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Note

Monitoring single-cell bioluminescence of *Arabidopsis* leaves to quantitatively evaluate the efficiency of a transiently introduced CRISPR/Cas9 system targeting the circadian clock gene *ELF3*

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Abstract The rapid assessment of gene function is crucial in biological research. The CRISPR/Cas9 system is widely used as a tool for targeted gene editing in many organisms including plants. Previously, we established a transient gene expression system for investigating cellular circadian rhythms in duckweed. In this system, circadian reporters and clock gene effectors—such as overexpressors, RNA interference (RNAi), and CRISPR/Cas9—were introduced into duckweed cells using a particle bombardment method. In the present study, we applied the CRISPR/Cas9 system at a single cell level to *Arabidopsis thaliana*, a model organism in plant biology. To evaluate the mutation induction efficiency of the system, we monitored single-cell bioluminescence after application of the CRISPR/Cas9 system targeting the *ELF3* gene, which is essential for robust circadian rhythmicity. We evaluated the mutation induction efficiency by determining the proportion of cells with impaired circadian rhythms. Three single guide RNAs (sgRNAs) were designed, and the proportion of arrhythmic cells following their use ranged from 32 to 91%. A comparison of the mutation induction efficiencies of diploid and tetraploid *Arabidopsis* suggested that endoreduplication had a slight effect on efficiency. Taken together, our results demonstrate that the transiently introduced CRISPR/Cas9 system is useful for rapidly assessing the physiological function of target genes in *Arabidopsis* cells.

Key words: *Arabidopsis thaliana*, circadian rhythm, CRISPR/Cas9, *ELF3*, single-cell bioluminescence monitoring.

The rapid assessment of the physiological function of an interesting gene candidate is important for the progress of research. The CRISPR/Cas9 system is a revolutionary gene editing tool that can be used to investigate gene function (Doudna and Charpentier 2014; Horvath and Barrangou 2010). The CRISPR/Cas9 system comprises two components: a Cas9 nuclease from *Streptococcus pyogenes* and an sgRNA. An sgRNA with a 20-bp sequence that specifically complements the target sequence adjacent to the "NGG" sequence—known as the PAM sequence—guides Cas9 to the target locus. The Cas9–sgRNA complex then induces a double-strand break (DSB) at the target site. Once Cas9 has created a DSB, the non-homologous end joining (NHEJ) repair machinery is triggered. This generally introduces insertions or deletions at the target site, which may cause disruption of the target gene. This gene disruption system has been used in a wide range of plant species (Bortesi and Fischer 2015; Osakabe and Osakabe 2015; Yin et al. 2017). Numerous studies have been conducted to improve the mutation induction efficiency by modifying the promoters of Cas9/sgRNA and by adjusting the length of the target sequences of sgRNA (Belhaj et al. 2013; Liang et al. 2016). In the model plant *Arabidopsis thaliana*, the CRISPR/Cas9 system is mainly delivered by *Agrobacterium tumefaciens* or polyethylene glycol (PEG)-mediated transfection of its protoplasts. A PEG-mediated protoplast transient transfection system is appropriate for a rapid evaluation of the efficiency of the CRISPR/Cas9 system. In one study, time-series gel image analysis was used to demonstrate the targeted mutagenesis of rice protoplasts 18 h after transfection, and the indel mutation frequency was estimated to be ~25% at the end of culture (72 h after transfection) (Shan et al. 2013). In a different study, the mutagenesis efficiency of another target gene of *Arabidopsis* protoplasts was estimated to be

Abbreviations: *CCA1*, CIRCADIAN CLOCK ASSOCIATED 1; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; *ELF3*, EARLY FLOWERING 3; FFT-NLLS, fast Fourier transform-nonlinear least squares; FRP, free-running period; PAM, protospacer adjustment motif; RAE, relative amplitude error; sgRNA, single guide RNA.

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only 6.5% (Lin et al. 2018). These mutation frequencies seem to be insufficient for investigating the physiological function of target genes in protoplast cells.

We have developed a particle bombardment transient transfection system to investigate clock gene functions in duckweed cells (Okada et al. 2017; Serikawa et al. 2008). In this system, circadian rhythms can be observed at the level of a single cell by monitoring the clock-driven luminescence of cells transfected with a bioluminescent reporter of a firefly luciferase driven by the circadian promoter *CCA1::LUC* (Isoda and Oyama 2018; Muranaka and Oyama 2016; Muranaka et al. 2013). This transient expression system is also useful for the functional analysis of clock genes by co-transfection of effector constructs: overexpressors and RNAi (Serikawa et al. 2008). CRISPR/Cas9 also works with this system (Okada et al. 2017). We performed co-transfection experiments with effector constructs for a CRISPR/Cas9 system targeting the *ELF3* gene in addition to the *CCA1::LUC* reporter using duckweed (*Lemna gibba*), and demonstrated that the circadian rhythms of the co-transfected cells were severely impaired. *ELF3* is essential for circadian rhythmicity, and the null *ELF3* mutant of *Arabidopsis* has an arrhythmic phenotype (Hicks et al. 1996, 2001). Although co-transfection of a CRISPR/Cas9 system targeting *ELF3* in duckweed severely affected the cellular circadian rhythms, there was a variation in the severity (Okada et al. 2017). In the present study, we applied the CRISPR/Cas9 system to *Arabidopsis*, which is commonly used as a model organism in plant biology. We based our evaluation of mutation induction efficiency on the proportion of cells with impaired circadian rhythms. Unlike in duckweed, endoreduplication is prevalent in *Arabidopsis* leaf cells (del Pozo and Ramirez-Parra 2015; Ferjani et al. 2007; Robinson et al. 2018; Van Hoeck et al. 2015), and the number of chromosomes is increased up to 16 (32). This means that the number of genes available for CRISPR/Cas9 targeting ranges from 2 to 16. In the present study, we demonstrated that this transiently introduced CRISPR/Cas9 system can be applied to polyploid cells in *Arabidopsis*, and its mutation induction frequency is sufficient to enable the investigation of the physiological function of the target genes in the cells.

We used *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) for the experiments. In addition to Col-0, we used a tetraploid *Arabidopsis thaliana* (Col_2-4-6) gifted by Dr. Tsukaya (Tsukaya 2013). The seeds were surface-sterilized and sown on 0.8% agar plates containing half-strength Murashige and Skoog medium with 1% sucrose, and were incubated in a growth chamber under continuous white fluorescent light ($\sim 50\,\mu E\,m^{-2}\,s^{-1}$) at 22±1°C for 2 weeks. We cut the petioles of the true leaves of the seedlings with scissors, and subjected the detached leaves to particle bombardment. The leaves were subsequently subjected to two 12-h light/12-h dark cycles followed by continuous dark (Figure 1A). We used the luciferase reporter gene *CCA1::LUC* [*CCA1::LUC* (a) in Nakamichi et al. 2004] to monitor the circadian cycle. The Cas9 expression vector, *pUC18-Cas9*, was constructed by subcloning the Cas9 expression cassette of pDe-Cas9 into pUC18 (Fauser et al. 2014). The *Arabidopsis* U6-26 promoter-driven sgRNA expression cassette of the pEn-Chimera vector (gifted by Dr. Puchta) was amplified by polymerase chain reaction (PCR) and cloned into a pENTR D-TOPO vector (named pENTR sgRNA). We designed three sgRNAs for the *ELF3* gene in the genome sequence coding for the N-terminal region of ELF3 (Figure 1B). We selected the target sequence of *ELF3* using CRISPRdirect software (https://crispr.dbcls.jp/). Three pairs of oligoDNAs specific to the ELF3 cleavage target sequence—ELF3_1 (+29–+51): 5′-attgTAT TGG AAC CTA TGT TTC CT-3′ and 5′-aaacAGG AAA CAT AGG TTC CAA TA-3′; ELF3_2 (+59–+81): 5′-attgTGA ATG ATG CAG ATA AAG GA-3′ and 5′-aaacTCC TTT ATC TGC ATC ATT CA-3′; and ELF3_3 (+87–+109): 5′-attgAGC TCC TCC TAG AAA CAA GA-3′ and 5′-aaacTCT TGT TTC TAG GAG GAG CT-3′—were annealed and subcloned into the BbsI-digested pENTR sgRNA vector to produce pENTR-sgELF3_1(ELF3_sg1), pENTR-sgELF3_2 (ELF3_sg2), and pENTR-sgELF3_3(ELF3_sg3), respectively. In the particle bombardment experiment, we used an 8-μl aliquot comprising a suspension of pre-washed gold particles (0.6 μm diameter) in 50% glycerol (13 mg ml^{-1}), 2 μg of reporter DNA, and 1 μg of effector DNA for each construct (Figure 1C). We used a PDS1000/He helium gun device (Bio-Rad) for particle bombardment according to the manufacturer's instructions [vacuum, 26 mmHg; helium pressure, 450 psi (rupture disc)]. We captured the bioluminescence images of the transfected leaves using an ImagEM C9100-13 cooled electron-multiplying charge-coupled-device (EM-CCD) camera (Hamamatsu Photonics), and selected a leaf with a high transfection efficiency (Figure 1C). The single-cell bioluminescence imaging of the leaf floating on the liquid medium was performed as described previously (Muranaka and Oyama 2016). To illuminate samples during single-cell monitoring, we used optical fiber-guided white light ($30\,\mu E\,m^{-2}\,s^{-1}$) from a PFB-20SW light-emitting diode device (CCS Inc.). A bioluminescence image was automatically captured every hour with 200-s exposure. The x–y coordinates of each luminescent spot in the images gradually changed throughout the monitoring with the expansion of the leaf drifting on the medium. Those coordinates were obtained by single particle tracking procedures (Yasui et al. 2014), and intensity of each luminescent spot was measured according to the coordinates.

We performed bioluminescence monitoring of the

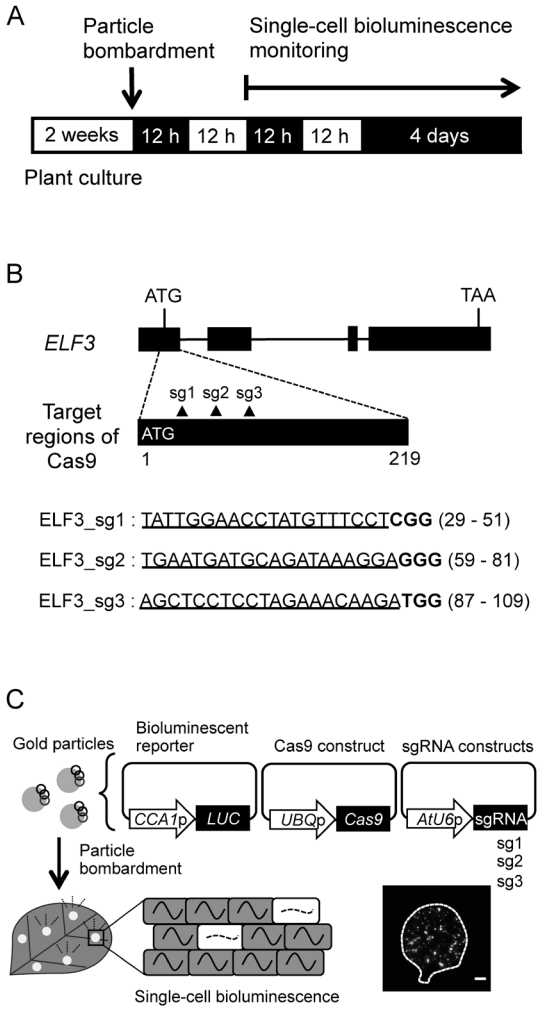

Figure 1. Experimental scheme of single-cell bioluminescence monitoring for the evaluation of CRISPR/Cas9 targeting of *ELF3*. (A) The experimental schedule for single-cell bioluminescence monitoring. Black and open bars indicate dark and light, respectively. (B) Design of the CRISPR/Cas9 constructs targeting the *ELF3* gene. The black boxes and lines indicate exons and introns, respectively. The start site of the coding region (+1) is indicated by ATG. The three CRISPR/Cas9 target regions (sg1, sg2, and sg3) in the first exon are indicated by triangles. The PAM sites and the target sequences of the three regions are shown in bold and underlined, respectively. The locus of each target region is indicated in parentheses. (C) A scheme of the circadian bioluminescent reporter system using CRISPR/Cas9. The plasmid constructs of the three elements (circadian bioluminescent reporter, Cas9 nuclease, sgRNA) are shown with the promoter information. *LUC*, *Cas9*, and sgRNA are driven by the promoters of *Arabidopsis CCA1*, a parsley *UBQ*, and *Arabidopsis U6-26*, respectively. A bioluminescence image of a detached leaf subjected to the gene transfection is shown (scale bar=1 mm). The dotted white line indicates the leaf shape.

cells in a detached leaf in two independent experiments (Experiments 1 and 2) for each construct. The experiment with the greater number of measured cells was designated Experiment 1. First, we monitored bioluminescence in a leaf transfected with the *CCA1::LUC* reporter and the *Cas9* construct without an sgRNA construct (control/2n in Figure 2A). The circadian rhythms of most of the cells appeared to be robust. We then quantitatively investigated the cellular circadian rhythms using the FFT-NLLS method (Plautz et al. 1997), and calculated the free-running period (FRP) and relative amplitude error (RAE) of each luminescence trace in constant darkness (48–120 h in Figure 2). The RAE value represents the degree of confidence of rhythmicity, ranging from 0 (complete sine-fitting) to 1 (arrhythmic) (Supplementary Figure 1). To evaluate the stability of the rhythmicity of each luminescence trace, we categorized the individual luminescence traces into three groups: "robust rhythm" (RAE <0.1), "unstable rhythm" ($0.1 \leqq \text{RAE} \leqq 0.15$), and "arrhythmia" (RAE >0.15). The cellular luminescence traces of most of the measured cells (89%) were categorized into the robust rhythm group (control/2n in Figure 2B, Table 1). Half of the remaining traces were categorized into the unstable rhythm (6%) and arrhythmia (6%) groups. As shown in Figure 2A and Supplementary Figure 1A, those in the unstable rhythm and arrhythmia groups still showed very low-amplitude rhythms with similar phases as the mean luminescence rhythm. Similar results were obtained in the duplicate experiment (control/2n in Supplementary Figures 2A and 2B, and in Table 1).

Next, we monitored the luminescence traces in a leaf co-transfected with the Cas9 construct and an sgRNA construct, ELF3_sg1 (sg1/2n in Figure 2A). The mean luminescence of the traces clearly showed a circadian rhythm throughout the monitoring; however, its amplitude looked slightly lower than that of the control/2n (control/2n in Figure 2A). At a single cell level, 46% of the measured cells exhibited robust circadian rhythms, and 44% of the cells were categorized into the arrhythmia group (sg1/2n in Figure 2B, Table 1). This suggests that co-transfection with ELF3_sg1 is capable of disrupting cellular circadian rhythms. Because the loss-of-function mutation in *ELF3* results in a recessive allele (Hicks et al. 1996; Zagotta et al. 1996), it is likely that the *ELF3* locus is disrupted in every chromosome in the arrhythmic cells. The relatively large proportion of arrhythmic cells in the measured cells resulted in the low amplitude of the mean luminescence rhythm. Similar results were obtained in the duplicate experiment (sg1/2n in Supplementary Figures 2A and 2B, and in Table 1). The sgRNA constructs ELF3_sg2 and ELF3_sg3 disrupted the *ELF3* gene more effectively than ELF3_sg1 (sg2/2n and sg3/2n in Figure 2A; sg2/2n and sg3/2n in Supplementary Figure 2A). The proportions of cells that were categorized into the arrhythmia group ranged from 47 to 91%, and the mean luminescence of the traces revealed low-amplitude rhythms or near arrhythmia (sg2/2n and sg3/2n in Figure 2B; sg2/2n and sg3/2n in Supplementary Figure 2B; Table 1). These results indicate that the mutation induction efficiencies differed depending on the target regions (sequences) of the sgRNA.

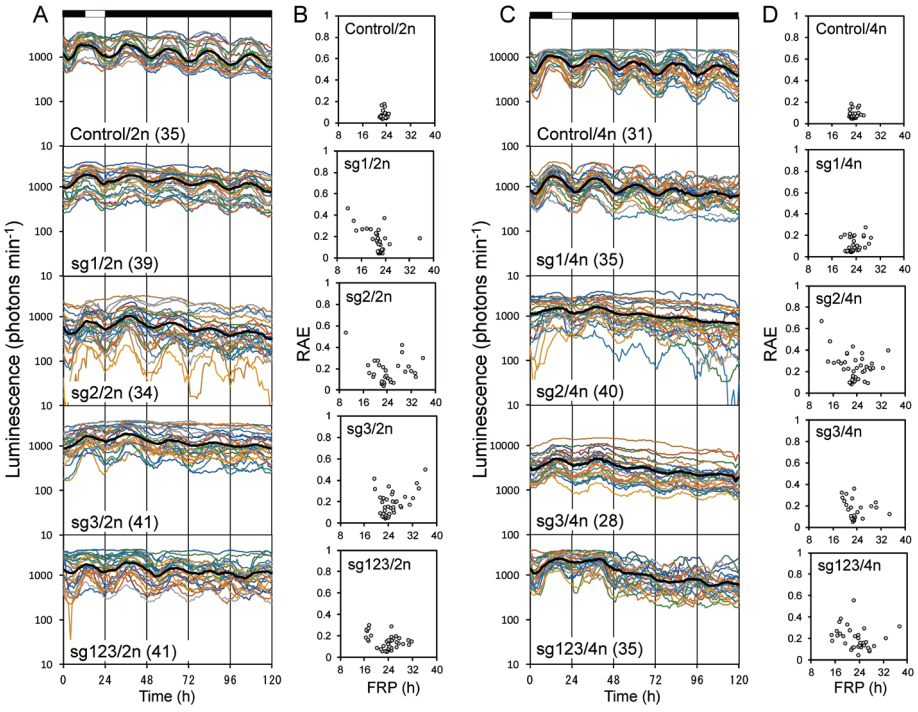

Figure 2. Bioluminescence traces of individual cells transfected with CRISPR/Cas9 constructs. (A, C) Mean luminescence (black lines) and luminescence traces of individual cells (lines in other colors) in diploid (A) and tetraploid (C) *Arabidopsis*. The CRISPR/Cas9 target region is indicated in each graph, and the number of measured cells is indicated in parentheses. Note that the *y*-axis scales differ between panels. (B, D) The free-running periods (FRPs) and the relative amplitude errors (RAEs) of individual cellular rhythms in diploid (B) and tetraploid (D) *Arabidopsis* are plotted. Fast Fourier transform-nonlinear least squares (FFT-NLLS) was performed using the data range 48–120 h shown in (A) and (C).

Table 1. Summary of circadian rhythmicity in cells transfected with a CRISPR/Cas9 system.

Effector	Diploid *Arabidopsis*				Tetraploid *Arabidopsis*			
	Number of analyzed cells	Number and percentage (in parentheses) of cells			Number of analyzed cells	Number and percentage (in parentheses) of cells		
		RAE<0.1	0.1≦RAE≦0.15	RAE>0.15		RAE<0.1	0.1≦RAE≦0.15	RAE>0.15
Control#1	35	31 (89)	2 (6)	2 (6)	31	26 (84)	0 (0)	5 (16)
Control#2	24	18 (86)	1 (5)	5 (10)	26	25 (96)	1 (4)	0 (0)
sg1#1	39	18 (46)	4 (10)	17 (44)	35	21 (60)	3 (9)	11 (31)
sg1#2	22	12 (55)	3 (14)	7 (32)	30	20 (67)	4 (13)	6 (20)
sg2#1	34	11 (32)	7 (21)	16 (47)	40	6 (15)	7 (18)	27 (68)
sg2#2	22	1 (5)	1 (5)	20 (91)	33	2 (6)	0 (0)	31 (94)
sg3#1	41	14 (34)	6 (15)	21 (51)	28	5 (18)	10 (36)	13 (46)
sg3#2	22	2 (9)	4 (18)	16 (73)	20	7 (35)	7 (35)	6 (30)
sg123#1	41	13 (32)	12 (29)	16 (39)	35	4 (11)	11 (31)	20 (57)
sg123#2	32	5 (16)	4 (13)	23 (72)	34	3 (9)	11 (32)	20 (59)

We then attempted to determine whether co-transfection with the three sgRNA constructs (ELF3_sg1, ELF3_sg2, and ELF3_sg3) resulted in more severe phenotypes than co-transfection with a single sgRNA construct. Co-transfection with the three sgRNA constructs disrupted the cellular circadian rhythms (sg123/2n in Figure 2A; sg123/2n in Supplementary Figure 2A), and the proportions of cells that were categorized into the arrhythmia group were 39% in Experiment 1 and 72% in Experiment 2 (sg123/2n in Figure 2B; sg123/2n in Supplementary Figure 2B; Table 1). The proportion of arrhythmic cells in Experiment 1 was comparable to that in the experiment involving the low-efficiency sgRNA construct ELF3_sg1. However, the proportions of cells with a robust rhythm were 32% in Experiment 1 and 16% in Experiment 2. The proportion was lower in the cells co-transfected with ELF3_sg1, and comparable to that in the cells co-transfected with ELF3_sg2 or ELF3_sg3, which exhibited high efficiency. As presented in Supplementary Figure 1B, the luminescence traces that were categorized into the unstable rhythm group exhibited circadian rhythms with aberrant periods/phases, suggesting that most of the cells were affected by co-transfection with ELF3_sg123. There was apparently little difference in the mutation induction efficiencies between the co-transfection of

ELF3_sg2, ELF3_sg3, ELF3_sg123. In our experiments, co-transfection of ELF3_sg123 had sgRNA constructs 3-fold larger than those of ELF3_sg2/ELF3_sg3, while the amount of Cas9 construct was the same between the single sgRNA transfection and the multiple sgRNAs. The larger amount of sgRNAs in the co-transfection of ELF3_sg123 might result in lower mutation induction efficiencies of individual sgRNAs. Nevertheless, these mutation induction efficiencies were high enough for the functional analysis of target genes in *Arabidopsis* leaves.

As shown in Figure 3A, even when the same sgRNA constructs were used for transfection, there were phenotypic variations between cells and variations in the mutation induction efficiencies between the experiments. Those variations may have resulted from differences in the ploidy level of the transfected cells, because the ploidy level of cells in an *Arabidopsis* leaf range from 2C to 16C (32C) owing to endoreduplication (del Pozo and Ramirez-Parra 2015; Ferjani et al. 2007; Robinson et al. 2018). A high level of ploidy in the transfected cells might result in lower complete gene disruption efficiency. To test this hypothesis, we used a tetraploid *Arabidopsis* strain to evaluate the CRISPR/Cas9 system. We first monitored bioluminescence in a leaf transfected with a Cas9 construct without an sgRNA construct (control/4n in Figure 2C; control/4n in Supplementary Figure 2C). The circadian rhythms of most of the cells appeared to be robust. In Experiment 1, the cellular luminescence traces of most of the measured cells (84%) indicated robust rhythms (control/4n in Figure 2D; Table 1). However, the rest of the cells were categorized into the arrhythmia group (16%). In Experiment 2, almost all the traces (96%) indicated robust rhythms, and only a single trace (4%) was categorized into the unstable rhythm group (control/4n in Figure 2D; Table 1). Although the proportion of cells with robust rhythms indicated a variation between the experiments, there was no obvious difference in the circadian behavior of the diploid and tetraploid *Arabidopsis* control leaves.

Next, we monitored luminescence traces in a leaf co-transfected with an sgRNA construct, ELF3_sg1 (sg1/4n in Figure 2C; sg1/4n in Supplementary Figure 2C). The mean luminescence of the traces clearly indicated a circadian rhythm throughout the monitoring. However, at the single cell level we observed cells that were categorized into the arrhythmia group: 31% in Experiment 1 and 20% in Experiment 2 (sg1/4n in Figure 2D; sg1/4n in Supplementary Figure 2D; Table 1). The proportions of arrhythmic cells were larger than those in control/4n. Although the proportions of arrhythmic cells were slightly lower than those in diploid *Arabidopsis*, co-transfection with ELF3_sg1 was also capable of disrupting the cellular circadian rhythms in tetraploid *Arabidopsis*. The sgRNA construct ELF3_sg2 was much more effective at disrupting the *ELF3* gene than ELF3_

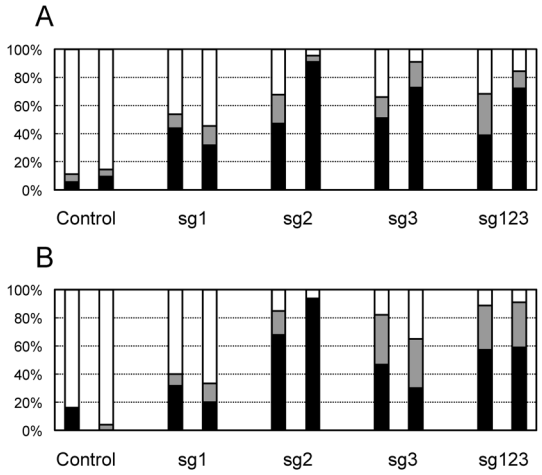

Figure 3. Comparison of the effects of CRISPR/Cas9 target regions on cellular circadian rhythms in diploid or tetraploid *Arabidopsis*. 100% stacked bar charts categorized by relative amplitude error (RAE) values of cellular bioluminescence traces are shown for mutation induction efficiencies in diploid (A) or tetraploid (B) *Arabidopsis*. The segments in white, gray, and black represent the proportions of robust rhythm (RAE$<$0.1), unstable rhythm (0.1$\leqq$RAE$\leqq$0.15), and arrhythmia (RAE$>$0.15), respectively. The introduced sgRNA is the stacked variable. The numerical data are summarized in Table 1.

sg1 (sg2/4n in Figure 2C; sg2/4n in Supplementary Figure 2C). The proportions of arrhythmic cells were 68% in Experiment 1 and 94% in Experiment 2 (sg2/4n in Figure 2D; sg2/4n in Supplementary Figure 2D; Table 1). The effects of the sgRNA construct ELF3_sg3 were milder than those of ELF3_sg2. The proportions of arrhythmic cells were 46% in Experiment 1 and 30% in Experiment 2 (sg3/4n in Figure 2D; sg3/4n in Supplementary Figure 2D; Table 1). Co-transfection of the three sgRNA constructs (ELF3_sg1, ELF3_sg2, and ELF3_sg3) resulted in almost arrhythmic mean luminescence with arrhythmic cell proportions of approximately 60%. In the series of experiments, the proportions of arrhythmic cells in tetraploid *Arabidopsis* were slightly lower than those in diploid *Arabidopsis* (Figure 3). Nevertheless, cellular gene disruption was obviously induced in tetraploid *Arabidopsis* with sufficient efficiency to enable functional analysis of the target gene in the leaves. This strongly suggests that the transiently introduced CRISPR/Cas9 system can not be greatly affected by the ploidy level of the transfected cells in *Arabidopsis* leaves.

We calculated the proportions of complete gene-disrupted cells based on the previous reports of ploidy levels in the cells of mature leaves (del Pozo and Ramirez-Parra 2015; Ferjani et al. 2007; Robinson et al. 2018) (Supplementary Table S1). Mesophyll and epidermal cells have been reported as the targets for gene transfection by particle bombardment (Muranaka et al. 2013). In our experiments, the proportions of cells that were categorized into the arrhythmia group were 32% or more in diploid *Arabidopsis* and 20% or more in

tetraploid *Arabidopsis* (Table 1). Based on the calculated proportions, the gene disruption rates in the target chromosome were estimated at 70% or more for any of the sgRNAs targeting *ELF3*. Our experimental results and this calculation indicate that with a highly efficient sgRNA, there could be no need to take into account the ploidy level of the transfected cells. Therefore, this mutation induction system is very useful for investigating the physiological function of target genes in the transfected cells of various plants, irrespective of their ploidy level.

The gene disruption by the transiently introduced CRISPR/Cas9 system in our experiments resulted in a relatively large variation of severity in cellular circadian phenotypes. This variation was likely due to variation of CRISPR-induced mutations in the *ELF3* region. Because the CRISPR/Cas9 system tends to induce indel mutations (Fauser et al. 2014), many of the induced mutations could result in complete loss-of-function of *elf3*. The phenotypic variation was likely to occur in transfected cells completely lacking the functional *ELF3* gene. Another possibility is involvement of some post-transcriptional events that would reduce the *ELF3* function in those cells with the functional *ELF3* in their genomes. Sequence information of individual transfected cells should clarify the various phenotypes. Because influence of cell–cell interaction on the circadian behavior of cells has been suggested (Muranaka and Oyama 2016), the phenotypic variation between cells may reflect the local variation of circadian behavior of neighboring cells in tissues. Protoplasts would be suitable for investigations of cell-autonomous gene function by using the transiently introduced CRISPR/Cas9 system. Recently, we succeeded in stably monitoring the long-term bioluminescence circadian rhythms of *Arabidopsis* protoplasts (Nakamura and Oyama 2018). By monitoring the bioluminescence rhythms of individual protoplasts for a long term, it would be possible to experimentally reevaluate the mutation induction efficiencies of protoplasts. Because DNA sequence of a genetic locus of a single protoplast is accessible (Lin et al. 2018), the phenotypic variation among cells would be directly interpreted based on genome editing information of individual cells.

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