the maternal genome.

Full Text

Mutation in pollen-specific[1] *ZmPHOSPHOLIPASE-A* gene named *ZmPLA1*[2] or *MATRILINEAL* (*MTL*)[3] or *NOT LIKE DAD* (*NLD*)[4] (hereafter *MTL/NLD/ZmPLA1*) was recently identified as the causative allele triggering *in planta* (*in vivo*) haploid induction (HI) in maize. However, the *MTL/NLD/ZmPLA1*-based HI system is so far only functional in staple monocot plants such as maize, rice, wheat, and foxtail millet[2–7]. Those *MTL/NLD/ZmPLA1* orthologous genes in monocots are pollen-specific and their mutation were reported to be able to trigger *in planta* haploid induction[5–7]. So far, no functional *MTL/NLD/ZmPLA1* orthologs have been identified in dicot plants. As an alternative, DOMAIN OF UNKNOWN FUNCTION 679 membrane protein (*DMP*) encoding genes were found to be conserved between maize and dicotyledonous plants, and mutations in orthologous *DMP* genes are able to trigger HI in *Arabidopsis thaliana, Brassica napus, Medicago truncatula, Nicotiana tabacum,* and *Solanum lycopersicum*[8–11].

Plant phospholipase A_2 (PLA_2) gene families are classified into two groups: secretory-type of PLA_2s ($sPLA_2$s) and patatin-related PLAs (pPLAs). The latter are further classified into three groups based on amino acid sequence similarity and gene structure: *pPLAI*, *pPLAIIs* (*pPLAIIα, -β, -γ, -δ,* -ε), and *pPLAIIIs* (*pPLAIIIα, -β, -γ, -δ*)[12–14]. A uniform nomenclature for plant pPLAs has been established[13], and will be used hereafter. *MTL/NLD/ZmPLA1* belongs to class II *pPLA* (*pPLAII*)[4] (Fig. 1a) and has clear orthologous genes in monocots[5–7]. However, based on phylogenetic tree and expression patterns (Extended Data Fig. 1)[15], it is not straightforward to identify functional orthologs in dicots because dicots orthologous genes are grouped together and weakly or not expressed in pollen (Fig. 1a and Extended Data Fig. 1). The closest *Arabidopsis pPLAIIα* homologue (*AtPLP2*, At2g26560) which shares 47.9% of its protein identity with MTL/NLD/ZmPLA1 is only expressed in vegetative tissues[16] but, the other closest *pPLAIIβ* is not only expressed in vegetative tissues but also in pollen. However, T-DNA insertion mutants of *pPLAIIα* and *pPLAIIβ* were not found to induce haploid plants (Extended Data Fig. 2). Among the other *pPLAIIs, pPLAIIγ, pPLAIIδ,* and *pPLAIIε*[15] that have not been functionally characterized, *pPLAIIε* and *pPLAIIδ* are expressed high in roots and not in sexual reproductive organs[15]. The only candidate gene left with possible role in sexual reproduction was *pPLAIIγ*[15] (also called *AtPLP3* or *AtpPLA IVC*, At4g37050), since it was reported to be expressed in gynoecium, the female reproductive structure of flowering plants.

In order to functionally characterized this gynoecium-expressed gene and to test the potential haploid inducibility of the loss-of-function mutant, knock-out mutants were searched. All the available T-DNA insertion lines (http://signal.salk.edu/cgi-bin/tdnaexpress) encoding *pPLAIIγ* were confirmed to be knock-downs (Extended Data Fig. 3), certainly because T-DNA insertions are not within exon (Fig. 1b). Thus, we generated RNAi transgenic and CRISPR-Cas9-mediated genome-edited lines. The RNAi transgenic lines were generated using 111 bp of coding sequence between the 3rd and 4th exon of *pPLAIIγ* that is less conserved among *pPLAIIs* (Fig. 1b and Extended Data Fig. 4a). Two independent RNAi lines were chosen for further analysis, which followed Mendelian inheritance of the RNAi cassette. The mRNA level was silenced up to 0.2 times on average, as compared to wild type (WT) plants (Extended Data Fig. 3b). To avoid method-specific-off-target effects, CRISPR-Cas9-mediated genome editing was also conducted using single-guide RNA (sgRNA). A 20-bp guide sequence (N_{20}) was designed, targeted on the 1st exon of *pPLAIIγ* (Fig. 1b and Extended Data Fig. 4a), and cloned into the pHAtC vector[17]. Two genetically edited lines, *sgpPLAIIγs*, were then selected by targeted deep sequencing, and T_2 lines were further confirmed by individual sanger sequencing (Extended Data Fig. 5). One edited line showed one nucleotide (A) addition and the others showed 4-bp (CGAT) deletions in the targeted region, followed by a premature stop at the 38th and 39th amino acid position, respectively (Fig. 1b and Extended Data Fig. 5). Haploids plants were first identified based on their smaller size as compared to diploid WT plants[18]. The fully grown putative haploid plant of RNAi and the edited lines exhibited a reduced size and height compared to diploid plant (Fig. 1c). Their ploidy status was further confirmed as haploid by flow cytometry and karyotyping (Fig. 1d and e). A close inspection of the *pPLAIIγ*-mediated haploid plants revealed partial sterility (Fig. 1c and Extended Data Fig. 6). The seed sets of selfed *pPLAIIγ* mutant lines were significantly reduced, by 39% on average in the gene-edited *sgpPLAIIγ* lines (Fig. 1f–h). An increase in aborted seeds was observed in the RNAi lines and more in the edited *pPLAIIγ* lines (Fig. 1f and i). Accordingly, the proportion of aborted seeds was significantly increased, up to 10% in the *sgpPLAIIγ* line #4 (Fig. 1i). The diploid homozygous *sgpPLAIIγ* plants also present a delay in stamen filament elongation (Extended Data Fig. 6). To access the haploid induction rate (HIR), haploid plants were screened among 251~301 offspring from self-pollinated plants from both RNAi lines and edited lines. The RNAi lines produced 1.12~1.66% of haploid seeds during self-pollination and the edited *sgpPLAIIγ* lines 1.45~1.59% (Fig. 1j). This result clearly shows that the loss-of-function mutants of *pPLAIIγ* is able to induce haploid plants *in vivo*. The *Arabidopsis* FAST-Red (*promOLEO1:OLEO1-RFP*) marker has been used as a simple and efficient method for high-throughput haploid identification, with >90% accuracy[8]. To investigate the HIR and the origin of haploid induction, crosses were carried out using *pPLAIIγ:RNAi* as either female or male parents, and FAST-Red marker was used to rapidly differentiate haploids (Extended Data Fig. 7). Diploid and haploid seeds are visually differentiated under fluorescent light; diploid WT seeds showed strong red fluorescent protein (RFP) signal in the embryo, whereas candidate seeds with haploid embryo showed weak RFP signal[8] (Extended Data Fig. 7). As a control cross, the *ms1* male sterile line was used as the female parent to limit contamination of self-pollination and other outcrossing. Two haploids were identified among 214 offspring plants screened when RNAi lines was used as female parent, whereas no haploid were identified among 515 offspring when RNAi lines was used as male parent (Fig. 1j). Haploidy was

confirmed by smaller sized plant phenotype and by flow cytometry analyses (Extended Data Fig. 7d and e). To verify the origin of haploid genome, PCR-based genotyping was performed and found that the induced haploid plants have a maternal origin (Fig. 1k).

The mRNA transcripts of *pPLAIIγ* was quantified in different flower developmental stages[19] and the analysis showed that *pPLAIIγ* expressed high in stage 12 and 17 (Fig. 2a). To further understand the spatial and temporal expression of *pPLAIIγ* in different flower developmental stages, two promoter::GUS constructs (full and Δ promoter of *pPLAIIγ,* Extended Data Fig. 4) were generated, containing whole intergenic regions between *pPLAIIδ* (At4g37060) and *pPLAIIγ* (At4g37050), with/without the 5'-UTR region of *pPLAIIγ* (Extended Data Fig. 4b). We found that the GUS expression showed the same expression patterns irrespective of using full or Δ promoter of *pPLAIIγ*. The GUS expressed in the funiculus during flower developmental stages 13 to 14 and 17 to 18 (Fig. 2b–d). The funiculus-specific expression was also reported previously[15] in which we found that the construct included C-term part of neighboring *pPLAIIδ* gene (Extended Data Fig. 4). However, we found that the *prompPLAIIγ*::GUS was not only expressed in the funiculus but also in other parts of floral organs (Fig. 2b–d) as well as in vegetative organs including the leaf and stamen filament (Extended Data Fig. 8). No GUS staining was observed in pollen grains (Extended Data Fig. 8h–j). To gain more insight regarding subcellular location of pPLAIIγ, the yellow fluorescence protein (YFP) was added to pPLAIIγ C-terminal part, and expressed under the control of the 35S promoter. A clear localization to plasma membranes (PM) in the root and gynoecium cells was observed (Fig. 2e–g). The PM localization co-localizes with the endocytic tracer FM4-64 (Fig. 2e). To double check the PM localization of pPLAIIγ-YFP, mannitol-induced plasmolysis was performed (Fig. 2f), which largely resulted in the preservation of the cell wall but also showed a good separation of the PM from the wall. Following the plasmolysis, the pPLAIIγ-YFP proteins were clearly visible in the PM physically separated from the cell wall. Interestingly, a substantial amount of pPLAIIγ-YFP signal remained attached to the wall, as well as stained thread-like structures between the PM and the cell wall: the Hechtian strands (Fig. 2f). Hechtian strands transduce cell wall integrity signals between the PM and the cell wall[20]. It is interesting to note that the polar domain of PIN-FORMED (PIN) proteins also connects with Hechtian strands[21]. Compared to the transcriptome of WTs, well-known auxin transporters including not only PINs but ABCB-types of transporters as well as auxin responses and signaling-related genes were down-regulated (Extended Data Table 1 and Supplementary Table 2). This indicates that *pPLAIIγ* is probably involved in the regulation of the auxin transport which is worthy of further studying.

Taken together, this study indicates that loss-of-function of gynoecium-expressed *pPLAIIγ* induces female organ-mediated haploid induction. Gynoecium-expressed *pPLAIIγ*-mediated haploid induction is comparable with the 2.1±1.1% of HIR in progeny of *ms1* × *dmp8dmp9* double mutants[8], where two *DMP8* and *DMP9* were used as male parent to induce maternal haploid. A single mutation of either *DMP8* or *DMP9* induced a low HIR frequency of 0.03 or 0.4% independently[8]. Further genetic studies on *DMP* genes and other groups of phospholipase family genes should shed more light on their additive or synergistic

effects, as well as on the functional redundancy of double or triple mutations in *pPLAIIγ:RNAi* and *sgpPLAIIγ* lines.

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Methods

Plant materials. The *Arabidopsis thaliana* Colombia ecotype (Col-0) was used as a control and background for the *pPLAIIγ* gene silenced and edited lines in this study. *Arabidopsis* seeds were sown in 1/2 MS medium [1% sucrose, 0.5 g/l 2-(N-morpholino) ethanesulfonic acid (MES), 0.8% phytoagar, pH5.7]. After vernalization treatment at 4°C dark conditions for 2 days, seeds were grown under long-day light (16 h light/8 h dark) at 23°C. *Arabidopsis* seedlings grown for 10 days *in vitro* were transferred to sterilized soil mixed with soil, vermiculite, and perlite in a 3:2:1 ratio. The *pplaIIγ* SALK T-DNA insertion lines (SALK_126029, SALK_074247, and SALK_024839) were purchased from the *Arabidopsis* Biological Resource Center (https://abrc.osu.edu/).

Transgenic construction and *in planta* transformation. To silencing of *pPLAIIγ* gene, *35S:pPLAIIγ-RNAi* was constructed. The 111 bp (sense) sequence and the complementary sequence (antisense) of the *pPLAIIγ* gene coding region were amplified and cloned into the XhoI/KpnI site and HindIII/XbaI site of the pHANNIBAL vector. Thereafter, the 35S:dsRNA cassette was recloned into the pART27 vector. *pPLAIIγ* gene editing was performed using CRISPR-Cas9 system. Single guide RNA (sgRNA) targeting exon1 of *pPLAIIγ* was selected using CRISPR RGEN Tools (http://www.rgenome.net/). The synthesized sgRNA oligomer of 20 bp excluding the PAM site was annealed with the complementary sequence and cloned into the AarI site of the pHAtC vector[17]. Edited types of *pPLAIIγ* gene were identified by targeted deep sequencing and sanger sequencing. To construct *35S:pPLAIIγ-YFP*, the *pPLAIIγ* coding sequence excluding the stop codon (1,284 bp) was amplified. Then, the SalI and SmaI sites were digested and cloned into the pCAMBIA1300 vector containing the *YFP* gene. For β-glucuronidase (GUS) histochemical analysis, the full length (1,949 bp; −1928 to +20 region based on the start codon) and delta (Δ) (1,797 bp; −1928 to −132 region based on the start codon) promoter of *pPLAIIγ* (Extended Data Fig. 4) were amplified and cloned in to the HindIII/BamHI site of the pCAMBIA1390 containing the *GUS* gene. To select haploid by fluorescence, seed specific expressed *Oleosin1* (*OLEO1*, At4g25140) gene was used as haploid selection marker[8,22]. *PromOLEO1:OLEO1* sequence (1,277 bp) was amplified and cloned into the

HindIII/AvrII site of the pCAMBIA1300 containing with the mRFP gene. Each construct was introduced into *Agrobacterium tumefaciens* C58C1 (pMP90), and transformants were obtained through *Arabidopsis* floral dipping transformation[23].

β-glucuronidase (GUS) histochemical analysis. The full length and Δ promoter of *pPLAIIγ* were used for GUS histochemical analysis. *PrompPLAIIγ*:GUS transgenic plants were incubated with GUS staining solution [1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexyl-ammonium salt (X-Gluc, Duchefa Biocheme, Haarlem, The Netherlands), 100 mM NaH_2PO_4, 10 mM EDTA, 0.1% (v/v) Triton-X, and 0.5 mM potassium ferri- and ferrocyanide] at 37°C in dark condition. After staining, 70% (v/v) ethanol, 100% (v/v) ethanol, 10% (v/v) glycerol/50% (v/v) ethanol, and 30% (v/v) glycerol/30% (v/v) ethanol were treated in this order to decolorize the plants except for GUS staining. GUS-stained plants were imaged using a digital single-lens reflex (DSLR) camera (D80, Nikon, Tokyo, Japan) and a microscope (M165FC and DM3000 LED, Leica, Wetzlar, Germany).

Observation of reporter gene expression. The fluorescence signals of pPLAIIγ-YFP protein and FM4-64 dye (SynaptoRed™ C2, 70021, BIOTIUM, Fremont, CA, USA) were observed using a confocal microscope (Model no. TCS SP5 AOBS/Tandem, Leica, Wetzlar, Germany). YFP and RFP (FM4-64) were detected using 514/>530 nm, 510/750 nm (excitation/emission) filter set, respectively. FM4-64 staining was carried out by treating with 2 μM FM4-64 for 5 mins and then washing with distilled water for 5 min. Plasmolysis in primary root cells was induced by immersing roots in 0.5 M mannitol for 30 min. Fluorescence images were taken and analyzed using the Leica LAS X program. The images were acquired at the Korea Basic Science Institute, Gwangju, Korea.

Total RNA extraction and real-time quantitative PCR (qPCR). Total RNA extraction was performed using *Arabidopsis* tissues from Col-0, vector control, *pPLAIIγ* silenced, and edited lines according to the user manual of the TaKaRa MiniBEST Plant RNA Extraction Kit (Takara, Shiga, Japan). The extracted total RNA was quantitatively and qualitatively analyzed using a UV spectrophotometer (Nano-MD, Scinco, Seoul, Korea). Then, total RNA was quantified at the same amount between samples up to 4 μg, and a total of 20 μl of cDNA was synthesized using RevertAid Reverse transcriptase. qRT-PCR was performed using TB Green™ Premix Ex Taq™ (Takara, Shiga, Japan) and Thermal Cycle Dice real-time PCR system (Takara, Shiga, Japan). Subsequently, the derived C_t value was standardized using the C_t value of *β-actin*, a house keeping gene, and using the $2^{-\Delta\Delta Ct}$ method, the expression level of the target gene was calculated as a comparison value with the control. The analyzed gene-specific primers are listed in Supplementary Table 3.

Ploidy analysis using flow cytometry. Sample preparation for flow cytometry was performed with simple modifications to the instructions of CyStain™ PI Absolute P (05-5022, Sysmex, Görlitz, Germany). *Arabidopsis* rosette leaves (0.5 cm x 0.5 cm size) are collected, treated with 100 µl of nuclear extract solution, and chopped using a razor blade. To remove the debris, the homogenate was filtered through a 30 µm CellTrics™ nylon filter (04-0042-2316, Sysmex, Görlitz, Germany). 400 µl of propidium iodide (PI) staining solution was added to the filtered homogenate and stained in the dark for 20 min. After PI staining, DNA content analysis was performed using a flow cytometry (Guava® Muse® Cell Analyzer, Luminex, TX, Austin). The G1 peak, which appeared by analyzing the DNA content of diploid Col-0, was set as the diploid (2n) peak, and the peak showing the DNA content of half the size of the diploid peak was determined as the haploid (n) peak.

Karyotyping. To visualize *Arabidopsis* chromosomes, karyotype analysis was carried out as described in a previous report[24,25]. *Arabidopsis* rosette leaves and flower bud were collected and treated with Carnoy's fixative (ethanol-acetic acid, 3:1) and fixed at room temperature for 12 h. The fixed samples were washed twice with distilled water and twice with citric acid-sodium citrate buffer (40 mM citric acid-monohydrate, 60 mM trisodium citrate dihydrate, pH 4.8). Leaves were incubated in an enzyme mixture consisting of 2% (w/v) cellulase R-10 (C80001, Duchefa Biocheme, Haarlem, The Netherlands), 3% (w/v) pectinase (P4716, Sigma-Aldrich, Saint Louis, MO ,USA) in citric acid-sodium citrate buffer at 37°C for 2 h. The enzyme solution was removed from the digested sample and washed with citric acid-sodium citrate buffer. Put the samples on a slide glass, 10 µl of 60% (v/v) acetic acid was added to allow the sample to be macerated well, and then tear the sample, spread it well on the slide glass, and air dry. Air-dried slides were stained by adding a 10 µl of 1 µg/ml of 4',6-diamidino-2-phenylindole (DAPI, D9542, Sigma-Aldrich, Saint Louis, MO ,USA) in McIlvain buffer (18 mM citric acid, 16.4 mM Na_2HPO_4, pH 7.0) and mounted with coverslip. The mounted slide was squashed between filter paper sheets to remove excess stain and mountant. DAPI-stained cells were photographed using a fluorescence microscope (M165FC, Leica, Wetzlar, Germany).

RNA-seq profiling and analysis. Total RNA was extracted from stage 17 siliques of Col-0 and *pPLAIIγ*:RNAi#14. After cDNA library was constructed using TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA), libraries sequenced using Illumina Hi-Seq, which created 151 bp paired-end sequencing reads. Raw reads were subjected to quality checks and trimming by Phred quality score ($Q \geq 20$) and read length (≥25 bp) with SolexaQA[26]. Clean reads were mapped to the *Arabidopsis* reference genome using HISAT2 software[27]. Only uniquely mapped reads were used to obtain read counts for each gene. The number of mapped clean reads for each transcript was counted using HTSeq (v.0.11.0)[28] and then normalized with DESeq package[29].

Data availability

The RNA-seq data that support the findings of this study have been deposited in the NCBI SRA BioProject database under accession number ( ). All other data of this study are available within the Article and the Supplementary Information.

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Declarations

Supplementary information accompanies the paper on www.nature.com/nplants/

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Competing interests

The authors declare no conflict of interest.

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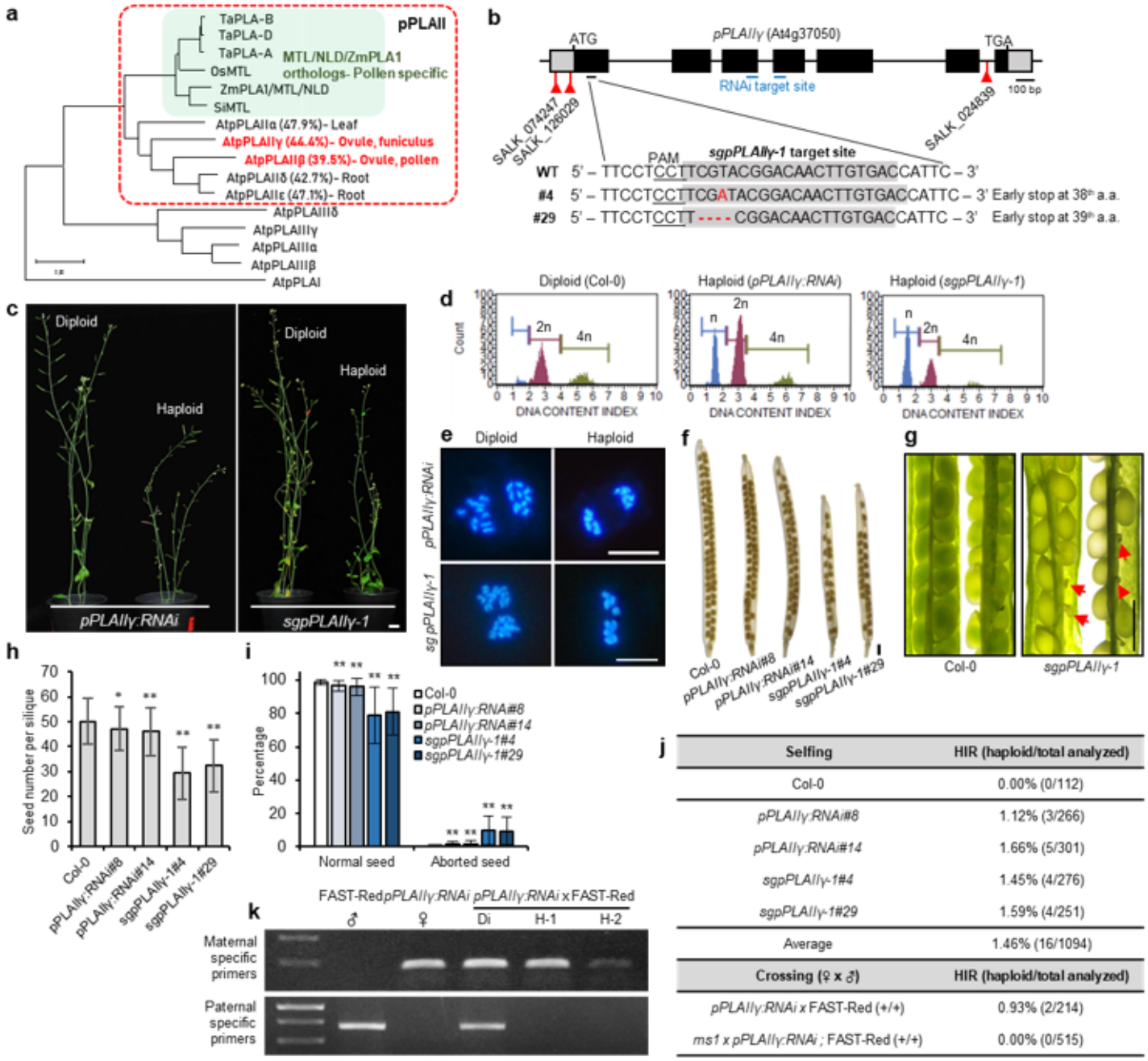
Figures



Selfing	HIR (haploid/total analyzed)
Col-0	0.00% (0/112)
pPLAIIγ:RNAi#8	1.12% (3/266)
pPLAIIγ:RNAi#14	1.66% (5/301)
sgpPLAIIγ-1#4	1.45% (4/276)
sgpPLAIIγ-1#29	1.59% (4/251)
Average	1.46% (16/1094)
Crossing (♀ x ♂)	**HIR (haploid/total analyzed)**
pPLAIIγ:RNAi x FAST-Red (+/+)	0.93% (2/214)
ms1 x *pPLAIIγ:RNAi* ; FAST-Red (+/+)	0.00% (0/515)

Figure 1

Mutation in *pPLAIIγ* triggers haploid plants. a, Phylogenetic tree of *Arabidopsis* pPLA family and MTL/NLD/ZmPLA1 orthologs. Phylogenetic tree was constructed by neighbor-joining method using MEGAX program. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Si, *Setaria italica*; Ta, *Triticum aestivum*; Zm, *Zea mays*. The amino acid sequences used to construct the phylogenetic tree are listed in Supplementary Table 1. Percentages (%) indicate protein identity with MTL/NLD/ZmPLA1. **b,** Types of *pPLAIIγ* alleles edited using CRISPR-Cas9, expected protein changes in the *sgpPLAIIγ-1* lines, *pPLAIIγ:RNAi*target site, and T-DNA insertion site (red triangles) of *pplaIIγ* mutants. **c,** Representative images of 5-week-old diploids and haploids of *pPLAIIγ:RNAi* and *sgpPLAIIγ-1*lines. **d,** Ploidy verification of haploid and diploid using flow cytometry. The x axes represent the DNA content index for the nucleus, whereas the y axes

represent the number of nuclei. **e,** Chromosome spread in the mitotic telophase of diploid and haploid. **f,** Representative images of cleared stage 18 siliques from selfed Col-0, *pPLAIIγ:RNAi* and *sgpPLAIIγ-1* lines. **g,**Representative images of dissected stage 17 siliques from selfed Col-0 and *sgpPLAIIγ-1*. Red arrows indicate aborted seeds. Quantification of **h,**seed number and **i,** seed phenotypes in selfed Col-0, *pPLAIIγ:RNAi*, and *sgpPLAIIγ-1* siliques. Data represent means ± SD at $*P < 0.05$, $**P < 0.01$ (Student's t-test). N=122 (Col-0), 100 (*pPLAIIγ:RNAi*#8, #14), 82 (*sgpPLAIIγ-1*#4, #29). **j,** Haploid induction rate (HIR) determined by self-pollination and crossing. **k,** PCR genotyping of diploid (Di) and haploid (H) plants to determine the origin of haploid genome. Scale bar = 1 cm (c), 1 mm (f and g), and 10 μm (e).

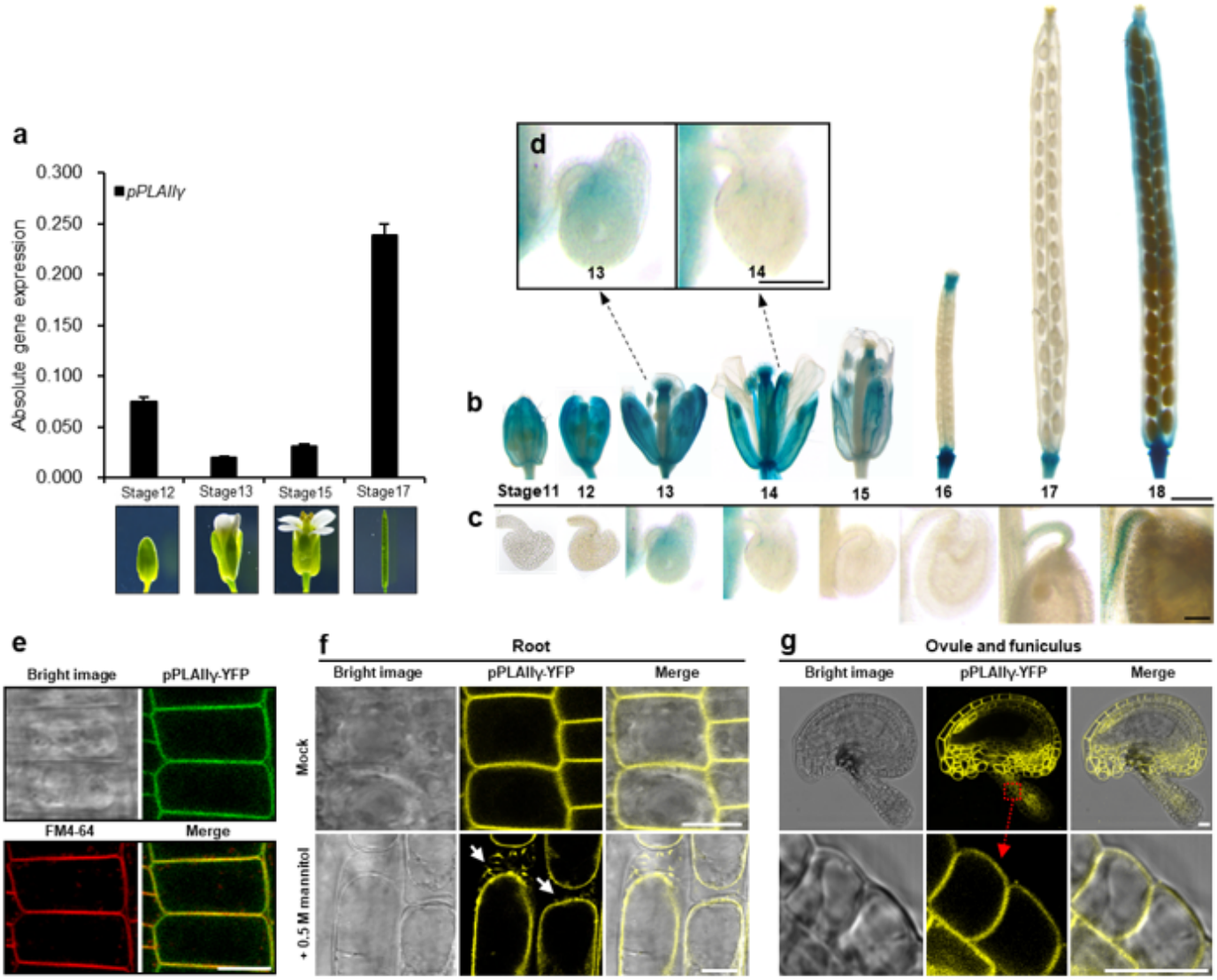


Figure 2

Expression pattern of *pPLAIIγ* and subcellular localization of pPLAIIγ-YFP. a, Transcript level of *pPLAIIγ* in flower developmental stage 12, 13, 15, and 17. Each data represents the average ± SE from three independent replicates. **b–d**, Histochemical analysis of full*prompPLAIIγ*:GUS plants. Expression patterns of the full*prompPLAIIγ*:GUS of **b**, flower buds, flowers, siliques and **c**, ovules at flower developmental

stages 11 to 18. **d**, Magnified image of ovule from flower developmental stage 13 and 14. **e–g,** Subcellular localization of pPLAIIγ-YFP expressed under 35S promoter. **e** and **f**, in 4-day-old primary root and **g**, in ovule. White arrows indicate Hechtian strands. Scale bars =1 mm (**b**), 100 μm (**c** and **d)**, and 10 μm (**e–g**).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SItable1.Aminoacidsequences.docx
- SItable2.RNAseqpPLAIIgRNAistage17silique.xlsx
- SITable3Primers.docx
- ExtendedData.docx