J. Plant Biol. (2017) 60:622-629
DOI 10.1007/s12374-017-0305-7

ORIGINAL ARTICLE

Production and Characterization of Polyclonal Antibody Against Arabidopsis GIGANTEA, a Circadian Clock Controlled Flowering Time Regulator

Laila Khaleda[1], Joon-Yung Cha[1,*], Min Gab Kim[2] and Woe-Yeon Kim[1,*]

[1]*Division of Applied Life Science (BK21Plus), Plant Molecular Biology and Biotechnology Research Center (PMBBRC), Research Institute of Life Sciences (RILS), Gyeongsang National University, Jinju 52828, Korea*
[2]*College of Pharmacy and Research Institute of Pharmaceutical Science, PMBBRC, Gyeongsang National University, Jinju 52828, Korea*

Received: August 8, 2017 / Accepted: September 21, 2017

Abstract Arabidopsis GIGANTEA (GI) is encoded by a single gene and highly conserved among vascular plants and its mutants display pleiotropic phenotypes involved in diverse biological processes such as light signaling, circadian clock, and sucrose metabolism as well as abiotic stress responses. However, molecular mechanisms of GI are largely unknown due to the lack of useful antibody. To date, the epitope tags have been widely used to detect GI in plants, but it needs to generate the transgenic plants which take a few months. Here, we produced polyclonal α-GI antibody using truncated variants of GI having amino-terminal (1-858 aa) and carboxyl-terminal (920-1173) regions as antigens. Both recombinant His-GI$^{1\text{-}858}$ and His-GI$^{920\text{-}1173}$ proteins were individually and successfully expressed in *E. coli* and immunized into rabbit. Anti-serum was purified by antigen-specific affinity purification method using both recombinant His-GI$^{1\text{-}858}$ and His-GI$^{920\text{-}1173}$ proteins. Purified polyclonal α-GI antibody not only detected endogenous GI proteins in wild-type Arabidopsis plants, but also reenacted its diel oscillations. Furthermore, the antibody showed cross-reactivity with the GI orthologs in other plants such as Chinese cabbage, rape and tomato. Our polyclonal GI antibody could help to determine the molecular mechanisms of GI involved in largely unknown pleiotropic responses in plants.

Keywords: Circadian clock; GIGANTEA; Polyclonal antibody; Truncation

*Corresponding author; Woe-Yeon Kim; Joon-Yung Cha
Tel : +82-55-772-1968; +82-55-772-1968
E-mail : kim1312@gnu.ac.kr; jycha@gnu.ac.kr

Introduction

Arabidopsis GIGANTEA (GI), encoded by a single gene and highly conserved among vascular plants, is controlled by circadian clock mediating photoperiodic flowering (Fowler et al. 1999; Park et al. 1999; Cha et al. 2017). GI interacts with and stabilizes the F-box protein ZEITLUPE (ZTL) playing as an evening-phased E3 ubiquitin ligase for targeted-proteasomal degradation of TIMING OF CAB EXPRESSION 1 (TOC1) and PSEUDO-RESPONSE REGULATOR 5 (PRR5), which are essential to clock function (Kim et al. 2007). GI also functions in diverse physiological processes including light signaling, hypocotyl elongation, sucrose signaling, starch accumulation, chlorophyll accumulation, transpiration, herbicide resistance, abiotic stress tolerance such as salt, cold and drought (Mishra and Panigrahi 2015). However, neither conserved domain nor molecular function of GI has been identified. Interestingly, several *gi* mutant alleles altering expression of *GI* transcripts displayed different flowering time and period length (Fowler et al. 1999; Park et al. 1999). Recent work revealed its biochemical function that large N-terminus (1-858 aa) of the GI recapitulates the activity of full-length GI for stabilizing ZTL protein as a chaperone. Furthermore it promotes the daily refolding of ZTL together with HEAT-SHOCK PROTEIN 90 (HSP90) as a co-chaperone whereas the C-terminus (920-1173 aa) of GI does not perform such fraction (Cha et al. 2017). In Arabidopsis, circadian rhythms are composed of transcriptional, translational and post-translational processes with feedback loops among morning- and evening-phased genes. GI diurnally oscillates both in transcriptional and translational levels with proteasome-dependent degradation under dark (David et al. 2006), and GI protein abundance is essential for regulating ZTL protein homeostasis in post-translational loop of Arabidopsis

circadian clock. Previous study also demonstrated that GI is degraded by salt in a proteasome-dependent manner which promotes activation of SOS (SALT-OVERLY SENSITIVE) pathway, suggesting that the absence of GI protein confers stress tolerance to salt (Kim et al. 2013). Although GI plays diverse pleiotropic functions in physiological and developmental processes as well as stress responses (Mishra and Panigrahi 2015), it has been difficult to explore the distinct molecular functions of GI due to the lack of specific antibody tools. Thus, the epitope-tags such as GFP, HA, and GFP fused to GI have been used to determine post-transcriptional modification of GI proteins regulated by environmental cues, but it takes several months to generate the transgenic plants. Here we expressed two recombinant truncated variants of GI having amino-terminal (1-858 aa) and carboxyl-terminal (920-1173 aa) region in *E. coli*, respectively. Using the mixture of two recombinant truncated GI variants as an antigen, polyclonal GI antibody was produced in rabbit and successfully reenacted the native full-length GI proteins in planta with diurnal oscillations in wild-type Arabidopsis plants. Our polyclonal GI antibody could help to determine the molecular mechanisms of GI involved in largely unknown pleiotropic responses.

Results and Discussion

Expression and Purification of Truncated His-tagged Recombinant GI$^{1-858}$ and GI$^{920-1173}$ Protein

Arabidopsis *GI* is a single gene encoding 1173 amino acids without any known conserved domain and is well conserved in vascular plants (Fowler et al. 1999; Mishra and Panigrahi 2015; Cha et al. 2017). His-tagged full-length GI had been expressed in *E. coli* and baculovirus, but the recombinant protein was poorly expressed and easily degraded with forming aggregates (Black et al. 2011). Our initial attempts to purify full-length GI were consistent having lower expression in *E. coli* regardless of affinity-tag such as GST, MBP and His-tag. Thus, we tried to express shorter-versions of GI to produce sufficient amounts of recombinant GI proteins. GI was randomly truncated with various lengths from amino- and carboxyl terminus, cloned into gRSET expression vector and expressed on a small-scale (3 mL) culture in *E. coli*. Among various truncated variants of GI, recombinant His-GI$^{1-858}$ (Fig. 1A) and His-GI$^{920-1173}$ (Fig. 1B) were expressed with significant quantities by induction of 1 mM IPTG while both proteins are not induced in the absence of IPTG. To identify the solubility of truncated His-GI proteins, cells expressing both proteins were disrupted by sonication followed by Triton X-100 treatment and centrifuged to separate soluble from insoluble (pellet) fractions. As shown in Fig. 1, both truncated GI proteins were abundantly expressed in insoluble fractions compared to soluble fractions. Expressed size of His-GI$^{1-858}$ and His-GI$^{920-1173}$ are estimated at approximately 105 kDa and 34 kDa, respectively. Previous works revealed that recombinant full-length His-GI was easily degraded to smaller fragments with approximately 90, 60 and 35 kDa wherein the bands at 90 kDa fragments represent the N-terminal GI peptides (Black et al. 2011). Together with our results, it indicates that GI$^{1-858}$ is a stable

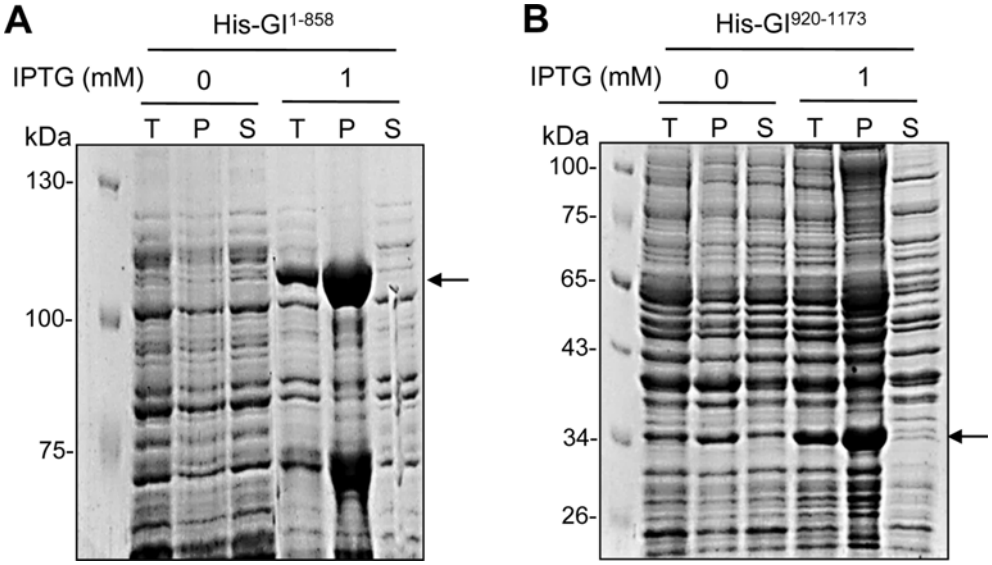

Fig. 1. Expression of recombinant truncated GI fragments in *E. coli.* Cells harboring the Arabidopsis *gRSET::GI$^{1-858}$* (A) and *gRSET::GI$^{920-1173}$* (B) were induced by 0 or 1 mM isopropyl-1-thio-β-$_D$-galactopyranoside (IPTG) in small scale culture (3 mL). Total extracts (T), pellet (P), and supernatant (S) fractions were separated in 6% (A) and 12% (B) SDS-PAGE. Molecular markers are represented on the left. Arrows show the expression of recombinant His-GI$^{1-858}$ (A) and His-GI$^{920-1173}$ (B).

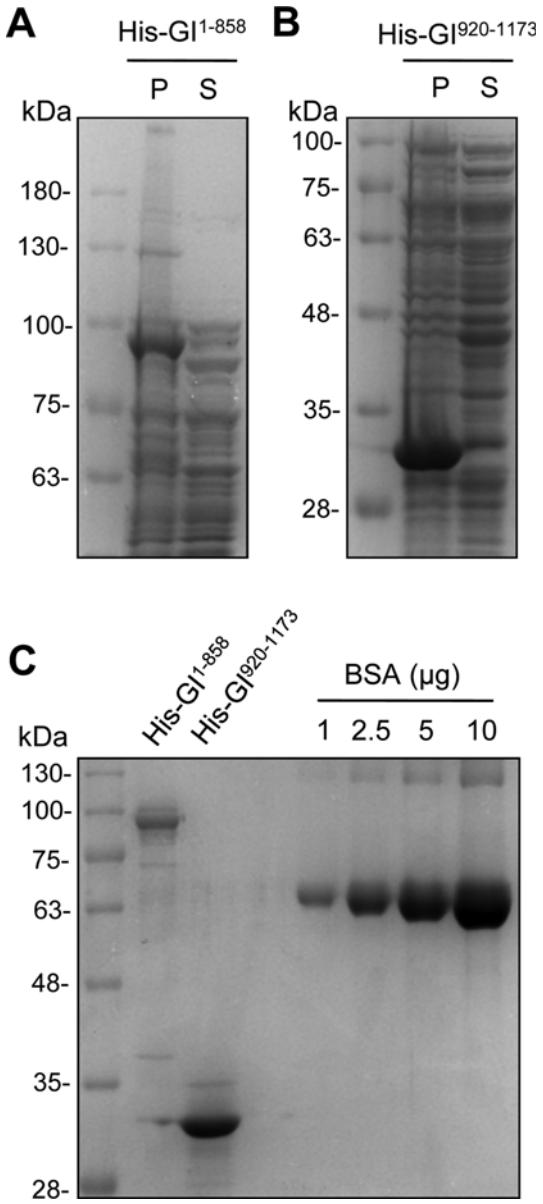

Fig. 2. SDS–PAGE of excised His-GI$^{1-858}$ and His-GI$^{920-1173}$. Cells expressing His-GI$^{1-858}$ (A) and His-GI$^{920-1173}$ (B) were cultured in a large scale (50 mL) and induced by 1 mM IPTG. Pellet (P) and supernatant (S) fractions were separated in 8% (A) and 12% (B) SDS-PAGE. (C) Excised His-GI$^{1-858}$ and His-GI$^{920-1173}$. Pellet fractions of recombinant proteins in A and B were re-run in SDS-PAGE and the bands corresponding His-GI$^{1-858}$ and His-GI$^{920-1173}$ were excised from gels. Both proteins were eluted from gels using the electro-eluter and run in 12% SDS-PAGE. BSA was used as a marker protein to estimate the protein concentrations.

N-terminal fragment of GI which is not further cleaved into smaller fragments and C-terminal GI$^{920-1173}$ fragment was also stable and well expressed in *E. coli*. To produce large amounts of recombinant truncated GI proteins for generating polyclonal antibody, the cells were cultured in a large-scale (50 mL). Proteins were extracted as same as the cultures in small-scale and confirmed His-GI$^{1-858}$ and His-GI$^{920-1173}$ mainly expressing in insoluble fractions which are consistent with Fig. 1 (Fig. 2A, 2B). Both insoluble fractions were re-run in SDS-PAGE individually and the parts corresponding to His-GI$^{1-858}$ or His-GI$^{920-1173}$ only were excised from the full gels. The proteins were eluted from gels and concentrated using the electro-eluter. As shown in Fig. 2C, the collected His-GI$^{1-858}$ and His-GI$^{920-1173}$ were confirmed and measured their concentrations using bovine serum albumin (BSA) with known concentrations. The estimated concentrations of His-GI$^{1-858}$ and His-GI$^{920-1173}$ were approximate 1 μg μL^{-1} and 3 μg μL^{-1}, respectively. Thus, we expressed sufficient amounts of recombinant truncated GI variants for generating polyclonal antibody.

Preparation of Antigens and Production of Polyclonal α-GI Antibody using Truncated GI$^{1-858}$ and GI$^{920-1173}$ Protein

Each truncated GI proteins was mixed to final concentration of 500 μg (250 μg His-GI$^{1-858}$ + 250 μg His-GI$^{920-1173}$) with Complete Freund's Adjuvant in a 1:1 (v/v) ratio and immunized into rabbit three times at intervals of 15 days. Anti-serum was collected from blood samples by centrifugation. To purify the polyclonal α-GI antibody from serum by the membrane-based antigen-specific affinity purification, the recombinant His-GI$^{1-858}$ and His-GI$^{920-1173}$ proteins were separated in SDS-PAGE, blotted onto PVDF membrane, and then the blots were incubated with anti-serum (Fig. 3A). Purified concentration of α-GI antibody was 0.38 mg mL^{-1}. Detailed procedures for antigen-specific affinity purification are described in Materials and Methods. Specificity of antibody was confirmed using native *GI* promoter driven GI-HA plant (*GI::GI-HA*), *CaMV 35S* promoter driven *HA-GI* overexpressing plant (*35S::HA-GI*) and T-DNA inserted *GI* knock-out plant (*gi-201*) harvested at zeitgeber time (ZT) 13 showing a peak of GI protein in long day condition (David et al. 2006; Kim et al. 2007; Martin-Tryon et al. 2007). Whole serum, post-binding serum after antigen-specific affinity purification and purified α-GI antibody with a dilution of 1:500 (antibody volume:5% skim milk volume) were used to detect GI proteins in Arabidopsis tissues. The GI proteins were clearly detected in *GI::GI-HA* and *35S::HA-GI* plants using purified α-GI antibody but not in *gi-201,* whereas the proteins were not detected by either whole serum or post-binding serum used as a primary antibody (Fig. 3B). HA-tag fused GI proteins migrated slower in *35S::HA-GI* plants compared to *GI::GI-HA* due to the difference of constructs. Immunoblot analysis using α-HA antibody also showed that the detected HA-tag fused GI proteins are consistent with the bands by α-GI antibody (Fig. 3B). To determine the recommended dilution, we have tried three different dilutions such as 1:250, 1:500 and 1:1000 (antibody:5% skim milk) using the plants mentioned above. In dilutions of 1:250 and

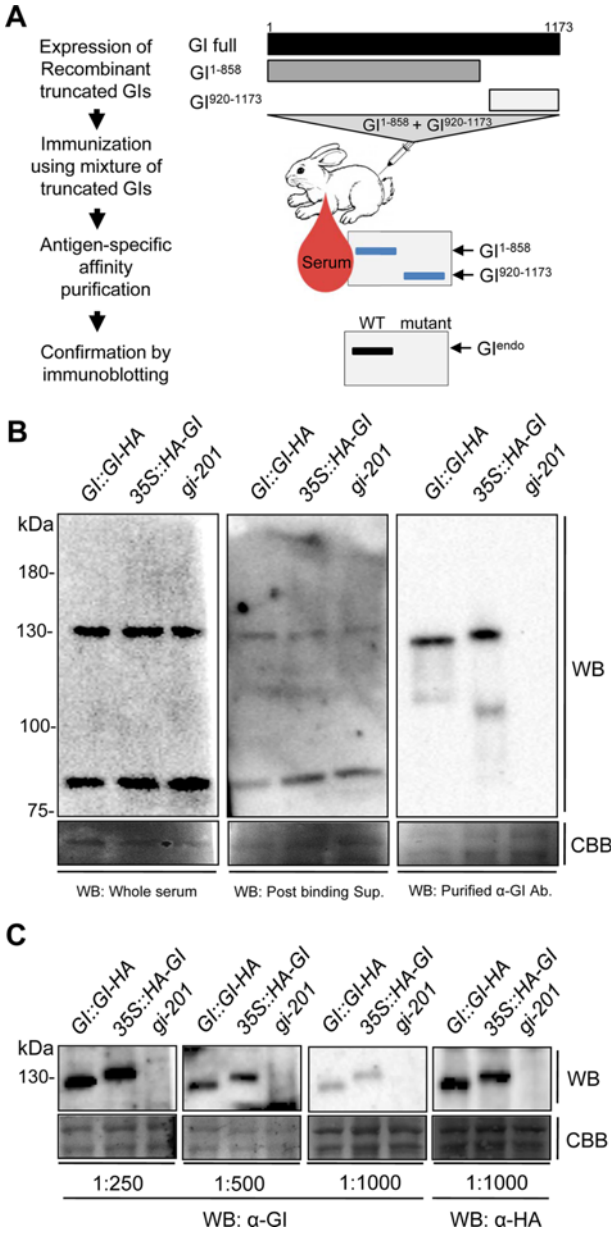

Fig. 3. Generation, confirmation and titration of purified polyclonal α-GI antibody. (A) Brief procedures for producing polyclonal α-GI antibody using truncated GI variants. Produced polyclonal α-GI antibody using His-GI$^{1-858}$ and His-GI$^{920-1173}$ in immunized rabbit were purified using antigen (His-GI$^{1-858}$ and His-GI$^{920-1173}$)-specific affinity purification method. (B) The specificity of whole serum, post binding supernatant after the purification, and purified α-GI antibody (1:500, antibody volume:skim milk volume) were used to detect GI proteins in plant tissues (two-week old *GI::GI-HA*, *35S::HA-GI* and *gi-201* plants) using western-blot analysis. CBB, coomassie brilliant blue-stained blot. (C) Purified α-GI antibody were titrated with 1:250, 1:500 and 1:1000 using same plant extracts shown in (A) and carried western-blot analysis. HA-tagged GI was detected by α-HA antibody (1:1000) using same plant extracts as a positive control. CBB, coomassie brilliant blue-stained blot.

1:500, GI proteins were strongly detected in both *GI* expressing plants (*GI::HA-GI* and *35S::HA-GI*), and the proteins were

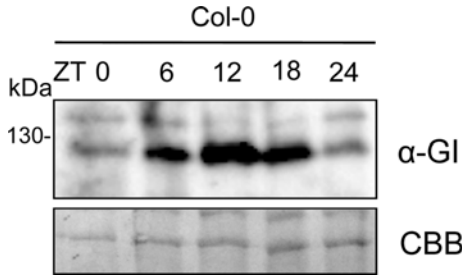

Fig. 4. Detection of oscillating endogenous GI protein in wild-type (Col-0) Arabidopsis plants. Two-week old wild-type (Col-0) Arabidopsis plants grown in 16 h light/8 h dark conditions were harvested at the indicated zeitgeber time (ZT) points. Proteins were separated in 6% SDS-PAGE and carried western blot analysis against α-GI antibody (1:500). CBB, comassie-brilliant blue-stained blot.

also visualized even in a dilution of 1:1000 with less background noise. Thus, we suppose to use our polyclonal α-GI antibody in a dilution of 1:500.

Characterization of Diel GI Protein Oscillations using Polyclonal α-GI Antibody

GI plays an essential role in photoperiodic flowering and is controlled by circadian clock both in transcriptional and translational levels (Fowler et al. 1999; Park et al. 1999; David et al. 2006). Both levels are regulated in a day-length dependent manner with peak in a day-time and trough during a night-time. Previous results demonstrated using *GI::GI-TAP* lines that *GI* mRNA is peaking at ZT9 and GI-TAP protein at ZT12 under long-day conditions (David et al. 2006). To identify the diel GI protein oscillations using our polyclonal α-GI antibody, wild-type (Col-0) Arabidopsis plants were grown in long-day conditions for 2 weeks and harvested from ZT 0 to ZT 24 with intervals of 6 h. Proteins were extracted in urea/SDS sample buffer, separated in 6% SDS-PAGE, and carried immunoblot analysis. As shown in Fig. 4, endogenous GI proteins in wild-type plants were clearly detected with strong oscillations and showed a peak at ZT 12 which is consistent with previous reports (David et al. 2006; Kim et al. 2007). It indicates that the diel oscillations of epitope-fused GI proteins are reenacted in wild-type plants using our polyclonal α-GI antibody which helps to reveal the unknown molecular mechanisms of GI in post-transcriptional levels. The increase of GI protein during day-time and the maximum abundance at the dusk is important to stabilize F-box protein ZTL triggering the proteasome-dependent degradation of TOC1 which is a core oscillator in circadian clock (Somers et al. 2000; Mas et al. 2003; Kim et al. 2007).

Cross-reactivity with GI Orthologs in Other Plants using Polyclonal α-GI Antibody

Next, we also tried to determine whether our α-GI antibody

have cross-reactivity with GI orthologs in other plants. Although the diel oscillations of GI protein have only been identified in Arabidopsis due to lack of useful α-GI antibody, those of *GI* transcripts were widely confirmed in various plant species such as *B. distachyon* (purple false brome), *B. rapa* (Chinese cabbage), *G. max* (soybean), *H. vulgare* (barley), *I. batatas* (sweet potato), *M. truncatula* (alfalfa), *O. sativa* (rice), *P. nil* (morning glory), *P. sativum* (pea), *P. alba* × *P. glandulosa* (poplar), *T. aestivum* (wheat) and *Z. mays* (corn) and regulated in a photoperiod-dependent manner (Hayama et al. 2002; Dunford et al. 2005; Zhao et al. 2005; Paltiel et al. 2006; Hecht et al. 2007; Hong et al. 2010; Higuchi et al. 2011; Bendix et al. 2013; Li et al. 2013; Kim et al. 2016; Ke et al. 2017; Tang et al. 2017). In Arabidopsis, the diel oscillation of GI protein is controlled by the expression of *GI* transcripts (Kim et al. 2007). To prove the diurnal oscillation of GI protein orthologs in other plant species, Arabidopsis, *B. rapa* (Chinese cabbage), *B. napus* (rape), *S. lycopersicum* (tomato), *Z. mays* (corn), and *F. arundinacea* (tall fescue) were grown in 12 h light/12 h dark for 2 (only for Arabidopsis) or 3 weeks and harvested at ZT 1 and 13. Aerial tissues were extracted in urea/SDS buffer and separated in 6% SDS-PAGE. Using polyclonal α-GI antibody, endogenous GI orthologs were detected in a size of around 127 kDa and the protein expressions in all plants examined were increased at ZT 13 compared to those at ZT 1, indicating that the protein expression of GI orthologs in other plants are also controlled by circadian clock like as Arabidopsis GI (Fig. 5). The amino acid sequences of GI orthologs in Chinese cabbage, corn and tomato have been identified and showed high sequence identity and similar molecular masses with Arabidopsis GI (Cha et al. 2017). Interestingly, amino acid sequences between 817 and 864 (based on Arabidopsis GI) were not well conserved among known GI orthologs in plants, whereas the others are highly conserved. This region having the sequence diversity among plants is close to the region for constructing our truncated variants of GI (1-858 and 920-1173). It may suggest that the $GI^{817-864}$ is the region which can be easily degraded in order to form a stable approximately 90 kDa N-terminal fragment of GI which is consistent with our GI^{1-858} and the previous report (Black et al. 2011). Although GI orthologs have not been identified in rape and tall fescue plants at present, our polyclonal α-GI antibody detected putative GI proteins with a similar size and a peak at ZT 13 similar to Arabidopsis GI (Fig. 5). To date, GI orthologs have not been found in prokaryotes, fungi, or animals. In the history of plant evolution, green algae is an ancestor of plants, but *GI* is absent in the green unicellular alga *Ostreococcus tauri*, *Chlamydomonas reinhardtii* and *Klebsormidium flaccidum* unlike *TOC1* and *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) which were known to be conserved (Corellou et al. 2009; Linde et al. 2017). Interestingly, *GI* is present in the earliest land plants, the bryophytes such as liverwort *Marchantia polymorpha* and hornwort *Anthoceros agrestis* (Kubota et al. 2014; Linde et al. 2017). Absence of *GI* in *M. polymorpha* abolished the long-day-dependent growth-phase transition and introduction of *M. polymorpha GI* ortholog into Arabidopsis *gi-2* mutant rescues the late-flowering phenotype (Kubota et al. 2014). In addition, the suppression of *GI* ortholog in *B. rapa* exhibits late-flowering, perturbation of circadian rhythms, increased freezing and salt tolerance, which are consistent with the phenotypes of Arabidopsis *gi* mutants, and complementation of *BrGI*, *BdGI*, *IbGI*, *PagGI* and *ZmGI* rescued the late-

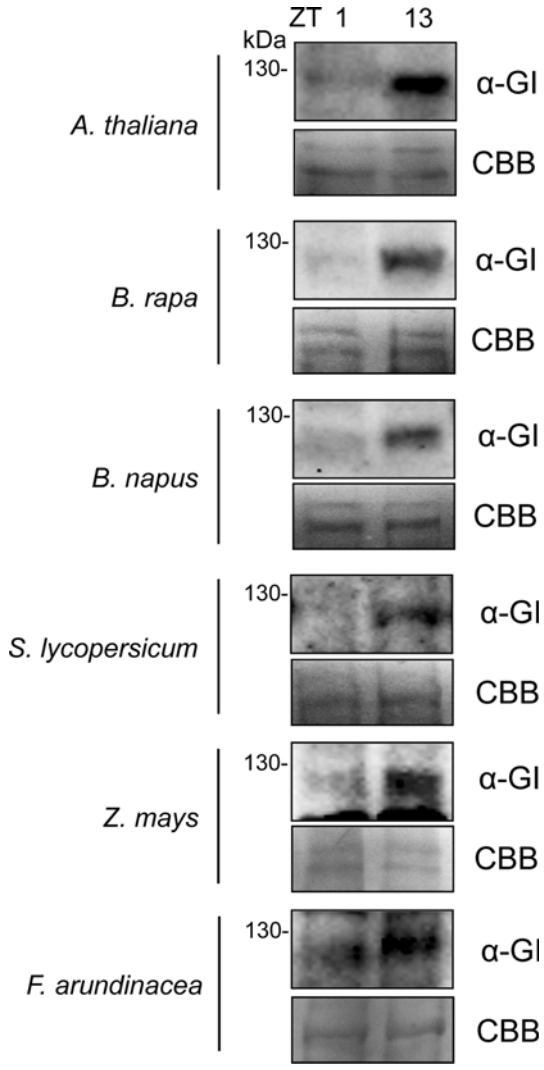

Fig. 5. Cross-activity of Arabidopsis α-GI antibody in other plants. Two-week old wild-type *Arabidopsis thaliana* (Col-0) and three-week old *Brassica rapa* (Chinese cabbage), *Brassica napus* (rape), *Solanum lycopersicum* (tomato), *Zea mays* (corn) and *Festuca arundinacea* (tall fescue) were harvested at ZT 1 and 13 and extracted in urea/SDS buffer. Proteins were separated in 6% SDS-PAGE and carried western blot analysis using α-GI antibody (1:500). CBB, comassie-brilliant blue stained blot.

flowering of Arabidopsis *gi-2* or *gi-201* mutant (Hong et al. 2010; Bendix et al. 2013; Mishra and Panigrahi 2015; Xie et al. 2015; Kim et al. 2016; Ke et al. 2017; Tang et al. 2017). Collectively, these suggest that *GI* has been evolved alongside with the emergence of land plants and has a functional redundancy among *GI* orthologs.

Applications for Immunoprecipitation Assay using Polyclonal α-GI Antibody

Antibody has been widely used to determine the post-transcriptional regulations, protein-protein interaction (for immunoprecipitation) and protein-DNA interaction (chromatin immunoprecipitation). As mentioned above, our polyclonal α-GI antibody detected the endogenous GI proteins in diverse plant species, and thus it helps to determine unknown molecular mechanisms of GI in post-transcriptional levels. Next we also tried to determine whether our α-GI antibody could be used for immunoprecipitation assays. Wild-type (Col-0) and *GI*-overexpressing (*35S::HA-GI*) plants grown in long-day conditions for 2 weeks were harvested at ZT 13 in the extraction buffer and extracts were mixed with protein-A agarose beads at 4°C for 3 h following to primary binding with α-GI antibody (4°C for 1 h). After washing, input and immunoprecipitated proteins probed by α-GI antibody were separated in 6% SDS-PAGE and detected by α-GI antibody. As shown in Fig. 6A, endogenous GI and HA-GI proteins in wild-type (Col-0) and *35S::HA-GI* plants, respectively, were immunoprecipitated by our α-GI antibody. Next, we examined co-immunoprecipitation (Co-IP) assay by co-infiltration of Agrobacterium cells transiently expressing *WT ZTL* with or without *GI-HA* constructs in tobacco epidermal cells. The leaf tissues were harvested at ZT13 of three days after the infiltration. Polyclonal α-GI antibody was conjugated with protein-A agarose beads at 4°C for 1 h, and then incubated with total extracts isolated from tobacco tissues at 4°C for 3 h. Co-IP samples were separated in SDS-PAGE and immunoblot analysis was carried using α-GI antibody and α-ZTL antibody to detect GI-HA and ZTL protein, respectively. Results showed that ZTL protein was co-immunoprecipitated with GI-HA bound α-GI antibody conjugates (Fig. 6B). Thus, our α-GI antibody can apply not only for immunoblot analysis but also for immunoprecipitation assay including Co-IP and chromatin IP.

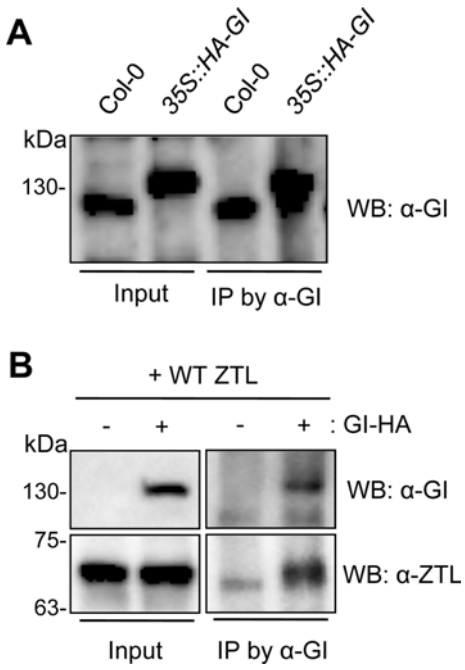

Fig. 6. Immunoprecipitation (IP) and co-immunoprecipitation (Co-IP) using purified α-GI antibody. (A) Primary binding capacity of purified α-GI antibody for immunoprecipitation (IP). Two-week old Arabidopsis plants (Col-0 wild-type and *35S::HA-GI* harvested at ZT13) were extracted in the buffer (input) and mixed with polyclonal α-GI antibody-bound protein A-agarose beads (IP). Proteins were separated in 6% SDS-PAGE, and carried western blot analysis against α-GI antibody (1:500). (B) *In planta* interactions between GI and ZTL by co-immunoprecipitation (Co-IP). Protein extracts from tobacco leaves transiently expressing full-length *ZTL* (*WT ZTL)* with or without *GI-HA* constructs were incubated with the protein-A agarose beads conjugated polyclonal α-GI antibody. Input and co-IP samples were separated in SDS-PAGE and immunoblot analysis was carried using α-GI antibody and α-ZTL antibody to detect GI-HA and ZTL protein, respectively.

Conclusions

GI is a circadian clock-controlled large single gene involved in diverse physiological processes as well as stress responses with transcriptional and post-transcriptional regulation. Proteostasis of GI is controlled by proteasomal degradation of GI in a dark- or salt-dependent manner and stabilization by SUMOylation under heat stress (David et al. 2006; Kim et al. 2013; López-Torrejón et al. 2013). However, full-length GI protein is rarely expressed in *E. coli* and easily degraded causing lack of useful α-GI antibody. Here, we respectively expressed two truncated variants of GI (1-858 and 920-1173 aa) with a His-tag and His-GI^{1-858} and His-$GI^{920-1173}$ proteins were used as an antigen to immunize into rabbit. Anti-serum was purified using antigen (His-GI^{1-858} and His-$GI^{920-1173}$ proteins)-specific affinity purification. Purified polyclonal α-GI antibody successfully detected the endogenous GI protein in wild-type Arabidopsis plants as well as HA-tag fused GI expressing plants. In addition, our α-GI antibody has cross-reactivity with GI orthologs in Chinese cabbage, rape, tomato, soybean, corn and tall fescue and furthermore its conjugates with Protein-A agarose beads precipitated endogenous GI proteins together with ZTL. Collectively, our polyclonal α-GI antibody is powerful to

determine unknown molecular mechanisms of GI involved in a wide-range of plant responses.

Materials and Methods

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* plants used were Columbia-0 (as a wild-type), HA-tagged *GI::GI-HA* (*gi-2* background), *35S:HA-GI* (*gi-2* background) and the *gi-201* mutant (Col-0 background) (David et al. 2006; Kim et al. 2007; Martin-Tryon et al. 2007). Sterilized seeds were prepared on MS agar media and grown in growth chamber under long-day condition (16 h light/8 h dark) with a fluorescence rate of 100 to 120 μmol m^{-2} s^{-1} of white light at 23°C. For cross reactivity of antibody, seeds of the Arabidopsis (Col-0), Chinese cabbage (*Brassica rapa* L.), rape (*Brassica napus* L.), tomato (*Lycopersicon lycopersicum* L.), corn (*Zea mays* L.) and tall fescue (*Festuca arundinacea* Schreb.) were surface-sterilized, prepared on MS, B5 agar media (for tomato) or soil (for corn and tall fescue) and grown in growth chamber under 12 h light/12 h dark condition at 23°C.

Construction of Truncated GI cDNAs and Expression of Recombinant Proteins

Construction of truncated GI variants was described previously (Cha et al. 2017). Briefly, 1-858 aa (GI^{1-858}) and 920-1173 aa ($GI^{920-1173}$) GI were cloned into the donor vector (pDONR-zeo) and subsequently moved into the gRSETA vector having 6xHis tag (His-) using the recombination-based Gateway cloning system (Invitrogen), according to the manufacturer's instructions. The plasmids were transformed into *E. coli* BL21 (DE3) pLysS for recombinant protein expression and the transformed cells were grown at 37°C (OD600 = 0.8) and induced by 1 mM isoprophyl-1-thio-β-$_D$-galactopyranoside (IPTG) for 3 h at 30°C. After harvest by centrifugation (6,000 rpm/10 min), cells were resuspended in 1xPBS and frozen at -20°C until use.

Preparation of Antigens and Generation of Polyclonal Antibody in Rabbit

Frozen cells were thawed and incubated for 20 min with 1% (v/v) Triton X-100 before disruption by sonication. Soluble and insoluble fractions of His-GI^{1-858} and His-$GI^{920-1173}$ were divided by centrifugation at 12,000 rpm, 4°C for 10 min and confirmed in 12% SDS-PAGE. Protein bands expressing His-GI^{1-858} and His-$GI^{920-1173}$ in insoluble fraction were excised, and proteins were collected using Electro-Eluter (Bio-Rad). Proteins (500 μg) were mixed with Complete Freund's Adjuvant in a 1:1 (v/v) ratio and the antigen was injected into rabbit with triple immunization. Blood samples were collected from immunized rabbit and centrifuged at 1,000 x *g* for 10 min at 4°C and the Anti-serum was purified using antigen-specific affinity purification with recombinant His-GI^{1-858} and His-$GI^{920-1173}$. Animals were housed in accordance with the ethical principles and experimental procedures to minimize animal suffering and followed a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Gyeongsang National University (GNU-151006-B0058). Insoluble His-GI^{1-858} and His-$GI^{920-1173}$ separated in 12% SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane and the membranes were only excised the portion of His-GI^{1-858} and His-$GI^{920-1173}$ to reduce the non-specific bindings. The blots were blocked with 1% (w/v) bovine serum albumin (BSA) in 1x TBS buffer and subsequently mixed with the antiserum (diluted 1 mL antiserum in 9 mL 1% BSA) overnight at 4°C. Blot strips were washed three times with 1x TBS, sliced into 1 mm (for helping antibody elution from blot strips), and bound polyclonal α-GI antibody was eluted with 900 μL 0.1 M glycine (pH 2.5) and immediately neutralized with 100 μL 2 M Tris-HCl (pH 8.0). The concentration of purified α-GI antibody was 0.38 mg mL^{-1}, and purified antibody was stored at -20°C until use.

Western Blot Analysis using Purified α-GI Antibody

Total proteins from plant tissues were extracted in urea/SDS buffer containing 8 M urea, 5% (w/v) SDS, 100 mM Tris-HCl (pH 6.8), 1 mM EDTA, 2% (v/v) β-mercaptoethanol, 0.00125% (w/v) bromophenol blue, and protease/phosphatase/proteasome inhibitors (1 mM PMSF, 5 μg mL^{-1} leupeptin, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} pepstatin, 5 μg mL^{-1} antipain, 5 μg mL^{-1} chymostatin, 2 mM Na_2VO_3, 2 mM NaF and 50 μM MG132). After centrifugation (12,000 rpm/10 min/4°C), supernatants were separated in 6% SDS-PAGE and transferred onto PVDF membrane. Immunoblot analysis was carried using rabbit α-GI antibody and GI protein was detected by chemiluminescence using an ECL-detecting reagent (Thermo).

Immunoprecipitation and Co-immunoprecipitation

Two-week-old Arabidopsis (Col-0 and *35S::HA-GI*) plants were extracted in the extraction buffer containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% (v/v) NP-40, 1 mM EDTA, 3 mM DTT, and protease/phosphatase/proteasome inhibitors as mentioned above and the supernatants were obtained after centrifugation (12,000 rpm/5 min/4°C). Rabbit α-GI antibody was incubated with Protein-A agarose (Sigma) for 1 h at 4°C and subsequently mixed with the supernatants (also save for an Input) for 3 h at 4°C. Beads were washed with the extraction buffer (without the inhibitors) three times and resuspended in cold 1xPBS. Input and immunoprecipitated proteins were mixed with the loading buffer and separated in 6% SDS-PAGE. Immunoblot analysis was carried as described above. GI-ZTL interaction was confirmed by co-immunoprecipitation (co-IP) assay. The constructs of *WT ZTL* and *GI-HA* were described previously (Kim et al. 2007; Kim et al. 2013). Agrobacterium cells expressing *WT ZTL* with or without *GI-HA* constructs were co-infiltrated in tobacco (*N. benthamiana*) epidermal cells. Tobacco leaves were harvested at ZT13 of three days after the infiltration. Protein extracts were isolated from tobacco leaf tissues using the extraction buffer as mentioned above. Polyclonal α-GI antibody was conjugated with protein-A agarose beads at 4°C for 1 h, and subsequently incubated with protein extracts at 4°C for 3 h. Input and co-IP samples were separated in SDS-PAGE and immunoblot analysis was carried using α-GI antibody and α-ZTL antibody to detect GI-HA and ZTL protein, respectively.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education (NRF-2015R1D1A1A02061979) and Next-Generation BioGreen21 Program (SSAC, PJ01106901), Rural Development Administration, Republic of Korea.

Author's Contributions

JYC and WYK designed the project and LK and JYC performed the experiments. LK, JYC, MGK and WYK analyzed the data and wrote

the paper. All authors discussed the results and approved the manuscript.

References

Black MM, Stockum C, Dickson JM, Putterill J, Arcus VL (2011) Expression, purification and characterization of GIGANTEA: a circadian clock-controlled regulator of photoperiodic flowering in plants. Protein Expr Purif 76:197–204

Bendix C, Mendoza JM, Stanley DN, Meeley R, Harmon FG (2013) The circadian clock-associated gene *gigantea1* affects maize developmental transitions. Plant Cell Environ 34:1379–1390

Cha JY, Kim J, Kim TS, Zeng Q, Wang L, Lee SY, Kim WY, Somers DE (2017) GIGANTEA is a co-chaperone which facilitates maturation of ZEITLUPE in the Arabidopsis circadian clock. Nature Commun 8:3

Corellou F, Schwartz C, Motta JP, Djouani-Tahri el B, Sanchez F, Bouget FY (2009) Clocks in the green lineage: Comparative functional analysis of the circadian architecture of the picoeukaryote *Ostreococcus*. Plant Cell 21:3436–3449

David KM, Armbruster U, Tama N, Putterill J (2006) Arabidopsis GIGANTEA protein is post-transcriptionally regulated by light and dark. FEBS Lett 580:1193–1197

Dunford RP, Griffiths S, Christodoulou V, Laurie DA (2005) Characterization of a barley (*Hordeum vulgare* L.) homologue of the Arabidopsis flowering time regulator *GIGANTEA*. Theor Appl Genet 110:925–931

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J (1999) *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J 18:4679–4688

Hayama R, Izawa T, Shimamoto K (2002) Isolation of rice genes possibly involved in the photoperiodic control of flowering by a fluorescent differential display method. Plant Cell Physiol 43:494–504

Hecht V, Knowles CL, Vander Schoor JK, Liew LC, Jones SE, Lambert MJM, Weller JL (2007) Pea *LATE BLOOMER1* is a *GIGANTEA* ortholog with roles in photoperiodic flowering, deetiolation, and transcriptional regulation of circadian clock gene homologs. Plant Physiol 144:648–661

Higuchi Y, Sage-Ono K, Sasaki R, Ohtsuki N, Hoshino A, Iida S, Kamada H, Ono M (2011) Constitutive expression of the *GIGANTEA* ortholog affects circadian rhythms and suppresses one-shot induction of flowering in *Pharbitis nil*, a typical short-day plant. Plant Cell Physiol 52:638–650

Hong SY, Lee S, Seo PJ, Yang MS, Park CM (2010) Identification and molecular characterization of a *Brachypodium distachyon GIGANTEA* gene: functional conservation in monocot and dicot plants. Plant Mol Biol 72:485–497

Ke Q, Kim HS, Wang Z, Ji CY, Jeong JC, Lee HS, Choi YI, Xu B, Deng X, Yun DJ, Kwak SS (2017) Down-regulation of *GIGANTEA-like* genes increases plant growth and salt stress tolerance in poplar. Plant Biotechnol J 15:331–343

Kim JA, Jung H, Hong JK, Hermand V, McClung CR, Lee YH, Kim JY, Lee SI, Jeong MJ, Kim J, Yum DJ, Kim WY (2016) Reduction of *GIGANTEA* expression in transgenic *Brassica rapa* enhances salt tolerance. Plant Cell Rep 35:1943–1954

Kim WY, Ali Z, Park HJ, Park SJ, Cha JY, Perez-Hormaeche J, Quintero FJ, Shin G, Kim MR, Qiang Z, Ning L, Park HC, Lee SY, Bressan RA, Pardo JM, Bohnert HJ, Yun DJ (2013) Release of SOS2 kinase from sequestration with GIGANTEA determines salt tolerance in Arabidopsis. Nature Commun 4:1352

Kim WY, Fujiwara S, Suh SS, Kim J, Kim Y, Han L, David K, Putterill J, Nam HG, Somers DE (2007) ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. Nature 449:356–360

Kubota A, Kita S, Ishizaki K, Nishihama R, Yamato KT, Kohchi T (2014) Co-option of a photoperiodic growth-phase transition system during land plant evolution. Nature Commun 5:3668

Li F, Zhang X, Hu R, Wu F, Ma J, Meng Y, Fu YF (2013) Identification and molecular characterization of *FKF1* and *GI* homologous genes in soybean. PLoS ONE 8:e79036

Linde AM, Eklund DM, Kubota A, Pederson ERA, Holm K, Gyllenstrand N, Nishihama R, Cronberg N, Muranaka T, Oyama T, Kohchi T, Lagercrantz U (2017) Early evolution of the land plant circadian clock. New Phytol doi: 10.1111/nph.14487

López-Torrejón G, Guerra D, Catalá R, Salinas J, del Pozo JC (2013) Identification of SUMO targets by a novel proteomic approach in plants. J Integr Plant Biol 55:96–107

Martin-Tryon EL, Kreps JA, Harmer SL (2007) *GIGANTEA* acts in blue light signaling and has biochemically separable roles in circadian clock and flowering time regulation. Plant Physiol 143:473–486

Mas P, Kim WY, Somers DE, Kay SA (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature 426:567–570

Mishra P, Panigrahi KC (2015) GIGANTEA-an emerging story. Front Plant Sci 6:8

Paltiel J, Amin R, Gover A, Ori N, Smach A (2006) Novel roles for GIGANTEA revealed under environmental conditions that modify its expression in Arabidopsis and *Medicago truncatula*. Planta 224:1255–1268

Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG (1999) Control of circadian rhythms and photoperiodic flowering by the Arabidopsis *GIGANTEA* gene. Science 285:1579–1582

Somers DE, Schultz TF, Milnamow M, Kay SA (2000) *ZEITLUPE* encodes a novel clock-associated PAS protein from Arabidopsis. Cell 101:319–329

Tang W, Yan H, Su ZX, Park SC, Liu YJ, Zhang YG, Wang X, Kou M, Ma DF, Kwan SS, Li Q (2017) Cloning and characterization of a novel *GIGANTEA* gene in sweet potato. Plant Physiol Biochem 116:27–35

Xie Q, Lou P, Hermand V, Aman R, Park HJ, Yun DJ, Kim WY, Salmela MJ, Ewers BE, Weinig C, Khan SL, Schaible DLP, McClung CR (2015) Allelic polymorphism of *GIGANTEA* is responsible for naturally occurring variation in circadian period in *Brassica rapa*. Proc Natl Acad Sci USA 112:3829–3834

Zhao XY, Liu MS, Li JR, Guan CM, Zhang XS (2005) The wheat *TaGI1*, involved in photoperiodic flowering, encodes an Arabidopsis *GI* ortholog. Plant Mol Biol 58:53–64