Down-regulation of GIGANTEA-like genes increases plant growth and salt stress tolerance in poplar

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Received 23 December 2015; accepted 21 August 2016.
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Keywords: poplar, Arabidopsis, PagGI, salt tolerance, RNAi, transgenic plants.

Summary

The flowering time regulator GIGANTEA (GI) connects networks involved in developmental stage transitions and environmental stress responses in Arabidopsis. However, little is known about the role of GI in growth, development and responses to environmental challenges in the perennial plant poplar. Here, we identified and functionally characterized three GI-like genes (PagGia, PagGib and PagGic) from poplar (Populus alba × Populus glandulosa). PagGIs are predominantly nuclear localized and their transcripts are rhythmically expressed, with a peak around zeitgeber time 12 under long-day conditions. Overexpressing PagGIs in wild-type (WT) Arabidopsis induced early flowering and salt sensitivity, while overexpressing PagGIs in the gi-2 mutant completely or partially rescued its delayed flowering and enhanced salt tolerance phenotypes. Furthermore, the PagGIs-PagSOS2 complexes inhibited PagSOS2-regulated phosphorylation of PagSOS1 under salt stress. Down-regulation of PagGIs by RNA interference led to vigorous growth, higher biomass and enhanced salt stress tolerance in transgenic poplar plants. Taken together, these results indicate that several functions of Arabidopsis GI are conserved in its poplar orthologues, and they lay the foundation for developing new approaches to producing salt-tolerant trees for sustainable development on marginal lands worldwide.

Introduction

The worldwide population is expected to reach between 9.6 and 12.3 billion by 2100, which presents challenges to energy security, economic growth and environmental protection (Gerrard et al., 2014). Environmental problems such as global warming, drought and salinity severely limit agricultural and forest productivity (Lobell and Gourdji, 2012; Sivakumar et al., 2005). There is an urgent need for plant breeders to develop novel plant varieties with increased growth and tolerance to various environmental stresses. Recent studies have revealed that genetic modification of flowering time, or circadian rhythm, represents a useful new strategy for crop and woody plant improvement (Grundy et al., 2015; Jung and Müller, 2009; Ni et al., 2009).

In the annual plant Arabidopsis, GIGANTEA (GI) was originally identified based on its roles in photoperiodic flowering and circadian clock regulation (Fowler et al., 1999; Martin-Tryon et al., 2007). First, the GI-CONSTANS (CO)-Flowering Locus T (FT) regulatory module controls flowering time under long-day conditions (Takada and Goto, 2003). GI and the FLAVIN-BING, KELCH REPEAT, F-BOX 1 protein form a complex that controls daytime CO transcription in a light-dependent manner by degrading a key CO repressor, CYCLING DOF FACTOR 1 (Sawa et al., 2007). Second, GI interacts with F-box protein ZEITLUPE (ZTL) through the amino-terminal flavin-binding LIGHT, OXYGEN or VOLTAGE domain of ZTL, which is necessary to sustain a normal circadian period by regulating the proteasome-dependent degradation of the central circadian oscillator, TIMING OF CAB EXPRESSION 1 (Kim et al., 2007). Recent findings indicate that GI also mediates responses to environmental stress. Among abiotic stress factors, salinity affects almost all aspects of plant development, including germination, vegetative growth and reproductive development, which threatens crop production on over 800 million hectares, or one-quarter to one-third of all agricultural land on earth (Rengasamy, 2010; Zhu, 2002). The salt stress response is controlled by many genes that function through complex genetic regulatory networks. One of the key responses to salt stress is maintaining cellular ion homoeostasis by restricting the accumulation of toxic sodium (Na+). The Salt Overly Sensitive (SOS) signalling pathway is a well-defined signalling pathway required for the control of ion homeostasis (Zhu, 2002). The SOS pathway modulates Na+ levels via three known components: calcium-binding protein SOS3, protein kinase SOS2 and plasma membrane Na+/H+ antiporter SOS1. Under salt stress conditions, the SOS2–SOS3 complex phosphorylates and activates the transport activity of the SOS1 antiporter (Guo et al., 2001; Ji et al., 2013; Zhu, 2000). The SOS pathway is functionally conserved in dicot plants Arabidopsis and Brassica nigra, the monocot plant rice, and the woody plant poplar (Tang et al., 2010; Yang et al., 2015). The role of GI in the salt stress response was recently documented. GI competitively binds to SOS2 kinase and prevents the phosphorylation-dependent activation of SOS1 under normal conditions. However, in the presence of high salt, GI is degraded by the 26S proteasome. Released SOS2 interacts with SOS3 to form an active SOS2–SOS3 protein kinase.
provides the first evidence that altering the expression of genes through genetic engineering could be used to develop expression of PagGIs like overexpressing the poplar Vinocur and Altman, 2005). Recently, transgenic poplar plants associated genes from other species (Harfouche et al., 2011; Janssen and Douglas, 2007). Most poplar cultivars are extremely sensitive to saline soils (Chen et al., 2014; Polle and Chen, 2014). Increasing the salt tolerance in crops and woody plants has become a major challenge for modern agriculture. Although much effort has focused on increasing salt tolerance in poplar through conventional breeding and genetic engineering, few studies have successfully produced transgenic poplar plants with enhanced salt tolerance using known abiotic stress-associated genes from other species (Harfouche et al., 2011; Vinocur and Altman, 2005). Recently, transgenic poplar plants overexpressing the poplar SOS2 gene were generated, which exhibit enhanced tolerance to salt stress (Yang et al., 2015). Since SOS2 is regulated by the photoperiodicity and circadian clock switch GI (Kim et al., 2013a), unravelling the mechanisms underlying the control of flowering time and its correlation with environmental stress tolerance in trees would greatly accelerate current tree-breeding programmes.

In this study, we identified three GI-like genes (named PagGla, PagGlb and PagGlc) from poplar (P. alba × P. glandulosa) and investigated their roles in circadian rhythm and flowering time control in transgenic Arabidopsis plants. In addition, we developed a salt-tolerant transgenic poplar line by down-regulating the expression of PagGls, as these genes negatively regulate salt stress tolerance via an SOS-mediated pathway. This study provides the first evidence that altering the expression of GI genes through genetic engineering could be used to develop woody plants with enhanced growth and increased salt tolerance.

Results

Cloning and characterization of poplar GIGANTEA (GI)-like genes

We isolated the cDNA fragments of poplar GI-like genes (PagGla, PagGlb and PagGlc) from P. alba × P. glandulosa by homology-based BLAST searches (DOE JGI, Walnut Creek, CA, USA) against the Joint Genome Institute Phytozone database (www.phytozone.com). Phylogenetic analysis of the deduced plant GI amino acid sequences from 12 species showed that PagGla, b and c are closely clustered with other dicot GI homologues and belong to the same subgroup as AtGI (Figure 1a). These results suggest that PagGla, b and c share a conserved role. Comparison of the deduced amino acid sequences of AtGI and the PagGI genes revealed that the regions for their subcellular localization are conserved among Arabidopsis and poplar GI, which contain four clusters of basic amino acids similar to the clusters identified in established bipartite NLSs in other nuclear proteins (Huq et al., 2000) (Figure 1b). To investigate the subcellular localization of PagGla, b and c, we transiently transfected N. benthamiana leaves with a 35S:PagGla/b/c:GFP construct. As shown in Figure 1c, GFP fluorescence produced by PagGla-, b- and c-GFP fusion proteins overlapped with blue fluorescence, as revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining. These results suggest that PagGla, b and c predominantly localize to the nucleus and may play a role in transcriptional regulation. GI function in photoperiodic flowering and circadian rhythms have been extensively studied from monocot to dicot plants (Mouradov et al., 2002), but have not yet been reported in perennial poplar. To verify whether poplar PagGIs are orthologues of Arabidopsis GI, we first characterized its original functions in regulation of circadian clock, flowering time.

PagGla, b and c are involved in the regulation of circadian rhythm in poplar

To determine whether PagGI mRNA levels fluctuate throughout the day, we examined the expression levels of the PagGI genes every 4 hours for 2 days. As shown in Figure 2a, PagGI (the sum of PagGla, b and c) transcripts were diurnally regulated, with a peak around zeitgeber 12 (ZT12) (Zerr et al., 1990) in mature leaves. Moreover, PagGI mRNAs were most abundant in young leaves among all tissues examined, including shoot tips, young leaves, mature leaves, bark, xylem and roots (Figure 2b). We then determined whether PagGI transcript levels were affected by salinity stress. As shown in Figure 2a, PagGI mRNA levels were significant up-regulated throughout the day and in all tissues examined in response to salt stress treatment. We also investigated the transcript levels of PagGla, b and c separately. Since it is difficult to distinguish between PagGla and PagGlc due to their high homology, we listed the expression data for PagGlc, the sum of PagGla and PagGlc. As shown in Figure S1a,b, PagGlc and PagGlb expression was induced by salt stress, and PagGlb was expressed at higher levels than PagGlc throughout the day and in all tissues examined.

Overexpression of PagGIs induces early flowering in Arabidopsis

To investigate whether PagGla, b and c play a functionally conserved role in regulating flowering time, we generated transgenic plants overexpressing PagGla, b and c in the WT (35S:PagGla/b/c Col-0) and gi-2 mutant (35S:PagGla/b/c gi-2) backgrounds. We first confirmed the expression levels of PagGls in transgenic Arabidopsis by RT-PCR, immunoblotting analysis, and confocal microscopy (Figure S2a–c). Next, we selected transgenic lines overexpressing the PagGI genes (two lines per gene) and subjected them to detailed phenotypic analysis under long-day conditions (LDs). The 35S:PagGla/b/c Col-0 plants flowered earlier than the WT (Figure 3a–c). Arabidopsis gi-2 mutant plants exhibit a strong late-flowering phenotype (Fowler et al., 1999). However, this late-flowering phenotype was partially or completely rescued in 35S:PagGla/b/c gi-2 plants. The 35S:PagGla/b/c Col-0 and 35S:PagGla/b/c gi-2 transgenic lines flowered from 15 to 17.8 days and from 19.7 to 38.2 days of seed sowing at the 3/4 (rosette) stage under LDs, whereas WT and gi-2 flowered within 19.3 and 41.3 days of seed sowing respectively (Figure 3c). These results suggest that PagGla, b and c play a role in flowering time regulation in plants.

Salt stress delays the floral transition in Arabidopsis (Kim et al., 2013a; Li et al., 2007). To determine whether PagGla, b and c transcript levels affect flowering time under salt stress, we observed the flowering time of WT and transgenic Arabidopsis.
plants under salt stress. As shown in Figure 3a–c, salt-induced late flowering was fully suppressed in 35S::PagGIa/b/c Col-0 and partially suppressed in 35S::PagGIa/b/c gi-2 plants, whereas WT displayed a late-flowering phenotype under salt stress.

Overexpression of PagGI genes confers salt sensitivity in Arabidopsis

To determine whether PagGIs a, b and c play a functionally conserved role in negatively regulating the salt stress response, we evaluated the roles of PagGla, b and c in transgenic Arabidopsis under salt stress condition. To examine the sensitivity of transgenic Arabidopsis seedlings to salt stress, we planted WT, 35S::PagGIa/b/c Col-0 and 35S::PagGIa/b/c gi-2 seeds on MS medium with or without the indicated concentration of NaCl. In the absence of NaCl, all lines exhibited similar seed germination rates. However, in the presence of 100 mM NaCl, 35S::PagGIa/b/c Col-0 and 35S::PagGIa/b/c gi-2 seeds exhibited delayed germination compared to WT and gi-2 seeds respectively (Figure S3a,b). We next examined the root growth phenotype of salt stress-treated plants. Root elongation was significantly suppressed in 35S::PagGIa/b/c Col-0 and 35S::PagGIa/b/c gi-2 compared to WT and gi-2 plants, respectively, on MS medium containing 125 mM NaCl. The 35S::PagGIa/b/c Col-0 and 35S::PagGIa/b/c gi-2 transgenic plants lost more root fresh weight (78%–85.9% and 47.6%–68%) than WT (59.7%) and gi-2 (38.3%) plants respectively (Figure S3c,d).

We also evaluated the salt sensitivity of the transgenic Arabidopsis plants grown in soil. Compared to WT plants, 35S::PagGIa/b/c Col-0 transgenic lines had reduced tolerance to NaCl-induced osmotic stress (Figure 4a,b). In addition, the increased salt tolerance of gi-2 mutant plants was fully or partially reversed in the 35S::PagGIa/b/c gi-2 transgenic lines (Figure 4a,b). These results suggest that PagGla, b and c also function as negative regulators of the salt stress signalling pathway.

PagGIs inhibit PagSOS2-dependent phosphorylation of PagSOS1

We next examined how PagGla, b, and c participate in the regulation of the salinity stress response. GI appears to be involved in salt stress tolerance through the SOS pathway in Arabidopsis (Kim et al., 2013a). To determine if PagGIs are also involved in the SOS pathway, we isolated genes encoding components of the SOS pathway in poplar, that is, PagSOS1 and PagSOS2 (Figure S4 and S5). We next examined the interaction between PagGIs and PagSOS2 via His pull-down assays in vitro. His-PagGIs and GST-PagSOS2C fusion proteins were purified as described in Supporting information. His-PagGla, b and c were incubated with GST-PagSOS2C, and the pull-down extracts were analysed by immunoblotting using the respective anti-GST antibody. GST-SOS2 was pulled down with His-PagGla, -b and -c (Figure 5a), suggesting that PagGla, b and c interact with...
Figure 2  PagGIs, b and c are Involved in the Regulation of Circadian Rhythms. (a) Time course of PagGI expression in mature leaves of poplar plants under LDs. (b) Expression levels of PagGIs in different tissues at ZT12. Total RNA samples were collected every 4 h from 2-month-old poplar plants under LDs. The mRNA abundance was quantified by quantitative RT-PCR, which was performed in triplicate with three independently harvested samples. Actin expression was used as an internal control. Detected tissues including shoot tip (S), young leaf (Y), mature leaf (M), bark (B), xylem (X) and root (R). White and black bars above the graph indicate day and night periods respectively. Error bars represent SD of three independent experiments. Asterisks indicate significant differences at P < 0.05.

To determine whether PagGI proteins interact with PagSOS2, predominantly in the nucleus and c-VN/PagSOS2C-VC were used, indicating that PagGI proteins negatively regulate salt stress tolerance by inhibiting PagSOS2-dependent phosphorylation of PagSOS1 via PagGIs-PagSOS2 interaction.

Generation of transgenic poplar with down-regulated expression of PagGIs

The above results encouraged us to generate transgenic poplar plants with improved growth and salt tolerance by down-regulating PagGI genes. An RNA interference (RNAi) construct was produced and introduced into poplar plants (P. alba × P. glandulosa; referred to as RB) using Agrobacterium-mediated transformation (Figure 8a). Nine independent RB plants were confirmed by genome PCR analysis with Int F/Nos R- and Bar-specific primers, and three lines (RB5, RB16 and RB32) were selected by RT-PCR and Southern blot analysis for further characterization (Figure S8a–c). To evaluate the morphological phenotypes of non-transgenic (NT) and RB plants, poplar plants grown on rooting medium (RM) for 1 month were transferred to pots. Two-month-old pot-cultured RB plants exhibited elongated petioles and large leaves with downward curvature (Figure 8b,c). Moreover, RB plants had thick stems and increased biomass (1.29–1.58-fold higher biomass than NT plants), but no significant difference in height compared to NT plants (Figure 8d–i). Plant viability and developmental processes, including cell division, cell growth and differentiation, are precisely monitored and determined by auxin (Dhonukshe et al., 2008). Auxin regulates the expression of numerous auxin-responsive genes, such as Aux/IAA genes (Hagen and Guilfoyle, 2002; Kim et al., 2011). Interestingly, the transcript levels of early auxin-response genes including IAA1, IAA2, IAA4 and IAA5 were significantly higher in all RB plants under normal growth conditions compared to the control (Figure 8j). These results indicate that the phenotypes of RB plants are positively correlated with the transcript levels of auxin-response genes.

RB plants exhibit increased tolerance to salt stress

To evaluate the salt stress tolerance of RB plants, we cultured poplar stem cuttings (NT, RB5, RB10 and RB16) with double nodes in RM tubes supplemented with or without the indicated concentration of NaCl. In the presence of 50 mM NaCl, RB plants
exhibited less loss (33.2%–44.1%) of root fresh weight than NT plants (74.7%), whereas very little rooting of NT plants occurred in the presence of 75 mM NaCl after 1 month of treatment (Figure S9a,b). Furthermore, we subjected 2-month-old pot-cultured NT and RB plants of the same health status to 200 mM NaCl for 6 days. NT plants displayed faster wilting and more chlorosis than RB plants under salt stress conditions. The differences in wilting symptoms between NT and RB plants became even more pronounced after normal irrigation was resumed (Figure 9a). As expected, reduced levels of PagGI transcripts were detected in RB plants (Figure 9b). RB plants maintained higher photosystem II efficiency (Fv/Fm) and higher levels of chlorophyll compared to NT plants under salt stress, which is consistent with their salt-resistant phenotypes (Figure 9c). All of these results indicate that down-regulating PagGI genes confers tolerance to salt stress.

**Discussion**

*PagGIs* are functional orthologues of *Arabidopsis* *GI*

*GI*GANTEA (GI) regulates several signalling pathways, but only its role in photoperiodic flowering and circadian rhythms have been
extensively studied (Mouradov et al., 2002). Poplar differs from annual Arabidopsis plant in many ways (Barlowe and Dean, 2006; Jansson and Douglas, 2007). Elucidating how perennial plants adapt their growth and development in response to perceived environmental stress at the molecular level would greatly accelerate current tree-breeding programmes.

The GI gene has undergone several intraspecific gene duplications: soybean has at least four GI paralogs (GmGI 1α, GmGI 1β, GmGI 2 and GmGI 3), and onion has two GI-like genes (AcGIa and AcGIb) involved in flowering promotion (Taylor et al., 2010; Watanabe et al., 2011). In this study, we identified three GI homologs (paralogs) in poplar. PagGIa and PagGIc are predicted to be localized to chromosome 5, while PagGIb is found on chromosome 2 (data not shown). Although the overall expression profiles of PagGIa, b and c were similar, slight but interesting differences were observed among the three GI paralogues. The peak transcript levels were higher for PagGIb than the total PagGIa and PagGIc transcript levels throughout the day and in all investigated tissues (Figure S1a,b). Gene duplication initially generates two identical copies of a gene that are functionally redundant. The original function may be conserved in both genes, providing extra amounts of protein or RNA products, or one gene may evolve freely without much pressure from natural selection until it becomes a non-functional pseudogene or acquires a novel function (Teichmann and Babu, 2004; Zhang, 2003). Although we detected no significant differences in the physiological functions of these genes, such as the induction of early flowering and salt sensitivity in transgenic Arabidopsis (Figure 3a and 4a), the GI paralogues appear to be differentially regulated in poplar.

Several lines of evidence indicate that PagGia, b and c are ArGI orthologues in poplar (Figure 10). PagGIs regulate circadian rhythms via direct protein–protein interaction with PagZTLs (orthologues of Arabidopsis ZTL; Figure S10 and S11), which is necessary to sustain a normal circadian period by controlling the proteasome-dependent degradation of the central clock protein TIMING OF CAB EXPRESSION 1 (TOC1) (Kim et al., 2007, 2013b). PagGIs appear to regulate flowering (at least in part) by influencing PagCO2 (orthologue of Arabidopsis CO; Figure S12 and S13), and PagGI-PagCO-PagFT (in the photoperiodic pathway) might play a role in regulating both flowering time and the timing of growth cessation (Böhle et al., 2006). PagGIs negatively regulate salt stress tolerance by caging PagSOS2 to the nucleoplasm and cytoplasm, which might influence the activities of the plasma membrane Na⁺/H⁺ antiporter PagSOS1 (Figure 6). These results provide strong evidence that these three poplar genes are (at least in part) functionally conserved between the herbaceous plant Arabidopsis and the woody plant poplar.

Figure 4 Overexpression of PagGI Genes Confers Salt Sensitivity in Transgenic Arabidopsis. (a) 35S:PagGIa/b/c Col-0 (overexpression lines) and 35S:PagGIa/b/c gi-2 (complemented lines) plants were grown in soil under LDs for 3 weeks (first row, before) and then (after) watered with water (0 mM NaCl) for 1 week (second row) or 150 mM NaCl solution for 1 week (third row) or 2 weeks (fourth row), and (b) relative fresh weights at the end of the treatments (shown in a) were measured. More than 10 plants were measured per data point. Error bars represent SD of three independent experiments. Asterisks indicate significant differences at P < 0.05.
PagGIs play the negative regulatory roles in the salt tolerance response

Among abiotic factors, soil salinity represents an increasing global agricultural and environmental challenge, particularly in irrigated lands (Zhu, 2002). High salt stress disrupts the homeostasis of water potential and ion distribution. Maintaining ion homeostasis is critical for plants combating high salinity stress (Zhu, 2000). The initiation of flowering involves components of the circadian clock and is precisely monitored by multiple environmental cues (Andrés and Coupland, 2012; McClung, 2006). A number of components of the circadian clock are transcription factors, which play dual roles in ensuring the functioning of the central oscillator and controlling the rhythmic expression of downstream genes, such as abiotic stress-responsive genes (Grundy et al., 2015). As GIs are large proteins, they possess various functional domains and can participate in multiple signalling pathways. In Arabidopsis, AtGI negatively regulates the salt stress response by caging SOS2 to the nucleoplasm and cytoplasm under normal growth conditions, but is degraded in response to salt stress (Kim et al., 2013a). Released SOS2 then interacts with SOS3 to form an active SOS2-SOS3 protein complex, which regulates the expression and/or activities of various ion transporters, including the plasma membrane Na+/H+ antiporter SOS1, thereby promoting salt stress tolerance (Guo et al., 2004; Zhu, 2000, 2002). The SOS pathway, which functions in the salt stress response, is conserved in poplar: PtSOS1, PtSOS2 and PtSOS3 are functional homologues of their Arabidopsis counterparts, reconstitution of poplar SOS pathway in yeast cells revealed that PtSOS2 and PtSOS3 act coordinately to activate PtSOS1 (Tang et al., 2010). Transgenic poplar plants overexpressing PtSOS2 or PtSOS2TD (constitutively active form of PtSOS2) have improved salt tolerance (Yang et al., 2015; Zhou et al., 2014). Although the exact mechanism in poplar remains unknown, our findings suggest that PagGIs function in the regulation of the salt stress response. Wild-type Arabidopsis plants overexpressing PagGia, b and c exhibited early flowering and enhanced salt sensitivity, while overexpressing these genes in the gi-2 Arabidopsis mutant rescued the delayed flowering phenotype and increased salt tolerance (Figure 3a, b, Figure 4a, b). His pull-down assays and BIFC experiments revealed that PagGI proteins interact with PagSOS2 in poplar. PagGI-PagSOS2 complexes inhibit the PagSOS2-based phosphorylation of PagSOS1 (Figure 5 and Figure 6a), which subsequently weakens Na+/H+ ion exchange under normal growth conditions (Figure 6b). PagGIs might play negative roles in salt response either by interfering with the PagSOS2-regulated phosphorylation of PagSOS1 in cytosol or regulating cytosolic amount of PagSOS2 proteins by sequestering PagSOS2 to the nuclear (Figure 5 and Figure 6a). However, it remains to be elucidated. These inhibitions were counteracted by the degradation of PagGI proteins in a proteasome-dependent manner under salt stress (Figure 7). Down-regulation of PagGI genes confers enhanced salt tolerance in poplar, further confirming that a link exists between salt stress responses and flowering control, and that PagGI genes may play pivotal roles in this process.

Down-regulation of PagGIs by RNA interference led to vigorous growth, higher biomass and enhanced salt stress tolerance in transgenic poplar plants

RNA interference (RNAi) is an effective tool used to manipulate gene expression experimentally and to probe gene function on a genome-wide scale in both plants and animals (Hannon, 2002). Altering flowering time regulation by overexpressing or suppressing gene activity can be used in crop breeding (Jung and Müller, 2009). Suppressing flowering time-related gene activity by RNAi has been used to delay bolting and flowering in Arabidopsis, rice, and wheat (Curtis et al., 2002; Peng et al., 2008; Yong et al., 2003). The development of salt-tolerant crops is urgently needed to sustain agricultural production. However, conventional tree-breeding techniques alone are no longer
supplemented with 75 mM NaCl. Plates were incubated at 28 °C of serial decimal dilutions were spotted onto plates of the same medium or of genes were grown overnight in liquid selective medium. Five microlitres cells transformed with an empty vector (control) or indicated combination decrease the NaCl tolerance of yeast expressing the auto-phosphorylated staining (bottom panel) of a gel containing resolved reactions. (b) PagGIa, b and c are degraded upon exposure to salt in a in vitro proteasome-dependent manner. Whole 3-week-old, soil-grown 35S PagGIa/b/c inhibit PagSOS2-mediated PagSOS1 phosphorylation in vitro. An in vitro kinase assay was performed using purified bacterially GST-PagSOS1C, GST-PagSOS2TD and His-PagGia, b, and c proteins in the indicated combinations. Shown are autoradiogram (top panel) and CBB staining (bottom panel) of a gel containing resolved reactions. (b) PagGIs decrease the NaCl tolerance of yeast expressing the auto-phosphorylated PagSOS2 (PagSOS2TD) and PagSOS1 ion transporter. Yeast strain AXT3K cells transformed with an empty vector (control) or indicated combination of genes were grown overnight in liquid selective medium. Five microtiter plates of serial decimal dilutions were spotted onto plates of the same medium or supplemented with 75 mM NaCl. Plates were incubated at 28 °C and photographed after 4 days.

Figure 6 PagGI proteins negatively regulate salinity stress tolerance. (a) PagGia/b/c inhibit PagSOS2-mediated PagSOS1 phosphorylation in vitro. An in vitro kinase assay was performed using purified bacterially GST-PagSOS1C, GST-PagSOS2TD and His-PagGia, b, and c proteins in the indicated combinations. Shown are autoradiogram (top panel) and CBB staining (bottom panel) of a gel containing resolved reactions. (b) PagGIs decrease the NaCl tolerance of yeast expressing the auto-phosphorylated PagSOS2 (PagSOS2TD) and PagSOS1 ion transporter. Yeast strain AXT3K cells transformed with an empty vector (control) or indicated combination of genes were grown overnight in liquid selective medium. Five microtiter plates of serial decimal dilutions were spotted onto plates of the same medium or supplemented with 75 mM NaCl. Plates were incubated at 28 °C and photographed after 4 days.

Figure 7 PagGia, b and c are degraded upon exposure to salt in a proteasome-dependent manner. Whole 3-week-old, soil-grown 35S: PagGia/b/c Col-0 plants were treated with NaCl (100 μM), MG132 (100 μM) or NaCl plus MG132 at ZT1. PagGia, b and c protein levels were evaluated after 0, 12 and 24 h treatments via immunoblot analysis with anti-GFP antibody. Coomassie Brilliant blue (CBB)-stained blots are shown as a loading control. Molecular weight markers in kDa.

Experimental procedures

Plant materials and growth conditions

The Arabidopsis thaliana ecotype Colombia-0 (Col-0) was used as the WT for all experiments. The gi-2 mutant (Col-0 background), which was described previously (Fowler et al., 1999), was kindly provided by Dr. Dae-Jin Yun (Gyeongsang National University, South Korea). Arabidopsis seeds were stratified at 4 °C in darkness for 3 days and transferred to a growth chamber at 22 °C under a 16 h light/8 h dark photoperiod (light intensity ~120 μmol/m²/s), unless otherwise specified.

A hybrid poplar clone (P. alba × P. glandulosa) was used in this study. The plants were sub-cultured monthly by aseptically transferring shoot apices with double nodes to fresh RM comprising 1× Murashige and Skoog medium (MS, Murashige...
and Skoog, 1962) containing 0.2 mg/L indole-3-butyric acid. After 1 month culture in RM, the rooted plantlets were transplanted to pots and grown in a growth chamber at 25°C with 60% relative humidity under a light intensity of 150 μmol/m2/s and a 16/8 h (light/dark) photoperiod.

Gene cloning
The poplar GIGANTEA (PagGI) and ZEITLUPE (PagZTL) genes were identified by sequence comparison of Arabidopsis GI and ZTL, respectively, to the Populus trichocarpa genomic database. Poplar CO2 (PagCO2) and SOS2 (PagSOS2) were identified based on the cDNA sequences of PtCO2 and PtSOS2 respectively. The coding sequences of PagGIs, PagGib, PagGlc, PagZTL1, PagZTL2, PagCO2 and PagSOS2 were amplified from cDNA from P. alba × P. glandulosa leaves with Pfu-X DNA polymerase (Solgent, Daejeon, Korea) using the primer pairs listed in Table S1. GenBank accession numbers are shown in Table S2.

Phylogenetic analysis
For details, see Supporting information.

Plasmid construction
For details, see Supporting information.

RNA preparation and analysis of gene expression
Total RNA was extracted from the indicated plant tissues using a GeneAll Ribospin Plant™ kit (GeneAll, Seoul, Korea) according to the manufacturer’s instructions. For cDNA production, 2 μg of
total RNA was reverse transcribed using an RT-PCR kit (Enzynomics, Daejeon, Korea). The reaction mixture was diluted to 100 μL with sterilized water, and 2 μL was used for real-time quantitative RT-PCR. Gene-specific primers are listed in Table S1. All quantitative RT-PCR analysis was performed with a CFX real-time PCR system and CFX system software (Bio-Rad, CA) using Ever-Green 20 fluorescent dye (BioFACT, Daejeon, Korea). The following program was used for PCR: an initial denaturation step for 5 min at 95 °C, followed by 44 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. There were at least three biological repeats and three technical repeats per data point.

Arabidopsis and poplar transformation

The PGWB5-35S-PagGlb/b/c-GFP constructs were introduced into Agrobacterium tumefaciens strain GV3101 by the freeze-thaw method (Wise et al., 2006) and transferred into WT and gi-2 mutant plants by floral dip infiltration (Clough and Bent, 1998). For each gene, two independent transgenic lines (T3 generation), which were homozygous for single-copy insertions of transgene T-DNAs in the WT or gi-2 mutant background, were used for the experiments.

Salt stress treatment

For details, see Supporting information.

Transient expression in N. benthamiana and confocal microscopy

Agrobacterium tumefaciens GV3101 strains carrying the indicated constructs and P19-silencing plasmid were grown in

Figure 9

Down-Regulation of PagGI Genes Confers Salt Tolerance in Poplar. (a) Effect of salt stress on RB poplar plants. Two-month-old non-transgenic and RB plants were subjected to 200 mM NaCl treatment for 6 day and recovery for 15 day. (b) Transcript levels of PagGs in three individual RB poplar plants. The 10th leaves from 1-month-old plants entrained in LDs were harvested at ZT12. The mRNA abundance was quantified by quantitative RT-PCR and normalized to the level of poplar actin transcript. (c) Fv/Fm and total Chl contents in the leaves (10th) of poplar plants were determined at 4 day after treatment. Data represent means ± SD of three independent experiments. Asterisks indicate significant differences at P < 0.05 respectively.

Figure 10

Model of the Roles of PagGI Genes in Regulating Circadian Rhythms, Flowering Time and the Salt Stress Response.
YEP medium supplemented with the appropriate antibiotics overnight. Cultures were spun down and resuspended in infiltration solution (10 mM MES, 10 mM MgCl₂ and 100 µM acetosyringone). Agrobacterium carrying the indicated construct or combinations of constructs and P19 were co-infiltrated into 2-week-old *N. benthamiana* leaves for subcellular localization analysis. The cultures were grown in infiltration solution to a final OD₆₀₀ = 0.5. After 3 days of growth in a greenhouse at 25 °C under LDs, the infiltrated parts of leaves were cut and immersed in DAPI solution for nuclear staining and subjected to fluorescent signal detection under a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany) with proper filter sets, as described by Gehl et al. (2009).

**Protein expression and purification**

For details, see Supporting information.

**Immunoblot analysis**

Total protein was extracted from whole plants of 3-week-old soil-grown *PagGIs*-OX plants, which were treated with NaCl (100 mM), MG132 (100 mM) or NaCl plus MG132 at the indicated time point. The extraction solution contained 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 mM DTT and one protease inhibitor tablet (Roche, Mannheim, Germany). The extracts were separated by SDS-PAGE. Immunoblot analysis was performed with anti-GFP antibody.

**His pull-down assay**

First, 5–10 µg of GST or GST fusion protein (GST-*PagSOS2F* or GST-*PagSOS2C*) was incubated with 30–50 µg of His fusion protein (His-*PagGlc*, His-*PagGib* or His-*PagGic*) in lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.1% Tween20, 1 mM DTT and one tablet of protease inhibitor) at 4 °C for 4 h. Next, 50–100 µL of Ni-NTA agarose was added to the sample, followed by incubation overnight at 4 °C. The beads were washed three times with lysis buffer. Bound proteins were eluted from the beads by boiling in SDS sample buffer and analysed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue or Western blotting using the appropriate antibodies.

**Kinase assay**

For the *in vitro* kinase assay, kinase reactions were performed in 20 µL of kinase buffer (20 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 5 mM EGTA, 100 mM NaCl, and 1 mM DTT) containing 5 µg of fusion protein with 1 µL of [γ-³²P] ATP at room temperature for 1 h. The reactions were stopped by adding 4× SDS loading buffer. The phosphorylation of fusion proteins was analysed by autoradiography after separation by 10% SDS-PAGE.

**Yeast experiments**

The *AXT3K* (Δ*en1::HIS3::en4, Δ*nh1::LEU2, Δ*nhx1::KanMX4*), which was described previously (Quintero et al., 2002), was kindly provided by Dr. Dae-Jin Yun (Gyeongsang National University, South Korea). The ion uptake experiment was performed as described previously (Ardie et al., 2009). Yeast *AXT3K* cells transformed with an empty vector or expressing combinations of *PagSOS1*, *PagSOS2*, *PagSOS2TD* and *PagGia*b/c were incubated in the 1/4 strength SD plate supplemented with or without 75 mM NaCl. Plates were incubated at 28 °C and photographed after 4 days.

**Measurement of flowering time**

Flowering time was measured by counting the number of rosette and cauline leaves or days to bolting when floral buds were visible (1 cm long) at the centre of the rosette. Flowering time was measured at least twice with similar results.

**Analysis of photosynthetic activity and chlorophyll contents**

Photosynthetic activity in leaves was estimated based on chlorophyll fluorescence determination of photochemical yield (Fv/Fm), which represents the maximal yield of the photochemical reaction in photosystem II (PSII), using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England) after 30 min of dark adaption. Chlorophyll contents were measured with a portable chlorophyll meter (SPAD-502; Konica Minolta, Japan). Total chlorophyll contents after stress treatment were compared with those under normal conditions. Both of these values were detected using the 5th–10th intact, fully expanded leaves (counting from the shoot apical meristem) of individual plants.

**Statistical analysis**

Data were statistically analysed with Statistical Package for the Social Sciences (SPSS 12.0, SPSS Inc., Chicago, IL). Means were separated using Duncan’s multiple range test at *P* = 0.05.

**Acknowledgements**

This work was supported by grants from the KRIBB Initiative Program, the Korea-China International Collaboration Project, National Research Foundation of Korea (NRF), Korea and the 111 Project of the Ministry of Education, China (no. B12007).

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Supporting information
Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 PagGI genes are involved in the regulation of circadian rhythms
Figure S2 Generation of transgenic Arabidopsis plants overexpressing PagGI genes
Figure S3 Sensitivity of transgenic Arabidopsis seedlings to salt stress

Figure S4 SOS1-like gene in poplar
Figure S5 SOS2-like gene in poplar
Figure S6 Nuclear and cytosolic distribution of PagGI proteins
Figure S7 PagSOS2 phosphorylates PagSOS1 in vitro
Figure S8 Generation of transgenic poplar plants by down-regulating PagGI genes
Figure S9 Effects of salt stress on NT and RB plants grown under in-tube conditions
Figure S10 ZEITLUPE (ZTL)-like genes in poplar
Figure S11 The interaction between PagGI proteins and PagZTL proteins in vivo
Figure S12 CONSTANS(CO)-like gene in poplar
Figure S13 The interaction between PagGI proteins and PagCO2 in vivo
Table S1 Gene-specific primers used in this study.
Table S2 GenBank accession numbers of genes described in this study.