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Report

ELF4 Regulates GIGANTEA Chromatin Access through Subnuclear Sequestration

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SUMMARY

Many organisms, including plants, use the circadian clock to measure the duration of day and night. Daily rhythms in the plant circadian system are generated by multiple interlocked transcriptional/translational loops and also by spatial regulations such as nuclear translocation. GIGANTEA (GI), one of the key clock components in *Arabidopsis*, makes distinctive nuclear bodies like other nuclear-localized circadian regulators. However, little is known about the dynamics or roles of GI subnuclear localization. Here, we characterize GI subnuclear compartmentalization and identify unexpected dynamic changes under diurnal conditions. We further identify EARLY FLOWERING 4 (ELF4) as a regulator of GI nuclear distribution through a physical interaction. ELF4 sequesters GI from the nucleoplasm, where GI binds the promoter of *CONSTANS* (*CO*), to discrete nuclear bodies. We suggest that the subnuclear compartmentalization of GI by ELF4 contributes to the regulation of photoperiodic flowering.

RESULTS AND DISCUSSION

GI Makes Dynamic Subnuclear Organelles

Many of the *Arabidopsis* circadian clock components are localized both in the nucleus and the cytosol. As one of the important regulators in the *Arabidopsis* circadian system, GIGANTEA (GI) is present in both compartments and associates with a range of different partners (Huq et al., 2000; Kim et al., 2007). GI protein interacts with the cytosolic F box protein ZEITLUPE (ZTL), which acts to regulate endogenous rhythms through phase-specific proteolysis (Kim et al., 2007). FKF1, CDF1, and GI form a nuclear protein complex at the promoter of *CONSTANS* (*CO*), a flowering time integrator, to regulate *CO* expression (Sawa et al., 2007). Additionally, TEM1/TEM2 and SVP associate in the nucleus with GI to modulate *FT* expression (Sawa and Kay, 2011). GI also interacts with a nuclear ELF3/COP1 complex that destabilizes GI protein (Yu et al., 2008).

Interestingly, the GI/ELF3/COP1 complex exhibits a specific subnuclear localization in onion epidermal cells (Yu et al., 2008). To test whether GI localizes to nuclear bodies in *Arabidopsis* cells, we constitutively expressed GI-GFP transiently in *Arabidopsis* protoplasts and in transgenic plants. In both systems, GI distribution in the nucleus showed two typical patterns: a dispersed type, and a punctate type (Figure 1A). The characteristics of nuclear bodies can also be defined by their numbers, size, and dynamic movements (Lamond and Sleeman, 2003). In our experiments, the number of GI nuclear bodies ranged widely, from a few to tens of bodies both in transient expression and transgenic plants. Additionally, the size of GI nuclear bodies varied from 0.5 ± 0.025 μm in transient assays to 0.29 ± 0.0016 μm in transgenic plants (Table S1).

Compartmentalization of proteins in the nucleus is a well-known means to regulate processes such as transcription, DNA replication, splicing, and degradation (Lamond and Sleeman, 2003; Shaw and Brown, 2004). There are well-established markers for subnuclear organelles in *Arabidopsis*, including DAPI for chromatin, H2B for chromatin and nucleoli, SCL28 for spliceosomes, and U2B for Cajal bodies (Lorković et al., 2004). To test whether GI nuclear bodies colocalize with any of these markers, we transiently expressed GI-GFP in the presence of DAPI and also coexpressed GI-GFP with H2B-RFP, U2B-YFP, or SCL28-RFP. DAPI, H2B, and SCL28 display distinct and several to tens of nuclear bodies, whereas U2B is present as a single nuclear body (Figures 1B–1E). When we quantified the fluorescence intensities from GI-GFP and each marker, none of these markers colocalized with GI-GFP (right plots in Figures 1B–1E). These results suggest that GI nuclear body localization is distinct from these subnuclear compartments, and they may have little functional relationship with the processes indicated by these markers.

Nuclear bodies are also known to change dynamically (Lamond and Sleeman, 2003; Misteli, 2001). Thus, we tested the nuclear dynamics of GI over a diurnal time course by transiently expressing GI and following the changing distribution of the dispersed and punctate bodies in single-living cells. After

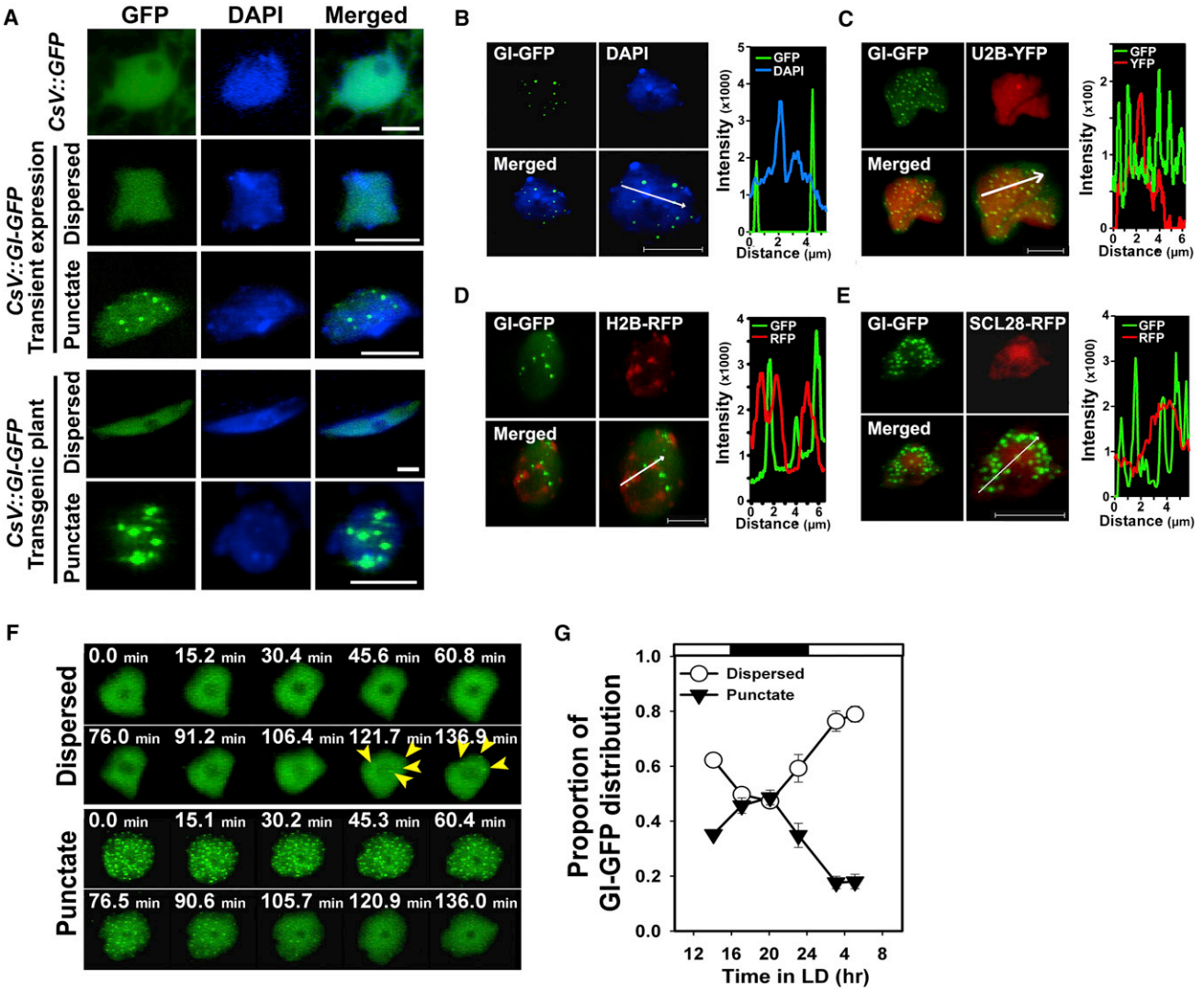

Figure 1. GI Forms Dynamic Subnuclear Structures

(A) Nuclear distributions of GI in a transient expression system using *Arabidopsis* mesophyll cell protoplasts and in transgenic plants. Two prominent distribution types (dispersed and punctate) of GI-GFP were imaged. GFP was used as a control vector in the transient expression system, and DAPI was used as a nuclear marker.

(B–E) Subnuclear colocalization of GI with other subnuclear marker proteins: DAPI for chromatin (B), U2B for Cajal bodies (C), H2B for chromatin and nucleoli (D), and SCL28 for spliceosomes (E). *Arabidopsis* protoplasts were transfected with *GI-GFP* and stained with DAPI for 1 min before imaging. *Arabidopsis* protoplasts were transfected with a *GI-GFP* and *H2B-RFP*, *U2B-YFP*, or *SCL28-RFP* and kept in dim white light for 8 hr before examination. Intensity plots obtained across the nucleus (white arrows) are shown in each image set.

(F) Association and dissociation of GI nuclear bodies. Fluorescence intensities from GI-GFP in the nucleus were tracked over 2 hr on a microscope stage. Yellow arrowheads in the dispersed type of GI localization indicate newly emerged nuclear bodies.

(G) Variation in the proportion of dispersed and punctate types of GI subnuclear localization under LDs (16L/8D). Protoplasts isolated from plants grown under LDs at ZT 0 were transfected with GI-GFP and kept under LDs. White and black boxes represent day and night, respectively. The proportions of cells that express GI-GFP with each of the two nuclear distribution patterns were counted every 3 hr. Data were normalized to total cells counted. Open circle and black inverted triangle represent proportions of the dispersed and the punctate type, respectively. Error bars represent the SEM from four independent replicates.

Scale bars represent 5 μm. See also Figure S1.

transfection, we observed dispersed GI-GFP in the nucleus for 1 hr, with a few small nuclear bodies starting to form after 2 hr of observation (Figure 1F, upper panel). For punctate-type bodies, numerous GI nuclear bodies were present at the outset and then started to dissociate after 45 min of observation, with most of them absent after 2 hr (Figure 1F, lower panel). Thus, the dispersed and punctate types of GI nuclear distribution appeared to be interchangeable, indicating the dynamic nature of their assembly and disassembly.

We next measured the dynamics of the changing proportion of the dispersed and punctate types of GI nuclear expression under diurnal conditions to test the relationship of GI subnuclear

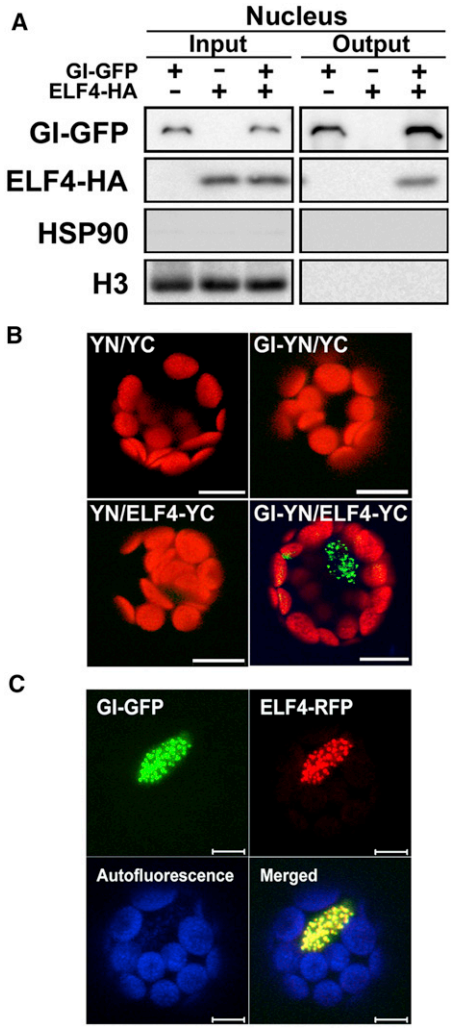

Figure 2. ELF4 Interacts with GI within Nuclear Bodies

(A) CoIP of GI and ELF4 in the nucleus. *CsV::GI-GFP* and *CsV::ELF4-HA* were transiently expressed in *N. benthamiana*, and GI-GFP and ELF4-HA were localized to both the cytosol and the nucleus. CoIP was performed with GFP antibody using the nuclear fraction. HSP90 and H3 were used as a marker for the cytosol and nucleus, respectively.

(B) BiFC analysis of the GI/ELF4 interaction. Green represents YFP from the GI/ELF4 interaction. Chloroplast autofluorescence is red.

(C) Subnuclear colocalization of GI and ELF4. GI-GFP and ELF4-RFP were transiently expressed in *Arabidopsis* protoplasts. Green indicates GI-GFP, red indicates ELF4-RFP, and blue indicates chloroplast autofluorescence.

Scale bars represent 10 μm. See also Figure S2.

distribution to its function. We counted the number of cells that expressed the two types every 3 hr under long days (LDs) and normalized these with total number of cells expressing GI-GFP at each observation point (Figure 1G). The relative proportion of the dispersed and punctate types of GI-GFP varied throughout the day. The dispersed type was high during the photoperiod and comprised the lowest portion at the end of night. In contrast, punctate GI was low during the day and was at the highest level by the end of night. We further measured the change of the nuclear distribution of GI under short days (Figure S1). The proportions of the dispersed and punctate types were also anticorrelated, but the peaks and troughs were not clear as seen in LDs. Interestingly, the point at which each nuclear distribution type changed relative to the other was phase advanced in short days compared to LDs and correlated well in both conditions with same number of hours after the light-to-dark transition (Figure S1). These data imply that the subnuclear distribution of GI might be coupled to the light-dark cycles and may provide a further regulatory mechanism to understand the photoperiodism in *Arabidopsis*.

GI Directly Interacts with ELF4 at the Nuclear Bodies

Nuclear bodies are large protein complexes that contain proteins with similar functions (Lamond and Sleeman, 2003). We next asked which additional proteins that have functions related to GI might complex with it. Among known circadian clock components, we screened for potential GI interaction proteins using yeast two-hybrid assays and isolated EARLY FLOWERING 4 (ELF4) as a GI interaction partner (Figure S2A). Both GI and ELF4 have a similar circadian phase (David et al., 2006; Fowler et al., 1999; Khanna et al., 2003; Kikis et al., 2005; Kim et al., 2007; Kolmos et al., 2009; McWatters et al., 2007; Mizoguchi et al., 2005; Nusinow et al., 2011; Park et al., 1999; Sawa et al., 2007) and genetically interact to affect similar circadian outputs such as seedling growth, flowering time, and expression of clock genes in a phase-specific manner over a diurnal cycle (Kim et al., 2012). GI localizes both in the nucleus and cytoplasm (Kim et al., 2007), and we performed immunoblot analyses on cell fractions using *ELF4p::ELF4-HA* seedlings to establish its presence in both the nucleus and cytosol (Figure S2B). We next tested for potential interaction by transiently expressing GI and ELF4 in tobacco. Both proteins localized in the nucleus and the cytosol, which is consistent with previous reports (Kim et al., 2007) (Figure S2C). Additionally, in vivo coimmunoprecipitation (coIP) assays demonstrated both nuclear and cytosolic interactions (Figures 2A and S2C). We next used bifluorescence complementation (BiFC) assays to determine where subnuclear interactions occur and observed fluorescence at distinct nuclear bodies (Figure 2B). Additionally, GI-GFP and ELF4-RFP are able to form nuclear bodies independently and localize at the same bodies in which their interaction was observed (Figure 2C). Taken together, these data indicate that GI and ELF4 interact both in the nucleus and cytosol and within the same nuclear bodies.

ELF4 Regulates GI Nuclear Compartmentalization

Because GI and ELF4 interact within nuclear bodies, we tested whether they can regulate subnuclear localization of each other. *GI-GFP* and *ELF4-RFP* were transiently expressed in *elf4* and *gi-2* protoplasts, respectively, and their nuclear distribution patterns under LDs were observed. When we expressed *GI-GFP* in WT and *elf4* protoplasts, the proportion of punctate bodies was lower in the *elf4* background than in WT protoplasts, whereas the portion of the dispersed type was higher than in WT protoplasts at all time points tested (Figure 3A). This strongly increased proportion of the dispersed type and decreased proportion of the punctate type of GI nuclear

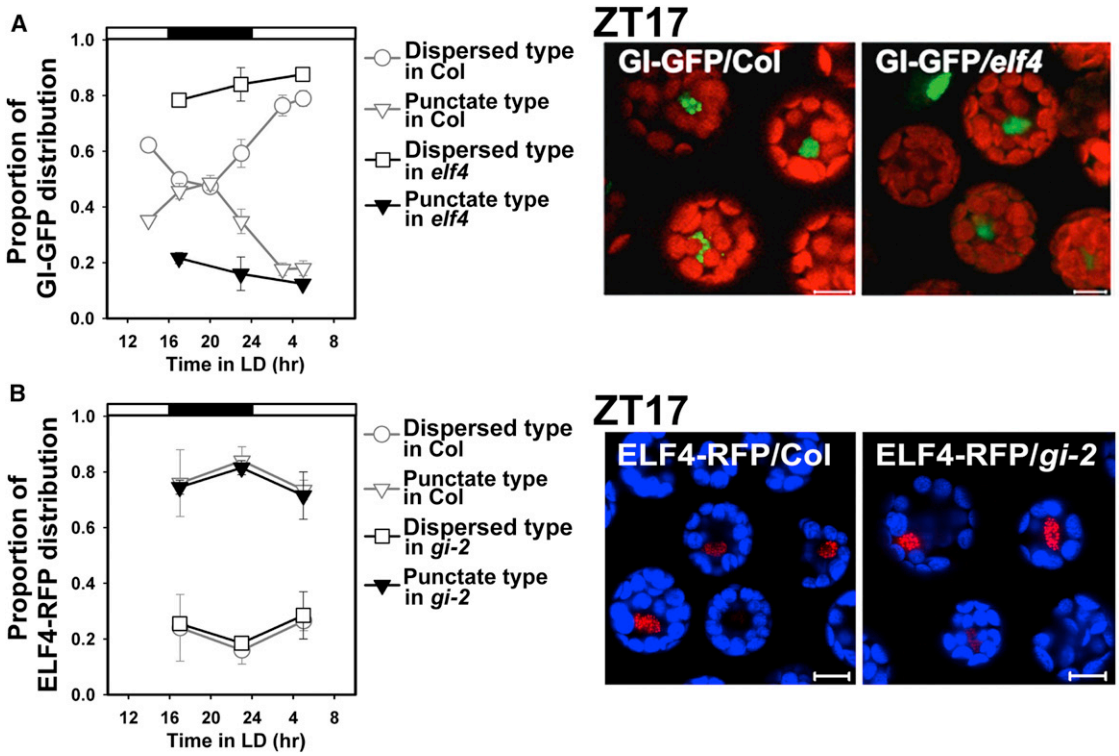

Figure 3. ELF4 Recruits GI to Nuclear Bodies

(A) Altered distribution of GI-GFP in *elf4* protoplasts. GI-GFP was expressed transiently in WT (Col) and *elf4* protoplasts, and the proportion of each distribution type of GI was observed (left). Typical image of subnuclear distributions of GI-GFP in WT and *elf4* protoplasts at ZT17 (right). Green indicates GI-GFP; red shows autofluorescence from chloroplasts.

(B) Subnuclear distribution of ELF4-RFP in WT and *gi-2* protoplasts. *ELF4-RFP* was transiently expressed in WT and *gi-2* protoplasts, and the proportion of each distribution type of ELF4 was observed (left). Typical image of subnuclear distributions of ELF4-RFP in WT and *gi-2* protoplasts at ZT17 (right). Red indicates ELF4-RFP; blue shows chloroplast autofluorescence. Data are mean ± SE from four biological replicates. Each dispersed or punctate form of GI-GFP and ELF4-RFP was counted and the relative proportion of each reported.

Scale bars represent 10 μm. See also Figure S3.

localization in *elf4*, especially at night, led to a dampening in the cyclic changes in the proportion of GI subnuclear distribution type (Figure 3A). We also measured the nuclear distribution of ELF4-RFP in WT and *gi-2* protoplasts. ELF4-RFP also displayed two prominent nuclear distribution types: dispersed, and punctate (Figure 3B). Approximately 80% of cells expressing ELF4-RFP formed nuclear bodies, and around 20% of these cells showed an even distribution of ELF4-RFP in WT nuclei with little change over a LD cycle. When we expressed ELF4-RFP in *gi-2* protoplasts, there was no significant change in the nuclear distribution of ELF4-RFP compared to those in WT protoplasts (Figure 3B).

We next expressed *GI-GFP* with *RFP* or *ELF4-RFP* in *elf4* protoplasts to test whether ELF4 could induce the localization of GI to nuclear bodies. The proportion of the dispersed type of GI-GFP when coexpressed with RFP was similar to the distribution pattern seen when GI-GFP was expressed alone in the *elf4* mutant (Figure S3). In contrast, GI-GFP coexpression with ELF4-RFP decreased the proportion of the dispersed type by about 20%, whereas the proportion of the GI-GFP punctate type increased (Figure S3). From these results, we conclude that the subnuclear localization of GI is strongly controlled by ELF4. These results suggest that ELF4 can modulate GI function by regulating the subnuclear localization of GI.

ELF4 Modulates the *CO* Promoter Binding Affinity of GI

We then asked what the functional significance of ELF4 control of GI localization might be. ELF4 is known to act upstream of GI in photoperiodic flowering time regulation (Kim et al., 2012), and transient translocalization of GI to the nucleus induces flowering (Gunl et al., 2009). GI protein is destabilized by a complex of ELF3 and COP1 (Yu et al., 2008), and nuclear GI can bind the promoter of *CO* and *FT* directly and regulate their expression (Sawa and Kay, 2011; Sawa et al., 2007). These previous reports led us to consider two possibilities: (1) ELF4 might regulate GI protein stability at the nuclear bodies within an ELF3/COP1 complex, and (2) ELF4 might regulate GI action as a transcriptional modulator of photoperiodic flowering integrators. To test these possibilities, we generated transgenic plants expressing *GI-GFP* under a constitutive promoter (*CsV::GI-GFP*) in an *elf4-209* background to avoid possible transcriptional regulation of *GI* by ELF4 because ELF4 is known to suppress *GI*

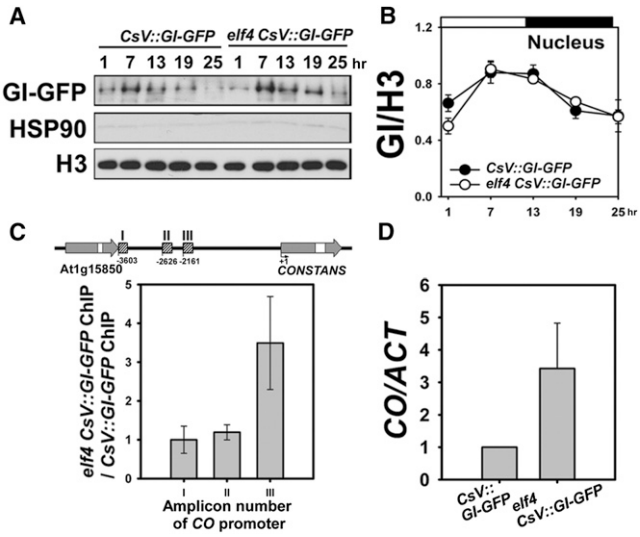

Figure 4. ELF4 Regulates Chromatin Accessibility of GI

(A and B) Nuclear GI-GFPs in *CsV::GI-GFP* and *elf4CsV::GI-GFP* under 12/12 hr light-dark cycles. GI proteins were detected by anti-GFP. HSP90 and H3 were used as cytosolic and nuclear markers, respectively. GI protein abundance was normalized to H3. Data are mean ± SE from four biological replicates.

(C) *CO* promoter binding affinity of GI in the *elf4* mutant relative to WT. Diagram for the *CO* amplicon in ChIP assay is indicated. Amplicons I, II, and III marked with hatched boxes correspond to amplicons 1, 3, and 4, respectively, as previously reported by Sawa et al. (2007). Ten-day-old seedlings were harvested 1 hr after lights on. The fold enrichment is calculated relative to *ACT2*.

(D) *CO* mRNA expression in *CsV::GI-GFP* and *elf4CsV::GI-GFP*. *CO* mRNA expression determined by quantitative RT-PCR with the same tissues used for ChIP. *CO* mRNA normalized relative to *ACT2*. Data are mean ± SE from six biological replicates.

See also Figure S4.

transcription (Kolmos et al., 2009). We first assayed the effects of the *elf4* mutation on GI protein stability in the nucleus to avoid possible complication of a cytosolic effect of ELF4 on overall GI abundance. Nuclear GI showed weak cycling under light-dark cycles, and GI nuclear protein levels were not significantly affected by the *elf4* mutation (Figures 4A and 4B). This result indicates that ELF4 has little effect on GI protein stability in the nucleus. Thus, the recruitment of GI to ELF4 nuclear bodies is unlikely to regulate GI protein abundance, and this complex seems to function differently from the GI/ELF3/COP1 complex, which affects GI protein levels (Yu et al., 2008). Cytosolic GI also showed very weak cycling under constitutive expression (Figure S4A), and cytosolic GI was decreased by the *elf4* mutation about 30% throughout the day, indicating that ELF4 stabilizes GI in the cytosol through an unknown mechanism (Figure S4A). These results additionally imply that ELF4 acts on GI differently in the nucleus and the cytoplasm.

We next tested whether ELF4 might affect the activity of GI as a transcriptional modulator of *CO*. We performed chromatin immunoprecipitation (ChIP) assays using *CsV::GI-GFP* and *elf4 CsV::GI-GFP* seedlings. Tissues were sampled at ZT1 (ZT: hours after lights on), corresponding to the time of the highest proportion of dispersed nuclear GI, both in WT and the *elf4* mutant (Figures 1G and 3A), and binding to amplicon III at the *CO* promoter was assayed (Figure 4C, diagram) (Sawa et al., 2007). When we compared *CO* promoter binding affinity of GI in these two backgrounds, GI-GFP was enriched 3-fold in the *elf4* mutant, relative to WT at amplicon III, whereas there were no significant differences at the control amplicons (Figure 4C). Consistent with this finding, *CO* expression level in *elf4* was significantly higher than WT (Figure 4D), and flowering time of *elf4 CsV::GI-GFP* plants was earlier than that of *CsV::GI-GFP* both in LDs and short days (Figure S4B). These results support the notion that ELF4 negatively regulates *CO* expression by sequestering GI from the *CO* promoter to specific ELF4-GI nuclear bodies.

Conclusions

Spatial regulation in cellular signaling is an important factor in understanding complex regulatory mechanisms (Herrero and Davis, 2012; Meier and Somers, 2011). Many circadian clock regulators can be localized at nuclear foci such as PRR5/TOC1 (Wang et al., 2010), ELF3/ELF4 (Herrero et al., 2012), ELF3/COP1/GI (Yu et al., 2008), and CKB4 (Portolés and Más, 2007). Diverse spatial regulations could, in principle, allow a single protein species to contribute to many signaling pathways. Specifically, subnuclear compartmentalization is characterized by a distinct set of resident proteins with dynamic changes in their size, shape, and number without the separation afforded by membranes (Lamond and Sleeman, 2003; Shaw and Brown, 2004). GI has been suggested to have differential roles in the nucleus and the cytosol (Gunl et al., 2009; Kim et al., 2007; Sawa and Kay, 2011; Sawa et al., 2007), and our results contribute to this notion by showing that GI forms a specific type of nuclear body into which it is sequestered by physical interaction with ELF4 from the *CO* promoter. GI nuclear body formation is induced by ELF4 under LDs (Figure 3), and this implies that the absolute levels of ELF4 protein can affect the proportion of GI nuclear body formation. Oscillation of ELF4 protein under diurnal conditions shows a broad peak at night that corresponds closely to that of GI (Figure S2B). This high expression of ELF4 at night may recruit GI to the nuclear bodies from the *CO* promoter, which is consistent with the cycling of GI subnuclear distributions (Figure 1F). However, *CO* transcript has a second peak at night (Suárez-López et al., 2001). This second *CO* peak does not correlate with GI/ELF4 nuclear body formation, and this discrepancy suggests that there are additional pathways to generate the *CO* waveform at night. Interestingly, ELF3/COP1/GI nuclear bodies have been suggested to form during darkness (Yu et al., 2008). Hence, there might be a competition between ELF4 and ELF3/COP1 to form a complex with GI. The ELF3/COP1 complex destabilizes GI proteins, which might act to shape the decreasing slope of the GI protein level during the night. In contrast, the GI/ELF4 nuclear body sequesters GI from the *CO* promoter, retaining GI within the nucleus for possible release in the morning, eliminating the need for de novo synthesis of GI. Taken together, the spatial regulation of GI with ELF4 in the nucleus can provide an additional mechanism that contributes to the currently known genetic and molecular interactions that control photoperiodic flowering.

EXPERIMENTAL PROCEDURES

Plant Materials and Transgenic Plants

The *gi-2* was reported previously (Park et al., 1999), and *elf4-209* (Kolmos et al., 2009) was backcrossed three times. *CsV::GI-GFP* was constructed using LR recombination (GATEWAY; Invitrogen, Carlsbad, CA, USA).

Preparation of Protoplasts

For transient expression in *Arabidopsis*, protoplasts were prepared from fourth, fifth, and sixth leaves of 1-month-grown Col-0 under LDs (16L/8D) or short days (8L/16D) at 22°C, as described previously (Kim et al., 2008). Transfected protoplasts were maintained and observed under the stated conditions of each experiment.

Vector Construction

The *CsV::GI-GFP*, *GI* cDNA clone was generated by PCR using the primers 5′-GGA TCC GAT GGC TAG TTC ATC-3′ and 5′-GAT GGA TCC TTG GGA CAA GGA TAT AGT ACA GCC GAG-3′, which resulted in removal of the termination codon. *GI* cDNA digested with EcoRI and BamHI was fused with *pCsV-eGFP-N-999* (Kim et al., 2008). For transient expression of *CsV::GI-GFP*, the gene cassette was moved to the pBlueSKII+ vector using NotI.

For *SCL28::mRFP*, *SCL28* cDNA was obtained by PCR using primers 5′-AGA GAA TTC ATG AGG GGA AGG AGC TAC-3′ and 5′-GAT GGA TCC TGC TTC TTC TAG GGC TGG-3′. *pCsV-mRFP-N-999* was generated by substitution of eGFP from *pCsV-eGFP-N-999* to monomeric RFP from pDsRed-Monomer-N1 (Clonetech Laboratories, Mountain View, CA, USA).

U2B::YFP was kindly donated by Drs. Yuda Fang and David L. Spector from Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, USA). The *CsVMV::ELF4-HA* vector was prepared by recombination of an entry clone that contained the *ELF4* cDNA and *pCsVMV-HA-1300*. The *ELF4-RFP* vector was also generated by recombination with *pCsVMV-HcRed1-999*.

To confirm the cellular localization of ELF4 in *planta*, we generated *ELF4p::ELF4-HA* plants. *ELF4p::ELF4* was amplified with primers 5′-CTT CTG CAG CTC ATG ATT TCC TGC GGTAAT-3′ and 5′-CTT AGG CCT AGC TCT AGT TCC GGC AGC A-3′ from *Arabidopsis* genomic DNA. This genomic gene cassette was moved to an entry vector and then transferred to an HA-tagged binary vector that has no promoter. The *ELF4p::ELF4-HA* clone was transformed into the *elf4-209* mutant.

Microscopic Analysis

Fluorescence images were obtained using an inverted confocal microscope (LSM5 *LIVE*; Carl Zeiss, Germany). Subcellular and subnuclear localizations of fluorescence fusion protein were observed with ×100 and ×63 oil-immersion apochromat objectives, respectively (Carl Zeiss). Images from each experiment were pseudocolored and plotted with LSM5 *LIVE* software.

Immunoblot Analysis and CoIP

Immunoblot analyses for GI, HSP90, and H3 protein detection were done as reported (Kim et al., 2007). Polyclonal HSP90 antibodies (rabbit) were made by W.Y.K. and were diluted 1:5,000 for detection. For cytosolic interaction between GI and ELF4, *CsV::GI-GFP* and *CsV::ELF4-HA* were transiently expressed in *N. benthamiana*, and immunoprecipitations were performed as reported (Wang et al., 2010).

Flowering Time Measurement/mRNA Expression

These experiments were performed as described (Kim et al., 2008).

ChIP

Chromatin binding affinity of GI on *CO* promoter was performed as reported by Sawa et al. (2007).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.021.

LICENSING INFORMATION

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