Identification of Arabidopsis phospholipase Ds involved in circadian clock alterations using CRISPR/Cas9-based multiplex editing

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Abstract:

Reciprocal regulation between circadian clock and lipid metabolism is emerging, but its mechanistic details remain elusive in plants. We previously reported that a lipid metabolite phosphatidic acid (PA) bound to the core clock transcription factors LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and chemical suppression of phospholipase D (PLD)-catalyzed PA formation perturbed the clock in Arabidopsis. Here, we investigated to identify, among 12 isoforms, specific PLD(s) important to the regulation of clock function. We generated a library of Arabidopsis plants bearing *PLD*s randomly mutated by multiplex CRISPR/Cas9 system. PCR and gel-based analyses showed that all *PLD*s, except for *β2*, were effectively edited and the mutations were heritable. Screening of T2 plants identified some with an altered rhythmic expression of *CCA1* and this trait was observed in many of their progenies. Genotyping by DNA sequencing revealed that at least two of six *PLD*s (*α1*, *α3*, *γ1*, *δ*, *ε* and *ζ2*) were mutated in the clock-altered T3 plants. This study identifies different combinations of two or more PLDs involved in altering clock outputs, and also suggests a functional redundancy of the six PLDs for the regulation of plant circadian clock.

Introduction:

The circadian rhythms are 24-hour periodic changes in cellular and physiological processes that are driven by an internal timekeeping mechanism known as the circadian clock, enabling organisms to anticipate and adapt to the cyclic environment. A growing body of recent evidence has suggested that the biological clock is interconnected with and reciprocally regulated by metabolism. While the clock regulates temporal metabolic activities by modulating the daily abundance of cellular factors involved in metabolism, some metabolites serve to provide input to the clock (Delezie and Challet, 2011; Pan et al., 2020; Grosjean et al., 2023). Despite the emerging pathways through which metabolic signals influence the plant circadian system (Buckley et al., 2023), however, mechanisms of how lipid metabolism affects the clock function are poorly understood in plants, mainly due to the scarce knowledge about molecular interactions between lipid metabolites and clock components. Recently, we reported that an important lipid mediator phosphatidic acid (PA) bound to and suppressed the ability of the core clock transcription factor LATE ELONGATED HYPOCOTYL (LHY) and its closely related CIRCADIAN CLOCK ASSOCIATED1 (CCA1) to bind the promoter of their target genes (Kim et al., 2019). We also found that cellular PA abundance directly correlated with the period of circadian rhythms, such as vertical movement of plant leaves and the oscillatory expression of a LHY/CCA1 target *TIMING OF CAB EXPRESSION1* (*TOC1*) (Kim et al., 2019). These findings suggest that PA is an important regulator for the plant circadian clock. Furthermore, given that PA is the pivotal metabolic intermediate formed during the conversion among many other lipids, the PA-LHY/CCA1 interaction may function as a cellular conduit to integrate lipid metabolism with the clock in plants.

Cellular level of PA is highly dynamic and tightly regulated by diverse enzymes that catalyze its formation and degradation (Kim and Wang, 2020). Moreover, many of them have multiple isoforms with different expression patterns, subcellular distribution, and catalytic properties, leading to the high complexity of PA metabolic routes. Phospholipase D (PLD) is one of the most extensively studied and best characterized PA-metabolizing enzymes with the largest number of family members in plants (Ali et al., 2022; Deepika and Singh, 2022). PLD hydrolyzes common membrane phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, to produce PA by releasing their alcohol moiety. Arabidopsis PLD

family comprises 12 members, designated as α1/2/3, β1/2, γ1/2/3, δ, ε, and ζ1/2, classified according to amino acid sequence and enzymatic properties (Qin and Wang, 2002). They can be activated differently in response to diverse stimuli, and these differences account for the highly dynamic nature of PA contents and compositions depending on environmental conditions (Hong et al., 2016; Kolesnikov et al., 2022). This is evidenced by, in addition to early studies, recent reports showing a rapid accumulation of PA by PLDα1 in response to magnesium stress and rhizobium infection, β2 during hypoxia, δ under heat and osmotic stress, and ε and ζ2 in nitrogen and phosphate deficit, respectively, with a few specific PA molecular species contributing to the accumulation of total PA in some cases (Su et al., 2018; Premkumar et al., 2019; Song et al., 2020; Kocourková et al., 2021; Liu et al., 2021; Zhang et al., 2021; Kim et al., 2022; Yao et al., 2022 a and b). Not only for plants to cope with the unfavorable conditions, PLDs are also required for normal physiological processes mediated by their direct interaction with effector proteins, regulation of cytoskeletal dynamics and vesicle trafficking, and membrane remodeling and degradation (Hong et al., 2016; Deepika and Singh, 2022).

All PLDs can use primary alcohols as a substrate to produce phosphatidylalcohol instead of PA, and 1-butanol has often been used to suppress PLD-catalyzed PA formation in plants (Munnik et al., 1995). We previously found ~40% reduction of basal PA levels in Arabidopsis treated with 1-butanol, and that this shortened the period of circadian rhythms (Kim et al., 2019). Since PLDs play different roles in stress responses, it is conceivable that specific PLD isoform(s) produces the cellular pool of PA that interacts with LHY/CCA and the reduction of the pool size leads to dissonance between the internal and external rhythms and eventually compromises plant response to stress. Thus, identification of the PLD(s) involved in this process is critical to determine how lipid metabolism communicate with the clock components to affect the circadian rhythms and stress responses. However, our preliminary data indicated that single T-DNA mutants of individual *PLD*s had no significant effect on the clock, indicative of functional redundancy of PLDs for the clock regulation. Moreover, LHY and CCA1 only bound to specific PA species, and neither their levels in Arabidopsis nor LHY-binding to its targets significantly changed in a double knockout mutant of the most abundant PLDs, *α1* and *δ* (Kim et al., 2019). This implies that PLD(s) other than or in addition to the two major ones is required for the formation of LHY/CCA1-binding PA species. Thus, here we generated and screened

Arabidopsis plants with various combinations of *PLD*s randomly mutated by multiplex CRISPR/Cas9 system. In this approach, we successfully produced a library of plants having mutations in a fairly wide spread of different *PLD*s and identified several PLDs important to proper functioning of the circadian clock.

Results:

Cloning of the CRISPR nuclease vector construct

To target the 12 *PLD*s by multiplex CRISPR system, we used a toolkit that allows assembly of nuclease constructs expressing up to 32 single guide RNAs (sgRNAs) in a single nuclease vector (Ordon et al., 2017; Grutzner et al., 2021; Stuttmann et al., 2021). This toolkit contains Dicot Genome Editing (pDGE) vector system that is composed of 'shuttle' vectors for the preparation of sgRNA transcriptional units, intermediate 'multi-multi' vectors for assembly of more than 8 sgRNAs, and pre-assembled 'recipient' vectors with *Cas9* (Figure 1A). We targeted two different sites in each *PLD* for enhanced editing efficiency (a total of 24 sgRNAs). Eight hybridized oligonucleotides for sgRNAs were first cloned into 8 different shuttle vectors (pDGE332-342) for each of three sets of the 8 oligonucleotides (1-8, 9-16 and 17-24 in Figure 1A). Since all the shuttle vectors contain two *BamHI* sites and one of them is within the cloning cassette to be replaced by the oligonucleotides, successful cloning can be verified by cutting the DNA constructs with *BamHI*: a large DNA fragment resulted from the cloned constructs (~3 kb) and an additional smaller fragment from empty vectors (~0.8-0.9 kb) (Figure 1B). The three sets of the shuttle vector constructs were then subcloned into three different multi-multi vectors (pDGE503-505 in Figure 1A). *BpiI* digestion of the multi-multi vector constructs from 5 randomly selected *E. coli* clones resulted in the correct size of the insert DNA (~1.7 kb; the 8 sgRNA transcriptional units with some flanking nucleotides) from at least one clone for each of the three constructs (Figure 1C). The three confirmed multi-multi vector constructs were finally cloned together into the recipient vector (pDGE668 in Figure 1A). Three of four randomly selected *E. coli* clones had the correct size of the insert DNA (5,007 bps; the 24 sgRNA transcriptional units with some flanking nucleotides), as revealed by *HindIII* digestion (Figure 1D). The final confirmed recipient vector construct was transformed into Arabidopsis for editing its *PLD*s.

Detection of deletion mutations in T1 transformants

The recipient vector contains a selection marker against kanamycin, and successful plant transformation was verified by PCR amplification of *Cas9* in the genomic DNA of 10 kanamycin-resistant T1 plants (Figure 2A). To see whether and which *PLD*s were edited in the transformants, we performed PCR to amplify a genomic DNA region spanning the two target sites for each *PLD* in six independent *Cas9*-positive plants, and checked the PCR products on a gel to detect large insertion, deletion or chimeric mutations that might have been introduced by the natural non-homologous end-joining. For all *PLD*s except for *β2* and *ε*, some of the plants had one or more PCR products smaller than and in addition to the one observed in wild-type (WT), indicative of heterozygous deletion mutations introduced (Figure 2B; the exact size of PCR products from WT in Figure 2C). PCR results from six additional transformants (a total of 12 individual plants) are shown as a diagram in Figure 2D. At least three plants had mutation in each *PLD* (>25% frequency), with a notably high frequency of *γ3* (100%), and no mutation was found in *β2* and *ε* (Figure 2D). In each plant, three up to nine *PLD*s were mutated, and no plant appeared to have all *PLD*s mutated (Figure 2D). It should be noted that these data are based on visual inspection of the DNA bands, and thus the actual mutation frequency is expected to be higher than revealed by the PCR/gel-based analyses. Collectively, the results here indicate that at least 10 *PLD*s were effectively edited by our multiplex CRISPR/Cas9 system.

Detection of mutations in T2 plants and sequencing analysis of PLDβ2 and *ε*

The vector system provides a gene encoding red fluorescence protein (RFP) expressed under the seed-specific promoter *OLE1p* as a counter-selection marker for non-transgenic seeds. We harvested and combined seeds from the 12 T1 transformants and selected the seeds without red fluorescence to obtain non-transgenic (*Cas9*-free) T2 plants. This was to avoid confounding effects due to the occurrence of novel somatic mutations caused by the transgene. Approximately a quarter of the screened T2 seeds exhibited no red fluorescence following the classic Mendelian model (Figure 3A). The absence of the transgene in the selected seeds was further confirmed by failure of PCR amplification of *Cas9* in the genomic DNA of their seedlings (Figure 3B). We then performed the same PCR analysis conducted to evaluate T1 plants for each *PLD* in 10 *Cas9*-negative T2 plants. As expected, the overall mutation frequency is much lower and some plants had a homozygous, in addition to heterozygous mutation, as demonstrated by a single

smaller PCR fragment without the one from WT (Figure 3C). Notably, some *PLD*s produced two different small fragments and no WT fragment, indicative of the 'biallelic' heterozygous mutation with the two alleles mutated differently (Figure 3C). Among the 10 plants, nine had a homozygous or heterozygous deletion mutation in one to three *PLD*s, and each *PLD* was mutated in one to three plants, again except for *β2* and *ε* that were unmutated in all plants tested (Figure 3D).

Since *PLDβ2* and *ε* appeared to have no mutation in both T1 and T2 plants, we carried out a more comprehensive analysis of 6 of the 10 T2 plants by amplicon sequencing of the two *PLD*s to identify their mutations unable to be detected by the gel-based method. We still found no mutation in *PLDβ2* at both target sites in all the 6 plants, indicating its very low efficiency for editing. However, two plants had a heterozygous insertion or deletion mutation in *PLDε* at the first target site, as identified in the sequencing chromatograms by a series of single peaks followed by two overlapping peaks continuing to the end (Figure 3E). Taken together, the data here indicate that the majority of our non-transgenic T2 plants have a mutation, either homozygous or heterozygous, in at least one of the 12 *PLD*s, except for *β2*.

Screening of T2 plants to select clock-altered ones

Based on our previous finding that inhibition of PLD-catalyzed PA formation shortened the period of *TOC1* expression (Kim et al., 2019), we screened the *Cas9*-negative T2 plants pre-transformed with luciferase reporter gene expressed by *TOC1* or *CCA1* promoter (*TOC1::LUC* or *CCA1::LUC*) to select the ones with a shorter period of the clock gene expression. PCR analyses confirmed the absence of *Cas9* and the presence of *LUC* in six RFP-negative T2 plants randomly chosen for each of the two *LUC* lines (Figure 4A). The luciferase activity was monitored over time to measure the rhythmic patterns of *CCA1* and *TOC1* expression in T2 seedlings. Among a number of plants displaying clear rhythmicity of the reporter activity, four with *CCA1::LUC* and two with *TOC1::LUC* showed an apparently shorter period when compared to their respective WTs (Figure 4B). We then measured the period of *CCA1* expression in ~20 progenies (T3) for each of the four T2 plants showing altered *CCA1* expression. Interestingly, most of the T3 plants appeared to have a shorter period of *CCA1* expression than WT, largely regardless of their parental lines. Figure 4C shows two

representative plants for each family displaying the shortest period. Computational period estimation (Zieliński et al., 2021) revealed that *CCA1* expression in WT was nearly 24-hour periodic (24.12 h) and the T3 plants had an average of 23.29-23.62 hours and differed from WT by up to 2.46 hours (Figure 4D). Our data suggest that the phenotypic change in the clock gene expression is heritable and likely caused by the CRISPR-induced *PLD* mutations.

Genotyping of the T3 plants with altered CCA1 expression to identify specific PLDs involved

To determine what and how PLDs were mutated in the clock-altered plants, we genotyped the 8 T3 plants shown in Figure 4C by amplicon sequencing. All *PLD*s in WT were intact as expected. Some of the selected plants had homozygous mutations in *PLDα1* and *γ1* at either or both of the two target sites, all of which were identified as an insertion or deletion of one nucleotide (Figure 5A). All homozygous mutations in the first target site of *PLDα1* were a deletion of 'A' at the same position (sixth nucleotide in the target site; Figure 5A). We also found heterozygous mutations in six *PLD*s (*α1*, *α3*, *γ1*, *δ*, *ε* and *ζ2*), as identified by the overlapping peaks extending in the chromatograms as in Figure 3E, and the other six (*α2*, *β1*, *β2*, *γ2*, *γ3* and *ζ1*) were unmutated in all plants (Figure 5B). Combinatorial analyses of the overlapping peaks revealed that all heterozygous mutations, like homozygotes, were a frame-shift mutation and not biallelic; *PLDα1* in plant 25-14 and *γ1* in 16-12 turned out to be mutated in both target sites in the same chromosome (Figure 5B). *PLDα1* in 25-12 and 25-14 had both deletion and insertion of one nucleotide, but the deletion mutation resulted in a premature stop codon at 37th amino acid (Figure 5C for 25-14). All the 8 plants had a homozygous or heterozygous mutation in at least two *PLD*s, with the equally highest frequency of *PLDα1*, *α3* and *γ1* (four plants) followed by *ε* and *ζ2* (two plants), and then *δ* mutated in only one plant (Figure 5B). The identification of the mutated *PLD*s here narrows down the number of candidates likely involved in the clock function, and indicates that the six PLDs may play a redundant role in the proper functioning of the circadian clock. Further interpretation and discussion on the genotyping data will follow in the next section.

Discussion:

While 1-butanol is often used to discern the contribution of PLD to PA production from that of other PA-producing enzymes, individual PLDs have been studied by classic reverse genetics

using available T-DNA null-alleles or higher-order mutants created by crossing them. The potential compensation effects of PLDs necessitate a more inclusive, unbiased approach to identify the clock-affecting PLDs. Although CRISPR-mediated genome editing provides a technical breakthrough for rapid and efficient gene alteration, its multiplex versions have been used only for simultaneous mutation of all desired genes in plants, mainly to decipher the function of a multigene family or metabolic pathway (Razzaq et al., 2019; Abdelrahman et al., 2021). A few mutant 'libraries' have been created using the CRISPR system, in tomato and rice for example, but they were generated by polyclonal transformation of plants with a genomic-scale mixture of sgRNA constructs (Jacobs et al., 2017; Lu et al., 2017; Meng et al., 2017; Van Huffel et al., 2022). The polyclonal method could be used but has not been attempted to produce a plant library containing random combinations of mutations in a subset of or all members of a gene family. Construction of such library could also be enabled by using a sgRNA with reduced specificity to target homologous genes, by which randomization may be challenging (Peng et al., 2015; Hyams et al., 2018). Recently, Stuttmann et al. developed a multiplex CRISPR/Cas9 toolkit for polycistronic assembly of sgRNA/Cas9 constructs for up to 32 sgRNAs (Stuttmann et al., 2021). Based on their finding that its editing efficiency was low in Arabidopsis, compared to tobacco plants, and limited with increasing numbers of sgRNAs, here we adapted the same vector system for one-shot generation of an Arabidopsis library mutated in various combinations of *PLD*s.

Our PCR and DNA sequencing analyses revealed that a surprisingly large number of *PLD*s (11 *PLD*s) were effectively edited by targeting two distinct sites, all of which were heritable as demonstrated in the non-transgenic T2 plants. The exceptionally low editing efficiency of *PLDβ2* could be due to a low on-target efficiency, compromised integrity (e.g. secondary structure), and/or low expression level of its sgRNAs (Javaid and Choi, 2021). Loss-of-function mutations of *PLDβ2* are unlikely to cause lethality because a knockdown mutant with 77% reduction of its transcript showed no visible phenotypic difference from WT (Premkumar et al., 2019). More importantly, at least one across a wide spread of 11 *PLD*s was mutated in all T2 plants tested, suggesting that all 11 *PLD*s were subject to germ-line mutation in a desirably, and fortunately, random manner. Our T2 plants were only partially genotyped because the gel-based analysis was limited to detection of an insertion or deletion large enough to be resolved by

electrophoretic separation of the PCR products. Despite the limitation, 1-3 out of 10 T2 plants (up to 30%) were found to bear mutations for each *PLD*. Although this value seems low and would be increased when analyzed by sequencing, a low frequency should be rather more desirable for screening with a high discrimination power of the library. More informative genotypic data could have been available through other complementary techniques, without sequencing, such as T7 endonuclease I (T7E1), Surveyor nuclease or PAGE-based genotyping assays (Yun et al., 2022). Nonetheless, we proceeded with screening our T2 plants to select clock-altered ones based on the effectiveness, heritability, and randomness of editing in all *PLD*s, except for only one.

Several plant circadian markers have been used for measurement of clock function, such as the rhythmic patterns in growth or organ movement (Tindall et al., 2015; Dakhiya et al., 2017). However, they require consistent positioning of individual plants and precise tracking of their organ positions on the time-course images, and so are not suited for screening large number of plants with a high timeseries resolution. One of the most widely used and reliable high-throughput methods is to monitor the activity of clock-controlled luciferase reporter, especially for plant species capable of stable transformation (Millar et al., 1992). Using this technique, here we measured the rhythmic patterns of the clock genes *CCA1* and *TOC1* expression in the T2 plants. It is noteworthy that the circadian clock is a self-sustaining molecular oscillator composed of transcription factors that regulate their genes reciprocally (McClung, 2019). In the core of the oscillator in Arabidopsis, the expression of *CCA1*/*LHY* and *TOC1* diurnally oscillates in an anti-phasic manner by the mutual repression mechanism. (Alabadí et al., 2001; Gendron et al., 2012). Thus, clock dysfunction is often associated with an altered rhythmic expression of the clock genes mis-synchronized with the 24-hour periodic Earth's rotation. Some of our T2 plants displayed a shorter period of *CCA1*/*TOC1* expression, suggesting that they have the clock misaligned with the environmental rhythm. While this is interesting, no statistical analysis with biological replicates is possible to determine significance of the period estimation due to different genotypes of the T2 plants. But, the similar phenotype in a large proportion of their progenies indicates heritability of the trait, thus validity of our T2 plant selection, and also a high dominancy of the *PLD*-induced trait as further evidenced by our genotyping.

The genotyping data of the 8 selected T3 plants are highly consistent with our previous observations. The mutations in multiple *PLD*s in all plants examined support no clock phenotype in single T-DNA mutants of individual *PLD*s. Also, the *PLDζ2* mutation in addition to *α1* and *δ* in one of the plants (20-7 in Figure 5B) explains why LHY/CCA1-binding to *TOC1* promoter was not altered in *pldα1pldδ* (Kim et al., 2019). Together with these previous findings, our data here also indicate that for the PA regulation of the clock 'specific' PLDs (possibly the six mutated ones) are required, rather than just as many of 'any' PLDs as possible even though all PLDs produce PA, because no altered clock phenotype was observed when the two major PLDs (α1 and δ) were deficient. The importance of specific PLDs, not just the number of PLD isoforms, is also supported by our phenotypic data that the T3 plants, which should have fewer *PLD*s mutated than their parental lines, still showed the altered clock phenotype.

The high frequency of mutations in *PLDα1*, *α3* and *γ1* in the selected T3 plants is intriguing. Among the 12 PLDs in Arabidopsis, α1 is the most abundant, highly ubiquitous, and functionally versatile isoform involved in responses to a variety of stimuli (Hong et al., 2016). *PLDα3* mutation is of interest because mass spectrometric lipid profiling in *pldα3* showed a dramatic decrease (~90%) in the level of one of LHY/CCA1-binding PA species, 34:1-PA (Hong et al., 2008). Also, PLDγ was found to be associated with the nuclear membrane, suggesting its potential role for PA formation in the nucleus where the clock transcription factors work (Fan et al., 1999). Thus, the three PLDs might be the major contributor to the clock function. Despite the identification of the specific *PLD*s mutated, potential off-target effects should not be ruled out. Whole genome sequencing of Arabidopsis and rice has revealed that CRISPR/Cas9 system is highly specific in plants, unlike in human cells (Feng et al., 2014; Zhang et al., 2014; Wolt et al., 2016). In addition, deep sequencing of a total of 178 off-target sites showed that multiplex targeting in Arabidopsis resulted in no detectable off-target mutations (Peterson et al., 2016). That said, it would be necessary to corroborate the on-target effect on the clock phenotype through genetic complementation, analysis of further generations, and/or next-generation sequencing. Also, it is worth noting that the altered *CCA1* expression observed in T3 plants with heterozygous mutations could be caused by the loss of potential oligomerization of PLD proteins and/or their interaction with other proteins. The biochemical and genetic basis for the clock output altered by the heterozygous, frame-shift mutations is under investigation.

Reciprocally, the circadian clock modulates lipid metabolism by regulating the expression of lipid metabolic genes, as evidenced by circadian profiles of the genes and oscillatory accumulation of some lipid metabolites, including PA, in plants (Maatta et al., 2012; Hsiao et al., 2014; Nakamura et al., 2014 a and b; Kim et al., 2019). Recently, we reported that LHY and CCA1 enhanced the expression of *KASIII* that encodes β-ketoacyl-ACP synthase III by directly binding to its promoter, and this binding was inhibited by PA (Kim et al., 2023). KASIII catalyzes the first condensation step in the biosynthetic pathway of fatty acids that are incorporated into myriad lipid species. The PA inhibition of LHY/CCA1-binding to *KASIII* promoter suggests the role of PA as a retrograde modulator for the clock regulation of lipid biosynthesis. Together with the PA effects on circadian rhythms, this indicates that the PA-LHY/CCA1 interaction may serve as a cellular conduit to transmit signals between the clock and lipid metabolism in plants. Given the highly dynamic and complex nature of PA metabolism, discovery of what controls the cellular levels of PA associated with the clock will help to unveil how the two important processes communicate to affect the plant performance.

Materials and methods:

Cloning of sgRNA/Cas9 vector constructs

We used zCas9i cloning kit: MoClo-compatible CRISPR/Cas9 cloning kit with intronized Cas9 (AddGene #1000000171). Sequence of all oligonucleotides and PCR primers used in this study are provided in Table S1. Two target sequences for each *PLD* (23 bps including a PAM sequence) were selected by CHOPCHOP (https://chopchop.cbu.uib.no). For each of them two complementary oligonucleotides for sgRNA were synthesized to have 5' (5'-ATTG-3') and 3' (5'-AAAC-3') overhangs to be ligated with *BpiI* (*BbsI*)-cut shuttle vectors. They were hybridized by incubating at 95°C for 5 min and slowly cooling down to room temperature. Each step of the sequential cloning was performed by Golden Gate cloning with type II restriction enzymes and T4 DNA ligase. It was in a single tube reaction by thermal cycling (20-30 cycles of 37 °C for 2 min and 16 °C for 5 min) followed by incubation at 50 °C for 10 min and then 80 °C for 10 min. For shuttle vector cloning, 0.1 pmol of the hybridized oligonucleotides were cut by *BpiI* (or *BbsI*) and ligated with 0.2 μg of shuttle vectors (pDGE332, 333, 335, 336, 495, 496, 341, and 342) in a final volume of 10 μl. 0.5 μl of the reaction product was transformed into *E. coli*

DH5α, and all transformed cells were grown together in 3 ml LB with carbenicillin at 37 °C overnight for polyclonal plasmid preparation. For multi-multi vector cloning, 1 μl of the 8 shuttle vector constructs were cut by *BsaI* and ligated with 0.2 μg of multi-multi vectors (pDGE503, 504, and 505) in a final volume of 20 μl. 2 μl of the reaction product was transformed into *E. coli* DH5α, and kanamycin-resistant clones were obtained. Plasmids were extracted from the *E. coli* clones selected to have all 8 sgRNAs. For recipient vector cloning, 2 μl of the three multi-multi vector constructs were cut by *BpiI* and *BsaI* and ligated with 0.3 μg of recipient vector (pDGE668) and 30 ng of end-linker vector (pDGE509) in a final volume of 20 μl. *E. coli* transformation was done to select spectinomycin-resistant clones. Plasmids were extracted from the *E. coli* clones with all 24 sgRNAs and used to transform *Arabidopsis thaliana* (Col-0) via Agrobacterium-mediated transformation by floral dipping.

Confirmation of cloning at each step

For confirmation of each of the 24 shuttle vector constructs, plasmid was extracted using High-Speed Plasmid Mini Kit (IBI Scientific) polyclonally from the 3 ml culture of pooled *E. coli* transformants, cut with 1 unit of *BamHI* (Ficher) at 37 °C for 15 min, and analyzed in 1.5% agarose gel. To confirm each of the 3 multi-multi vector constructs, 5 randomly selected *E. coli* colonies were grown individually in 3 ml LB at 37 °C overnight. Plasmid was extracted from each of them, cut with 1 unit of *BpiI* (NEB) at 37 °C for 1 hour, and analyzed in 1.5% agarose gel. For the recipient vector construct, plasmids from 4 randomly selected *E. coli* clones were cut with 1 unit of *HindII* (Fisher) at 37 °C for 15 min and analyzed in 0.8% agarose gel. All agarose gels were stained with ethidium bromide to visualize DNA under UV light.

PCR analysis and sequencing of genomic DNA

Genomic DNA was extracted from a small piece of Arabidopsis leaf using Extract-N-Amp extraction kit (Sigma-Aldrich) and used as a template for PCR. Reaction mixture was set in a final volume of 20 μl as 0.2 μM dNTP mix, 50 μM each primer, 0.5 μl template, 1 unit of Taq DNA polymerase (GenScript). PCR was conducted at 94 °C for 3 min for denaturation followed by 35-40 cycles of 94 °C for 30 sec, 50-55 °C for 45 sec and 72 °C for 60 sec, with final extension at 72 °C for 7 min. PCR products were analyzed in 1% agarose gel stained with ethidium bromide. For DNA sequencing, the PCR products were purified using Gel/PCR DNA

Fragments Extraction Kit (IBI Scientific) and sent with a 10 μM primer (forward and/or reverse depending on the chromatogram quality) for a commercial Sanger sequencing service (Eurofins Genomics). Sequencing results were processed and analyzed using Chromas software (v 2.6.6).

Selection of non-transgenic seeds

T2 seeds were harvested and approximately 200-300 seeds were evenly spread on a white paper. They were mounted and observed in a fluorescence microscope with RFP filter (Eclipse E800, Nikon). The seeds without fluorescence were carefully picked up using the tip of a wet tooth pick and transferred to water in a microtube for the sterilization as described below. This process was repeated until a sufficient amount of non-transgenic seeds was obtained.

Plant materials and growth conditions

All Arabidopsis plants used in this study were Col-0 ecotype of *Arabidopsis thaliana*. Transgenic plants with *CCA1::LUC* or *TOC1::LUC* were previously generated (Kim et al., 2019). Seeds were surface-sterilized with 70 % (v/v) ethanol and then with 20 % (v/v) bleach, washed with water 4 times, and sown on 1/2 strength of Murashige and Skoog (MS) media supplemented with 1 % (w/v) sucrose and 0.8 % (w/v) agar. After stratification at 4 °C for 2 days in the dark, plants were germinated and grown in a growth chamber (Percival, CU22L2) maintained at 22 °C and a relative humidity of 60% under light cycles of 12-h light/12-h dark with a photosynthetic photon flux density of 120-150 μmol/m^2/sec.

Time-course measurement of luciferase activity

The *CCA1::LUC* and *TOC1::LUC* constructs were previously described (Alabadí et al., 2001). Sterilized seeds were sown on 1/2 MS plate (10×10 cm) supplemented with 1% sucrose in lines with intervals of at least 1.5 cm. The plates were sealed with paper tapes, not Parafilm, to promote gas exchange and prevent condensation on the lid. The seeds were germinated and grown for entrainment in 12-h light/12-h dark cycles at 22 °C for 7 days. Under aseptic conditions, they were sprayed evenly with 5 μM luciferin dissolved in 0.01% Triton-X100 and transferred to a growth chamber equipped with PIXIS 1024B CCD camera (Princeton Instruments) and LED lights (70 μmol/m^2/s, wavelengths 400, 430, 450, 530, 630, and 660 at intensity 350; Heliospectra LED lights). Plant images were taken every 60 min after a 180-sec

delay for 4 min with 1×1 binning using µManager software (Edelstein et al., 2010, 2014), starting at Zeitgeber time (ZT) 0 hour for *CCA1::LUC* and 12 hour for *TOC1::LUC* to synchronize the phase of their expression. The luminescence intensity from each seedling on each image was measured with ImageJ (1.53e) using the ROI manager. Circadian parameters were estimated using BioDare2 (biodare2.ed.ac.uk) with linear detrend and the fast fourier transform non-linear least squares (FFT-NLLS) method (Zieliński et al., 2021).

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Author contributions:

S.K. (skim@danforthcenter.org) designed and performed all experiments, collected and analyzed all data, and wrote the manuscript. D.N. (dnusinow@danforthcenter.org) discussed the results and edited the manuscript. X.W. (swang@danforthcenter.org) proposed and supervised the study, discussed the results, and edited the manuscript.

Data availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends:

Figure 1. Cloning of oligonucleotides for 24 sgRNAs targeting 12 *PLD*s. A. Schematic overview of the cloning procedure. 24 sgRNAs (two target sites for each *PLD*) were cloned sequentially in shuttle vectors, multi-multi vectors, and finally recipient vector for Arabidopsis transformation, in order by Golden Gate cloning. Two cohesive ligation sites are connected with dashed lines, and restriction enzyme sites to confirm the cloning are in red (*BamHI* site removed by sgRNA is struck through). **B.** Confirmation of shuttle vector constructs. Empty vectors (332-342) and cloned DNA constructs (1-24) were cut with *BamHI*. Arrow head indicates the position of the small fragment resulted from *BamHI* cut in the empty vectors. Note that '2' was failed first (left lane) and retried later (right lane). **C.** Confirmation of multi-multi vector constructs. Plasmids from 5 random *E. coli* clones were cut with *BpiI* for each multi-multi vector. Arrow head indicates the position of the fragment containing all 8 sgRNAs. **D.** Confirmation of recipient vector constructs. Plasmids from 4 random *E. coli* clones were cut with *HindIII*. Arrow head indicates the position of the fragment containing all 24 sgRNAs. For C and D, the clone numbers with the desired fragments are highlighted in red.

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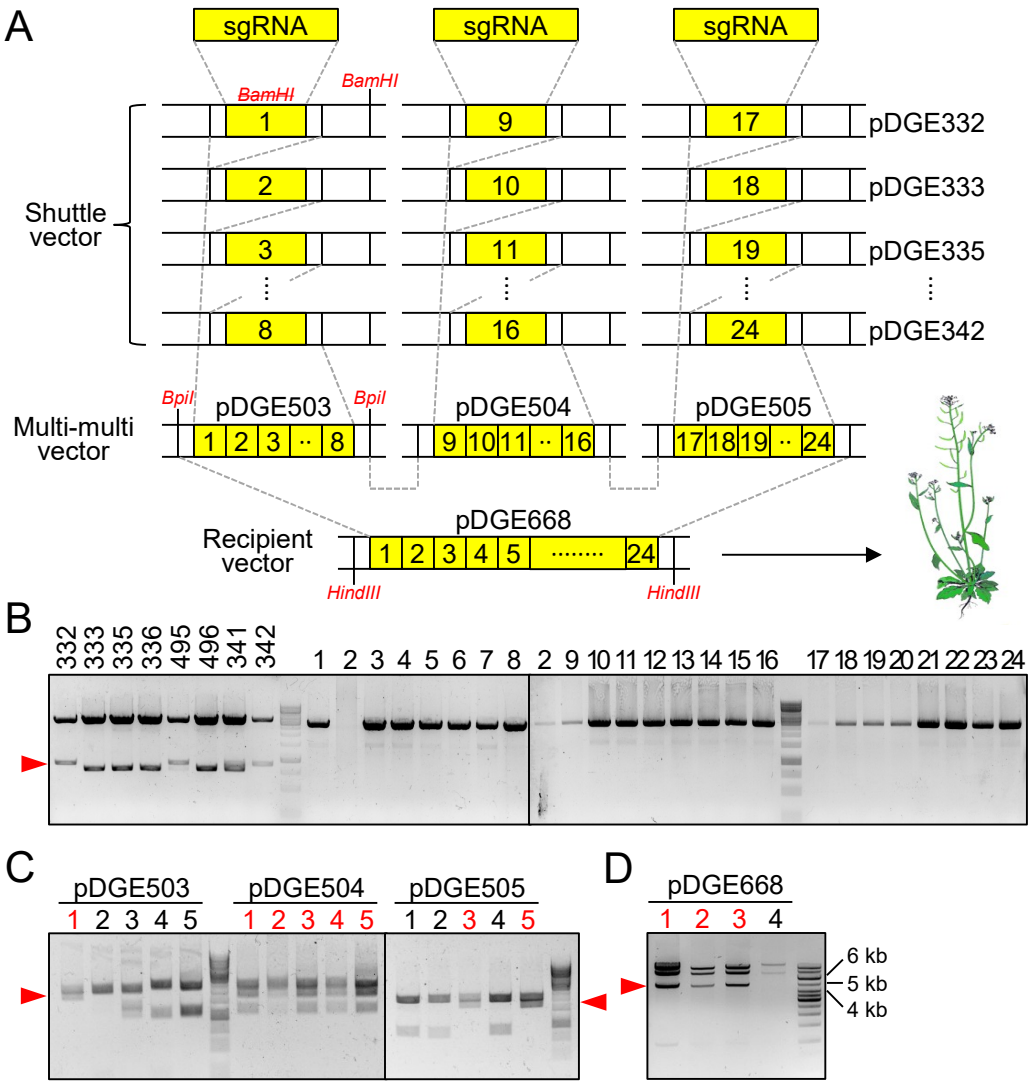

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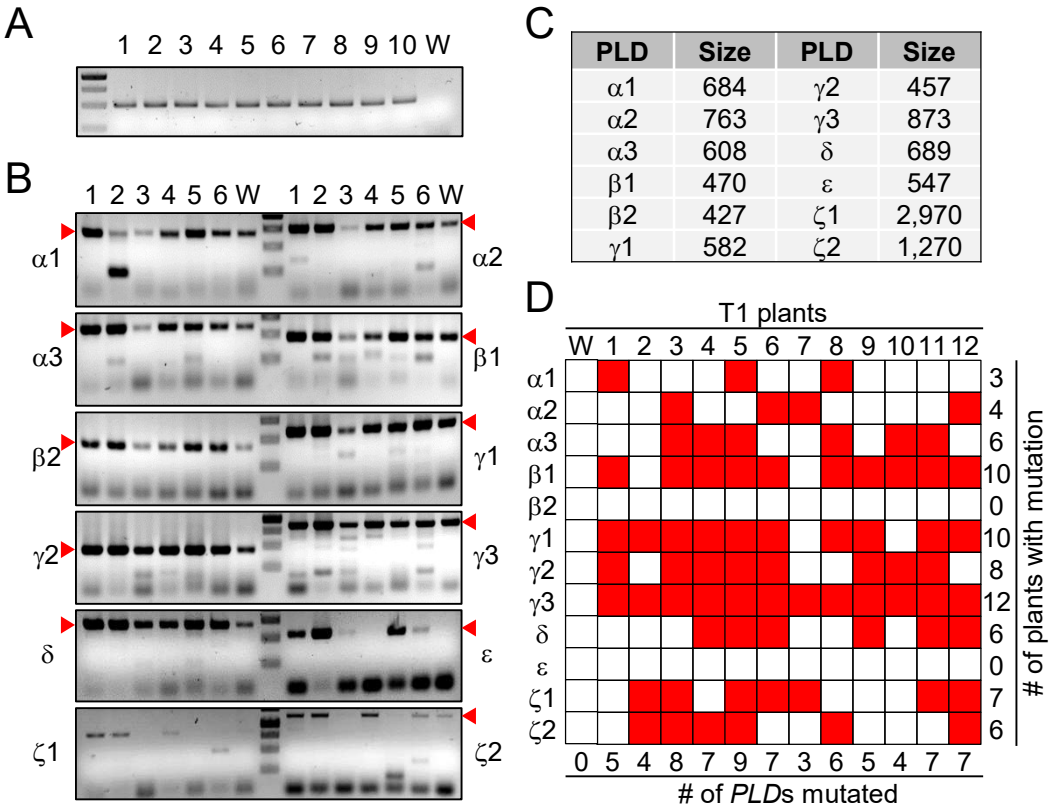

PLD	Size	PLD	Size
α1	684	γ2	457
α2	763	γ3	873
α3	608	δ	689
β1	470	ε	547
β2	427	ζ1	2,970
γ1	582	ζ2	1,270

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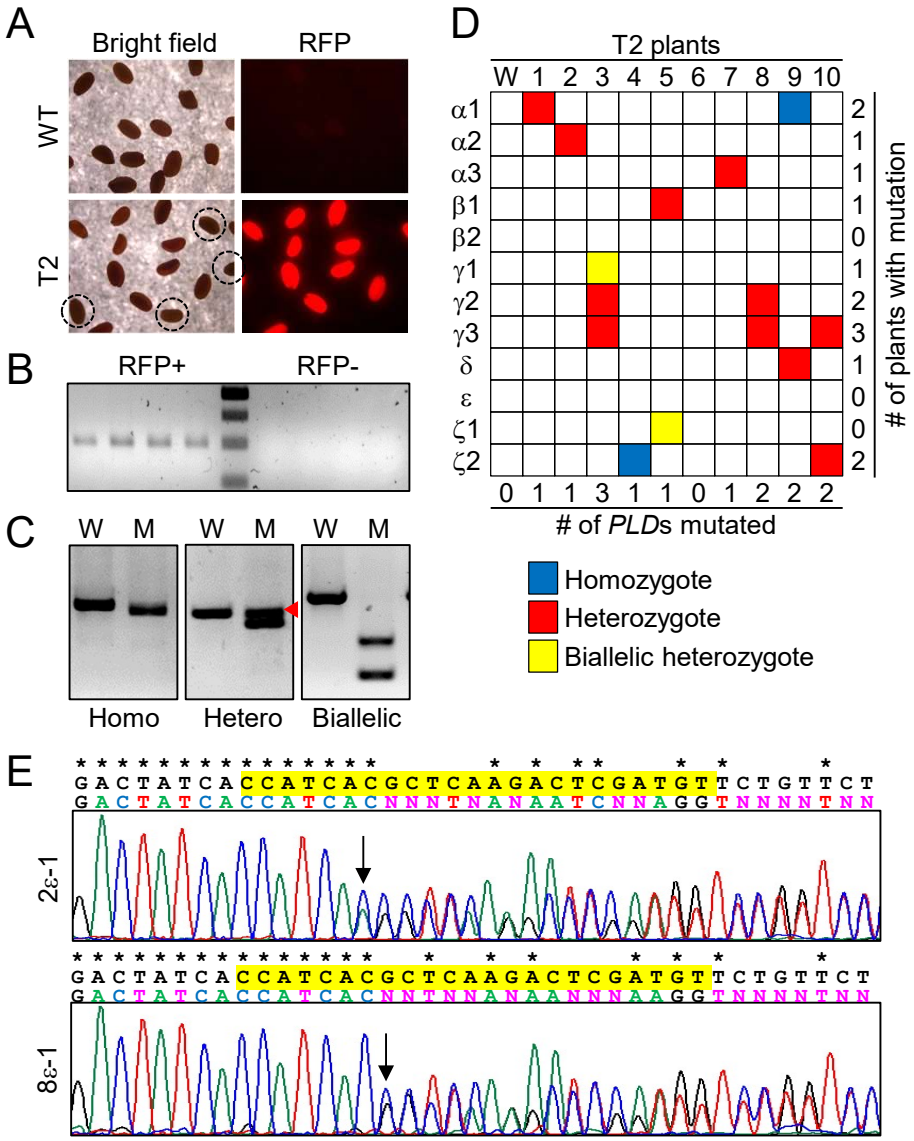

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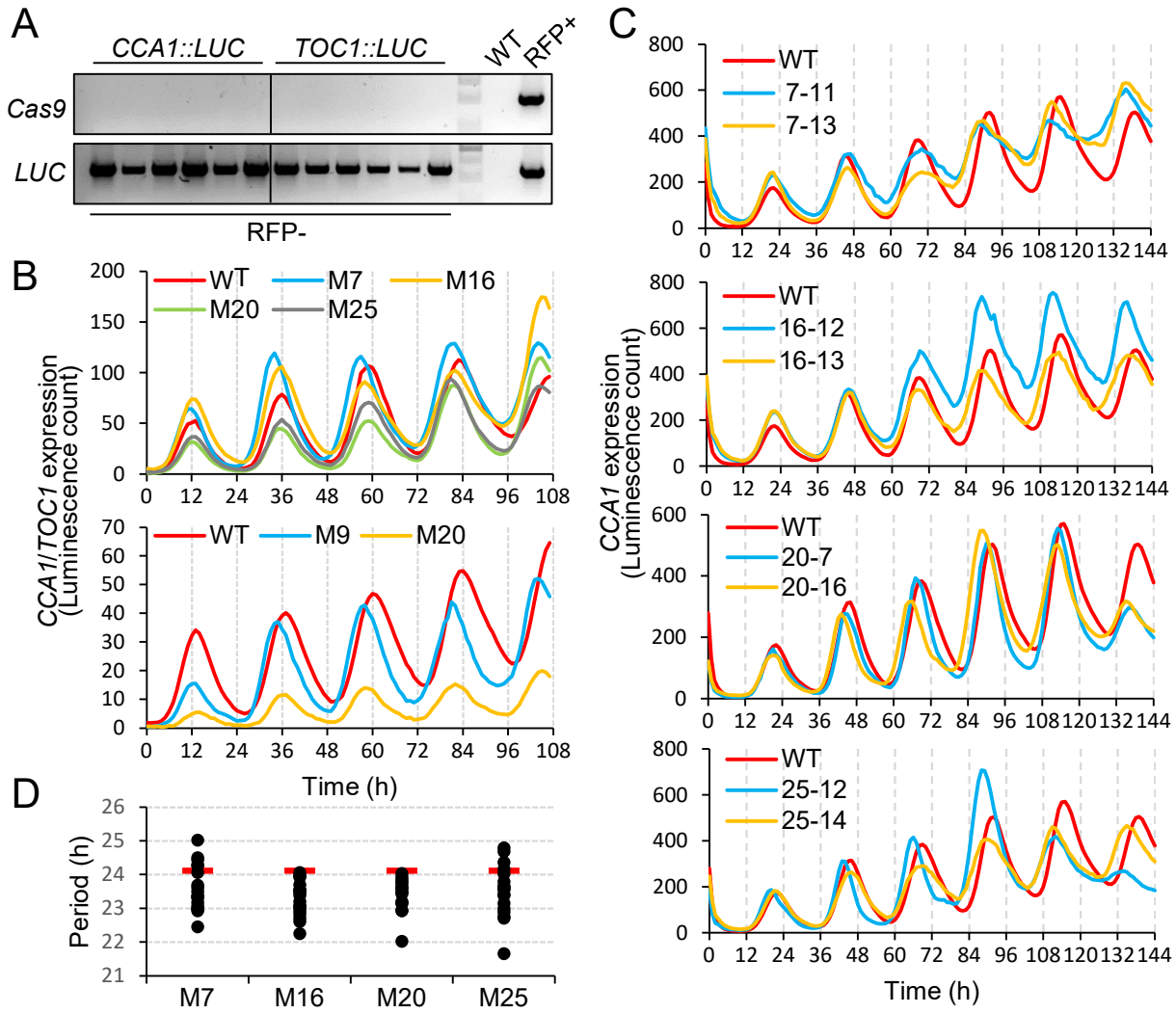

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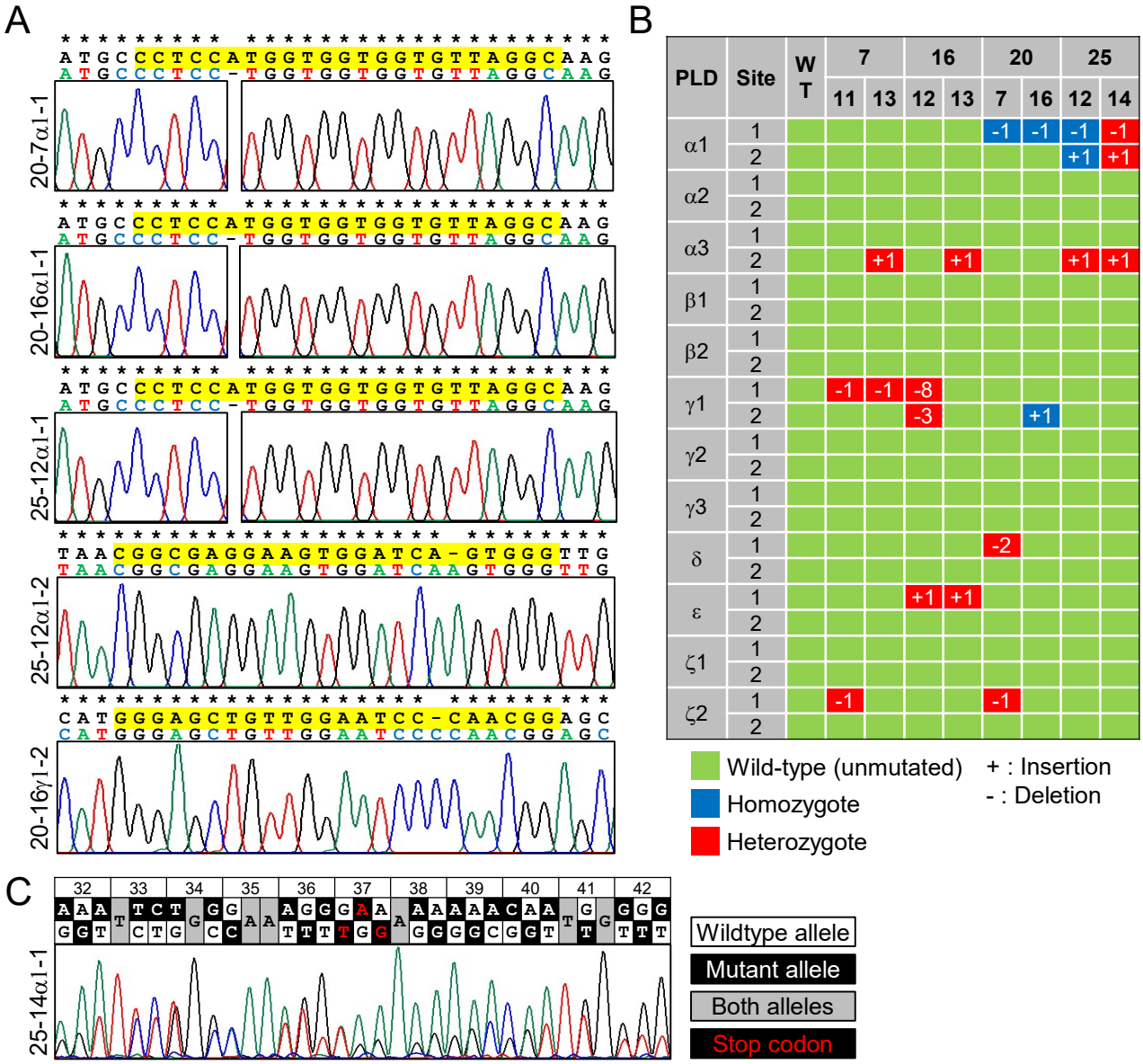

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