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ORIGINAL ARTICLE

Reduction of *GIGANTEA* expression in transgenic *Brassica rapa* enhances salt tolerance

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Key message **Here we report the enhancement of tolerance to salt stress in *Brassica rapa* (Chinese cabbage) through the RNAi-mediated reduction of *GIGANTEA* (*GI*) expression.**

Abstract Circadian clocks integrate environmental signals with internal cues to coordinate diverse physiological outputs. The *GIGANTEA* (*GI*) gene was first discovered due to its important contribution to photoperiodic flowering and has since been shown to be a critical component of the plant circadian clock and to contribute to multiple environmental stress responses. We show that the *GI* gene in *Brassica rapa* (*BrGI*) is similar to Arabidopsis *GI* in terms of both expression pattern and function. *BrGI* functionally rescued the late-flowering phenotype of the Arabidopsis *gi-201* loss-of-function mutant. RNAi-mediated suppression of *GI* expression in Arabidopsis Col-0 and in the Chinese cabbage, *B. rapa* DH03, increased tolerance to salt stress. Our results demonstrate that the molecular functions of GI described in Arabidopsis are conserved in *B. rapa* and suggest that manipulation of gene expression through RNAi and transgenic overexpression could enhance tolerance to abiotic stresses and thus improve agricultural crop production.

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Introduction

All eukaryotic and some prokaryotic species have circadian clocks. These circadian clocks have an endogenous periodicity of ~24 h and allow the organism to anticipate the daily transitions of dawn and dusk and consequently to coordinate its physiology temporally with the surrounding environment (Sanchez et al. 2011; Gehan et al. 2015). Plant circadian clocks are entrained to the environment, with daily cycles of light and temperature providing the strongest resetting cues (Salomé and McClung 2005). Identification of the molecular components of the circadian clock of the model plant *Arabidopsis thaliana* has revealed the basic workings of the plant circadian clock. The circadian clock consists of positive and negative elements that comprise a transcriptional-translational feedback loop (Bell-Pedersen et al. 2005; Sanchez et al. 2011). In higher plants, this system consists of multiple interlocked feedback loops (Harmer 2009). Also, post-transcriptional (e.g., alternative splicing and mRNA stability) (Staiger and Brown 2013; Romanowski and Yanovsky 2015) and post-translational processes (e.g., reversible phosphorylation, protein stability, and nucleocytoplasmic localization) are essential to correct clock function (Kim et al. 2013a, c; Hsu and Harmer. 2014).

The plant circadian clock gates many responses to environmental stimuli (Gehan et al. 2015). For example, the circadian clock regulates phytochrome gene expression and thus modulates its own sensitivity to light. Similarly, the responses to abiotic and biotic stimuli are a function of the time of day. For example, circadian-regulated gene sets overlap with ABA-regulated gene sets; ABA plays central roles in many environmental stress responses, including water use and responses to drought (Matsui et al. 2008; Mizuno and Yamashino 2008). The expression of *TIMING OF CAB EXPRESSION1* (*TOC1*), a member of the core oscillator, is induced by ABA and implicated in plant responses to drought by controlling stomatal aperture through the circadian clock (Legnaioli et al. 2009). Similarly, the circadian clock modulates the cold acclimation response to freezing temperatures (Fowler et al. 2005; Mikkelsen and Thomashow 2009; Dong et al. 2011). Alternative splicing of the *Arabidopsis thaliana* core clock gene CIRCADIAN CLOCK ASSOCIATED1 *(CCA1)* has been suggested to link the clock with cold acclimation (Seo et al. 2012). Recently, the circadian clock component GIGANTEA (GI) was demonstrated to play a key function in salt tolerance through the control of the SALT OVERLY SENSITIVE (SOS) pathway (Kim et al. 2013b; Qiu et al. 2003). GI interacts with the SOS2 kinase to prevent activation of the SOS1 plasma membrane-resident Na^+/H^+ antiporter in the absence of salt stress. However, under saline conditions, SOS2, released by proteolytic degradation of GI, can interact with the SOS3 sodium ion sensor, which activates SOS1 to establish salt tolerance (Kim et al. 2013b). GI overexpression increases salt sensitivity, whereas loss of GI function enhances salt tolerance (Kim et al. 2013b; Xie et al. 2015). These and other studies indicate that a circadian clock synchronized with the environment enhances fitness through elevated stress resistance as well as via enhanced photosynthetic capacity and carbohydrate metabolism (Dodd et al. 2005; Graf and Smith 2011; Sanchez et al. 2011).

The polyploid species *Brassica rapa* includes a variety of vegetable crops, such as Chinese cabbage, bokchoy, turnip, and broccoletto, as well as oilseed crops, such as turnip rape and sarson. Many of these varieties are agriculturally important worldwide. Among *Brassica* crops, *B. rapa* has been used as a model species (Mun et al. 2009). Comparative mapping of *Arabidopsis* and *Brassica* species has revealed considerable synteny (Parkin et al. 2005; Schranz et al. 2006), which facilitated identification of *B. rapa* homologs of *Arabidopsis* clock components (Kim et al. 2012; Lou et al. 2012). Here we identify and clone a *GI* homolog from the *B. rapa* Chinese cabbage genome and investigate its role in salt tolerance of this vegetable crop. We generated transgenic *B. rapa* DH03 RNAi suppressor lines that exhibited reduced expression of *GI*, resulting in enhanced salt tolerance.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* lines used were from the Col-0 background. Seeds were plated on MS medium and incubated at 4 °C for 3 days and then transferred to a growth chamber at 22 °C under LD conditions (16 h light/8 h dark). After the seeds had germinated, seedlings were transplanted into soil. The *B. rapa* ssp. *pekinensis* inbred lines ‘Chiifu’ and DH03 (Kim et al. 2007a) were used in this study. Plants were grown in a controlled environment growth chamber under LD or SD conditions (8 h light/16 h dark) at 22 °C with cool-white fluorescent illumination (150 μmol m^{-2} s^{-1}, FLR40D/A fluorescent tubes, Osram, Korea).

Isolation and phylogenetic analysis of *BrGI*

A bacterial artificial chromosome (BAC) clone containing the ortholog of *GI* was identified (http://www.brassica-rapa.org), and the *GI* structure and sequence were predicted using the web-based gene prediction software FGENE-SH Arabidopsis (http://www.softberry.com/berry.phtml). Primers

were designed based on the predicted *GI* orthologous region using the Primer3 software (http://frodo.wi.mit.edu/primer3/). A full-length *BrGI* cDNA sequence, consisting of 3552 bp, was amplified from total RNA using *BrGI*-specific primers. The PCR fragments were subcloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced using the ABI 3730 DNA Sequencer. Details of the primers used are listed in Supplement Table 1. A phylogenetic tree was constructed using the predicted amino acid sequences of *BrGI* (HQ615940.1), *GI* (NM_102124.2), *Populus trichocarpa* (*PtGI*; XM_002307480.1), *Triticum aestivum* (*TaGI1*; AF543844.1), *Hordeum vulgare* (*HvGI*; AY740524.1), *Ricinus communis* (*RcGI*; XM_002524295.1), *Ipomoea nil* (*InGI*; AB265781.1), *Allium cepa* (*AcGIa*; GQ232756.1), *Allium cepa* (*AcGIb*; GQ232757.1), and *Oryza sativa* (*OsGI*; NM_001048755.1). A phylogenetic tree was generated using the neighbor-joining method in the MEGA software, version 4.0 (Kumar et al. 2004). The significance of the phylogenetic lineages was assessed by bootstrap analysis with 500 replicates and expressed as percentages. A multiple sequence alignment was performed using the ClustalW (Thompson et al. 1994) and GeneDoc (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html) software.

Analysis of *BrGI* expression

Total RNA was isolated from leaf tissue using RNeasy Plant Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed using EcoDry™ Premix (Oligo dT) (Clontech Laboratories, Inc., Takara Bio, Mountain View, CA, USA). For cDNA synthesis, 5 µg total RNA was incubated with oligo(dT) primers at 42 °C for 60 min, followed by 70 °C for 10 min. To assess *BrGI* expression under LD and SD conditions, tissues were collected every 3 h for 24 h under light/dark conditions and for 72 h under continuous light, and expression was analyzed by qRT-PCR. The qRT-PCR reaction was performed in three technical replicates using a Bio-Rad system and the SYBR Green I master mix in a volume of 20 µl. To determine *BrGI* expression in transformants of *Arabidopsis* and *Brassica*, synthesized cDNA and 10 pmol each of the left and right primers were then amplified in PCR mixtures subjected to denaturation at 94 °C for 5 min, amplification for 36 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The relative expression level of each target gene was determined by calculating the ratio of the target gene intensity to that of the β-*ACTIN* gene (Hong et al. 2008; Bactin5′, 5′-TGGCATCACACTTTCTACAA-3′; and Bactin3′, 5′-CAACGGAATCTCTCAGCTCC-3′) from the same cDNA preparation.

Yeast two-hybrid screening and β-galactosidase activity assays

Protein–protein interaction assays were performed using the ProQuest™ Two-Hybrid System with Gateway® Technology (Invitrogen™, CA, USA). The yeast strain used was MaV203 (MATα, *trp1, leu2, his3*). The vectors used were pDEST™32 (GAL4 DNA Binding Domain containing Gateway® Destination Vector; BD, bait) and pDEST™22 (GAL4 Activation Domain containing Gateway® Destination Vector; AD, Prey). cDNAs encoding the proteins were PCR-amplified using gene-specific primer sets (Table S1), and the PCR products were subcloned into the GAL4 AD- or BD-encoding vector using the gateway technique. The bait and prey pairs were cotransformed into the yeast strain MaV203. β-galactosidase activity was determined using o-nitrophenyl-b-D-galactopyranoside as the substrate. The gene sequences were fused to a DNA sequence encoding the activation domain of GAL4 in the pDEST™22 vector. The plasmids were co-transformed into cells of the MaV203 yeast strain along with the pDEST™32-*BrGI* plasmid containing *BrGI* fused in-frame to the sequence encoding the binding domain of *GAL4*. A pDEST™32-*GI* plasmid was generated and analyzed for comparison.

Construction of plant expression vectors

To express *BrGI* in Arabidopsis, *BrGI* was inserted into the pB2GW7 binary vector together with the herbicide-resistance gene (*bar*; Interuniversity Institute for Biotechnology, http://www.psb.rug.ac.be/gateway) as a selectable maker using Gateway Technology (Invitrogen, https://www.invitrogen.com). To knockdown expression of *BrGI*, we developed the 10 *BrGI*_RNAi vectors from the pB7GWIWG2(II) binary vector, each targeting a distinct region of *BrGI* (Fig. 3a; Table S1).

Generation of transgenic plants

Arabidopsis thaliana Col-0 and *gi-201* (Martin-Tryon et al. 2007) were transformed with the *BrGI* constructs; flowers were sprayed with a suspension of *A. tumefaciens* GV3101 carrying the appropriate construct in 5 % sucrose. The plants were then incubated in a growth chamber at 25 °C and 100 % humidity for 1 day and transferred to a growth chamber under a 16-h photoperiod at 23 °C. Transformants were selected by application of Basta (0.3 % solution). Primary transformants were self-fertilized and homozygous lines were identified. All experiments used seed from homozygous T4 lines.

The Chinese cabbage DH03 transformation procedure was similar to the method of Kim et al. (2007a). Surface-sterilized seeds were placed on MSO medium (MS basal medium + 3 % sucrose + 0.8 % Phytagar) and incubated in a culture room under LD conditions at 25 °C for 6–7 days. Hypocotyls were cut into 0.5 cm segments and placed on pre-culture medium (MS basal medium + 1 mg/l NAA + 3 mg/l BA + 2 mg/l $AgNO_3$ + 3 % sucrose + 0.8 % Phytagar). The pre-cultured hypocotyls were inoculated with *Agrobacterium* suspension in MS liquid medium for 15–20 min. The inoculated hypocotyls were then placed on co-cultivation medium (MS basal medium + 1 mg/l NAA + 3 mg/l BA + 3 % sucrose + 0.8 % Phytagar) and incubated for 3 days in darkness at 25 °C. To remove *Agrobacterium*, the explants were washed three to five times in liquid cocultivation medium supplemented with 100 mg/l carbenicillin and 250 mg/l cefotaxime and transferred to selective medium

(MS basal medium + 1 mg/l NAA + 3 mg/l BA + 2 mg/l $AgNO_3$ + 10 mg/l hygromycin + 3 % sucrose + 100 mg/l carbenicillin + 250 mg/l cefotaxime + 0.8 % Phytagar). Calli that formed on the hypocotyls were subcultured on fresh selective medium, which was replaced every 2–3 weeks until the shoots regenerated. The regenerated shoots were transferred to MSO medium to induce root formation. The transformed plants were grown in a glasshouse after acclimatization.

Salt stress

Arabidopsis thaliana transformants were sown on basal medium (½ strength Murashige and Skoog (MS) salts amended with 2 % sucrose and solidified with 1 % agar) and transplanted to soil after germination. Plants were grown under LD conditions at 23 °C for 2 weeks. To examine tolerance to salt, 250 mM NaCl solution was supplied every 3 days for 2 weeks. We evaluated the salt resistance of *B. rapa* RNAi transformants in several ways. To examine effects at the germination stage, seeds were sown on ½ MS salts, 2 % sucrose, and 1 % agar with or without 150 mM NaCl and root elongation was assessed after 14 days. For hydroponic cultures, seeds were sown in sponge cubes and supplemented with nutrient solution (Lee et al. 2012) for 2 weeks. When two to three true leaves had developed, the nutrient solution was replaced with fresh solution with or without 150 mM NaCl every 2 days for 2 weeks. Finally, 1-week-old seedlings grown on MS medium were transferred to fresh MS medium amended with 0, 200, or 300 mM NaCl. After 1 week, the photosynthetic capacity (Fv/Fm) was measured using a FluorCam 800MF chamber (Photon system instruments, Czech Republic), and the chlorophyll contents of whole seedlings were determined (Ritchie 2006). Data were analyzed via Student's *t* test or by analysis of variance (ANOVA) followed by Tukey's test.

Protein immunoblotting

GI protein abundance in *B rapa* lines carrying different *BrGI* alleles was analyzed by Western blotting of protein extracts prepared from 2-week-old plants probed with a rabbit polyclonal antibody prepared against native *GI*. An HRP-conjugated secondary antibody and chemiluminescent substrate were used for detection. Arabidopsis wild-type plants (Col-0) harvested at ZT1 and ZT13 and 35S::GI-HA (a constitutive GI overexpre ssion line) were used as GI protein controls (Kim et al. 2007c, 2013b).

Results

Isolation of the *B. rapa GI* gene

As the initial step in evaluating the role of *GI* in salt tolerance of *Brassica rapa*, an ortholog of *GI* was identified and isolated from the Chinese cabbage *B. rapa* DH03. Using a comparative genomics approach (Kim et al. 2007b), we first identified a *GI* cDNA from *B. rapa* (*BrGI*) that was orthologous to the *GI* gene from *Arabidopsis thaliana* (*GI*). The putative *GI* cDNA sequence (GenBank accession no. HQ615940) and its encoded amino acid sequence were compared with those of known dicot and monocot *GI* genes available in the GenBank DNA sequence database. The coding sequence of *BrGI* showed 87 % nucleotide sequence identity to that of *GI*. *BrGI* encodes 1183 amino acids (Fig. S1a) and consists of 13 exons and 12 introns (Fig. S1b). The size and structure of *BrGI* were similar to those of *GI*. Southern blot hybridization using a probe specific to *BrGI* revealed a single *BrGI* gene in the *B. rapa* DH03 genome (data not shown), consistent with the reference *B. rapa* Chiifu genomic sequence (Wang et al. 2011). Multiple sequence alignments using the GI proteins from dicot and monocot plants showed that the BrGI protein is highly conserved (Table S2). The BrGI protein is most similar to GI, with a sequence identity of 91 %, and is more closely related to dicot GI proteins than to monocot GI proteins (Fig. S1c).

Circadian clock regulation of *BrGI* expression

In Arabidopsis, *GI* transcript abundance is regulated by the circadian clock (Fowler et al. 1999; Park et al. 1999). In *B. rapa,* many orthologs of circadian clock genes of Arabidopsis exhibit daily rhythmic changes in expression (Kim et al. 2012). We, therefore, expected that *BrGI* expression would be under the control of the circadian clock. To

examine this hypothesis, *B. rapa* seedlings were collected in triplicate every 3 h over a 24-h period comprising a long (LD; 16 h light/8 h dark) or short day (SD; 8 h light/16 h dark), and *BrGI* transcript levels were analyzed by quantitative RT–PCR (qRT-PCR). Sampling times were expressed as zeitgeber time (ZT), which is the number of hours after the onset of illumination (Zerr et al. 1990). We found that the *BrGI* transcript levels cycled under both light regimens (Fig. 1a, b). Under LD conditions, the transcript level was lowest at dawn (ZT0) and peaked at 9 h after dawn (ZT9). A similar pattern was observed under SD conditions, but the peak was reached at ZT6. *BrGI* transcript levels decreased rapidly in the evening. These oscillation patterns are similar to those of *GI* expression in Arabidopsis (Fowler et al. 1999; Park et al. 1999) and *Brachypodium distachyon* (Hong et al. 2010), which peaked at ZT12 and ZT8 under LD and SD conditions, respectively. This pattern was also similar to that of *BrGI* transcripts in the R500 and IMB211 *B. rapa* accessions, which peaked at ZT8 under a 12/12 h photoperiod (Xie et al. 2015). To determine if the rhythmic cycling of the *BrGI* transcript was under the control of the circadian clock, *B. rapa* plants entrained under either LD or SD conditions were transferred to continuous light conditions. The *BrGI* transcript levels continued to cycle with oscillation patterns similar to those observed under LD or SD conditions (Fig. 1c, d), indicating that *BrGI* mRNA abundance is regulated by the circadian clock.

BrGI is similar in function to Arabidopsis GI

The GI protein interacts with a variety of proteins related to circadian rhythm, light responses, and photoperiodic flowering (Kim et al. 2007c, 2013b). A series of yeast two-hybrid assays were carried out to determine whether BrGI also interacts with the GI-interacting partners AtCOP1, ZTL, FKF1, and LKP2 of *Arabidopsis thaliana*. Both GI and BrGI interacted with each of the other proteins examined (Fig. 2a,

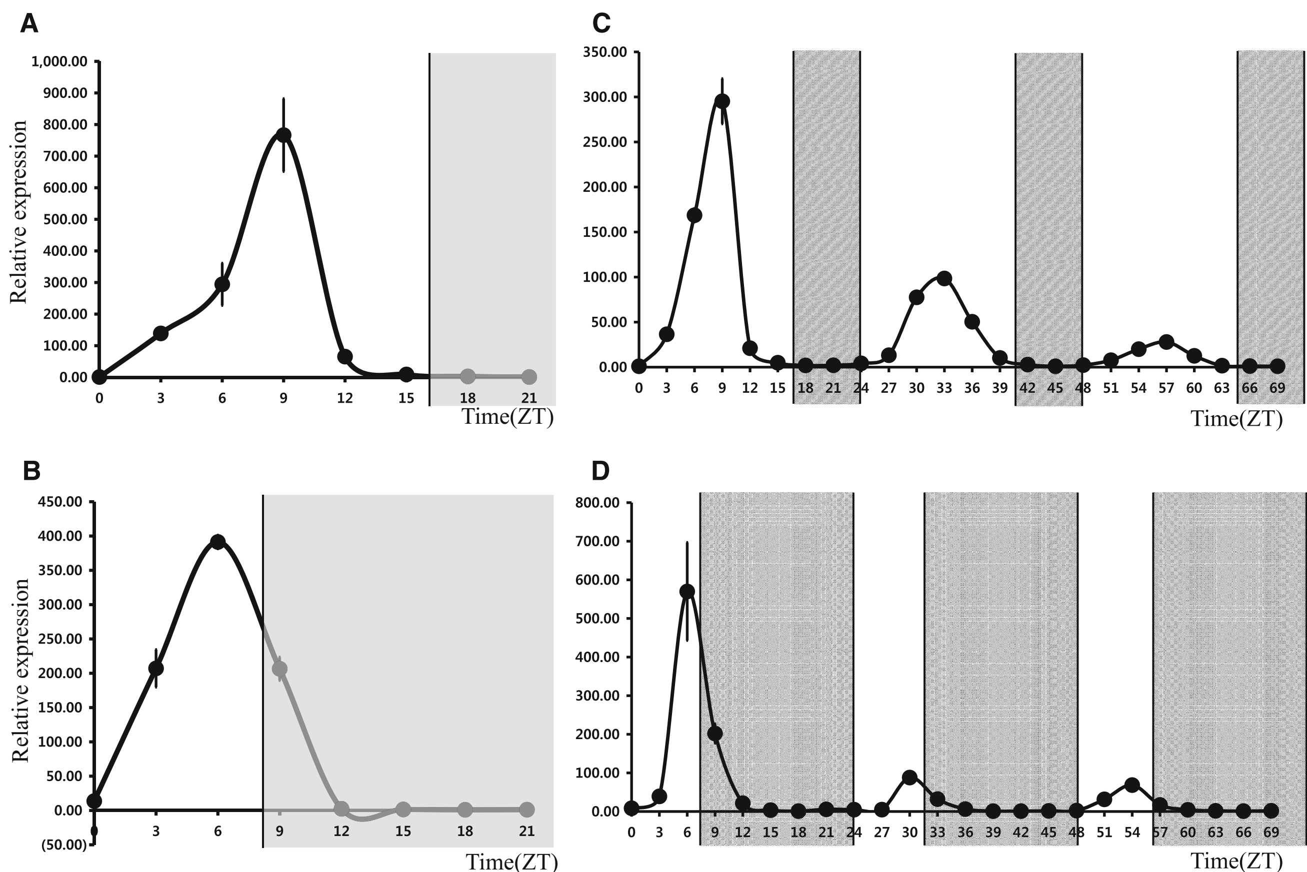

Fig. 1 Expression of *BrGI* under different light regimens. The leaves of 2-week-old plants grown on soil were harvested at the indicated times for total RNA extraction and qRT-PCR analysis. Data are presented as mean ± SEM of three biological replicates. Diurnal rhythm of *BrGI* mRNA abundance under long day (LD: 16 h day/8 h night) (**a**) or short day (SD: 8 h day/16 h night) conditions (**b**). Circadian rhythm of *BrGI* mRNA abundance in plants grown under LD (**c**) or SD conditions (**d**) and then transferred to constant light. Time is presented as zeitgeber time (ZT), in which the *dark* to *light* transition is defined as ZT = 0. *Shaded regions* in **a** and **b** indicate *dark*. *Shaded areas* in **c** and **d** indicate subjective night as defined by the entraining light–dark cycle

b). We found that the BrGI protein physically interacts with the GI-interacting partners related to circadian clock regulation. In addition to the circadian expression of the *BrGI* gene (Fig. 1), these observations further suggest that the BrGI protein is under the control of the circadian clock and is functionally similar to Arabidopsis GI. To examine the hypothesis that BrGI is similar in function to GI, we transformed the Arabidopsis *gi-201* null mutant, which exhibits extremely late flowering under LD conditions, with the *BrGI* gene under the control of the CaMV 35S promoter. The flowering phenotypes of homozygous transgenic plants were compared with that of the parental *gi-201* mutant. The resultant transgenic plants (*35S::BrGI* in *gi-201*) flowered earlier than did the *gi-201* mutant (Fig. 2c). These results demonstrate that the Br*GI* gene rescued the *GI* loss-of-function mutant in terms of flowering time.

Effect of reduced *GI* expression on Arabidopsis plants

In Arabidopsis, *GI* contributes to the flowering and abiotic stress response pathways (Fowler et al. 1999; Edwards et al. 2011; Xie et al. 2015). We generated eight *BrGI*-RNAi constructs (Fig. 3a) and transformed them into Arabidopsis Col-0 to assess their functionality. Semi-quantitative PCR and qRT-PCR established that each of the RNAi lines showed reduced *GI* expression relative to expression in Col-0 (Fig. 3b, c). However, flowering time was not delayed relative to that in Col-0, suggesting that the reduction in *GI* expression was insufficient to delay flowering. In contrast, several RNAi lines (GK5, GK7, and GK8) were more tolerant to NaCl stress than was Col-0 and exhibited similar salt tolerance to those of the *gi-201* null mutants (Fig. 3d).

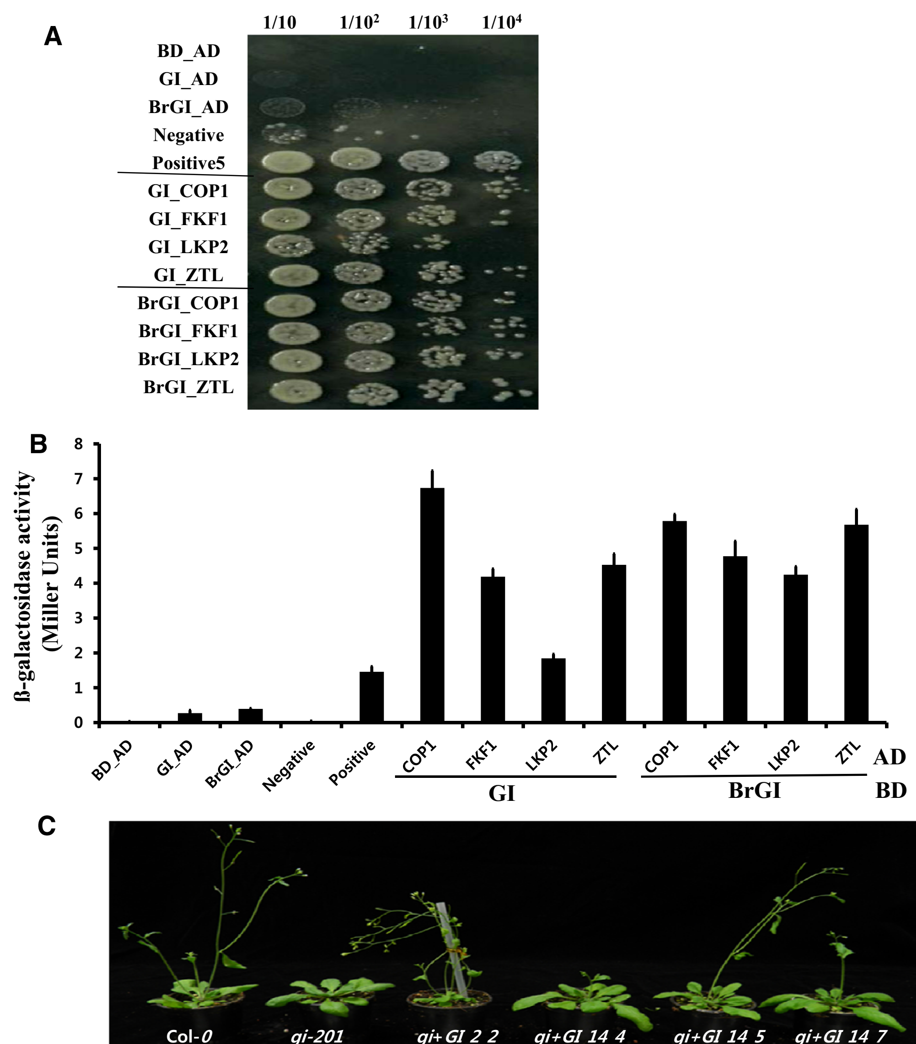

Fig. 2 Characterization of the BrGI protein. **a** Interaction of BrGI with ZTL/FKF1/LKP2 and COP1 in yeast two-hybrid assays. The pDEST^{TM}32 vector containing full-length *BrGI* was used as bait. The prey pDEST^{TM}22 constructs contained *COP1*, *ZTL*, *FKF1* or *LKP2*. The pDEST^{TM}22 vector containing the *GAL4* gene was used as a positive control. AD–BD indicates a control cotransformation using the pDEST^{TM}32 and pDEST^{TM}22 vectors, which contain only the binding domain (BD) or the activation domain (AD), respectively. **b** β-Galactosidase activity assays (mean ± SEM of three measurements). **c** Complementation of the late-flowering Arabidopsis *gi-201* mutant with *35S:BrGI*. Arabidopsis (*gi-201*) was transformed with the *BrGI* gene under the control of the CaMV 35S promoter. Four-week-old plants grown in soil under LD conditions were photographed

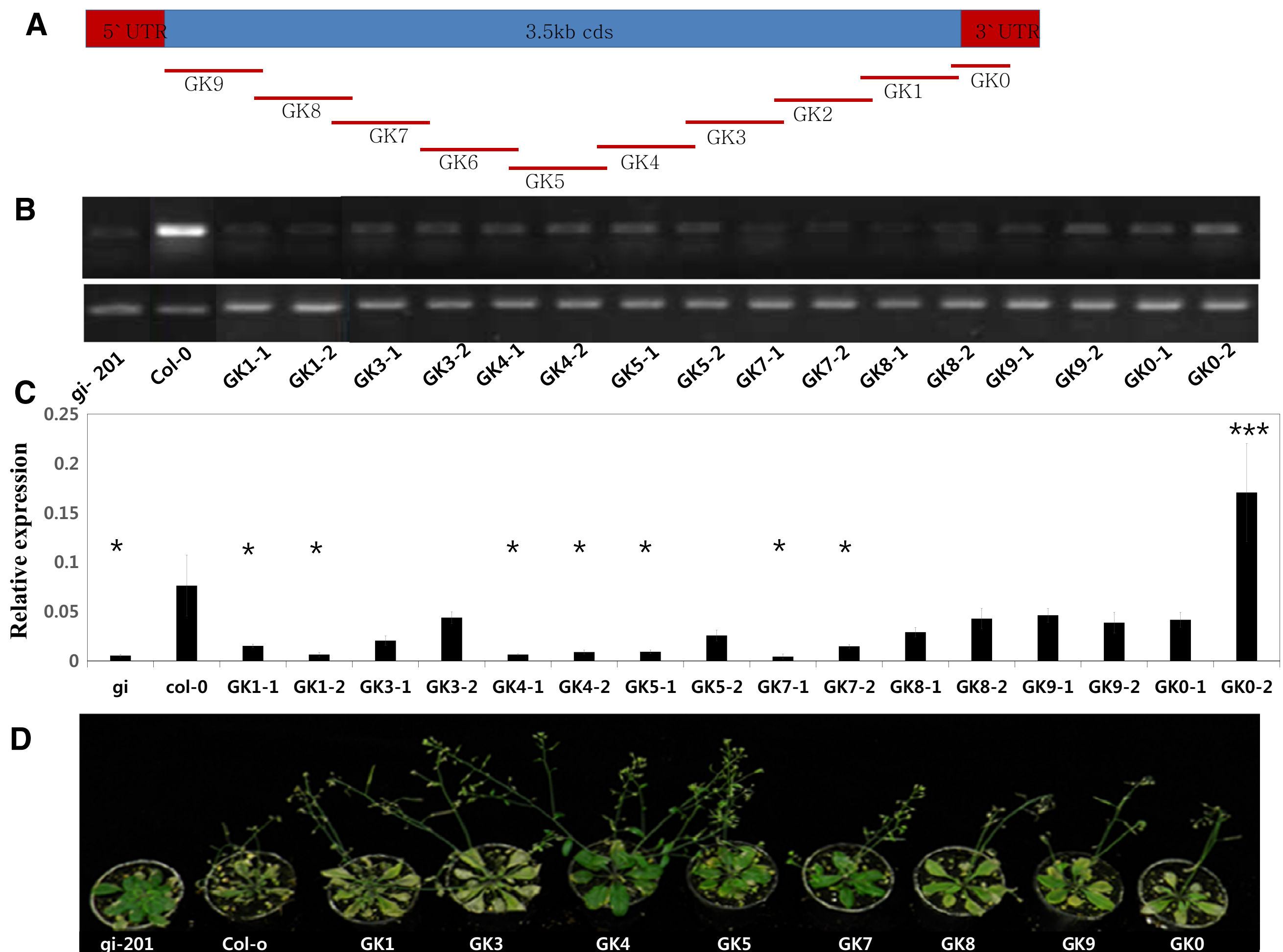

Fig. 3 Knockdown of *GI* expression in Arabidopsis using RNAi constructs derived from *BrGI* enhances salt tolerance. **a** Locations of the *BrGI* RNAi constructs inserted into the pB7GWIWG2(II) binary vector and transformed into Arabidopsis. **b** *GI* expression in transgenic lines as determined by semiquantitative PCR. **c** *GI* expression in *transgenic lines* as determined by qRT-PCR. Data are presented as mean ± SEM of three technical replicates. *Asterisk* indicates value that are significantly different ($p < 0.05$), as determined by Comparisons with a control using Dunnett's Method (Table S3). **d** The salt tolerance of transgenic T3 plants grown for 2 weeks at 23°C under LD conditions and then watered with 250 mM NaCl every 2 days for 2 weeks

Reduced expression of *BrGI* increases the tolerance to salt stress of transgenic Chinese cabbage

To evaluate the effect of *BrGI* expression in *B. rapa*, we transformed all of the *BrGI*-RNAi constructs into the *B. rapa* accession DH03, a Chinese cabbage. Using a tissue culture approach we obtained several transformants harboring the GK1 and GK4 RNAi constructs. BASTA-resistant transgenic plants regenerated from callus cultures were self-pollinated to obtain homozygous seeds, which were assessed for tolerance to salt stress. These transgenic plants could germinate and grow in the presence of 75 and 125 mM NaCl but failed to grow at 250 mM NaCl (Fig. S2). We further characterized four lines (GK1_11, GK-_12, GK4_4, and GK4_6) that showed stable tolerance to salt stress. Reduced *GI* expression resulted in increased root growth in the presence of 150 mM NaCl (Fig. 4a), supporting the increased tolerance to salt of our *GI* RNAi lines of *B. rapa*. Most transformants showed decreased *BrGI* mRNA levels compared with non-transgenic plants (Fig. 4b). We evaluated the effect of RNAi knockdown of *GI* mRNA on GI protein abundance; GI protein levels were reduced to a greater extent in GK1_11 and GK1_12 than in GK4_4 (Figs. 4c, S3). When 2-week-old hydroponically grown seedlings were exposed to 150 mM NaCl for 1 week, non-transformed *GI* wild-type plants withered, but most *GI* knockdown transformants survived. Thus the reduced *BrGI* expression enhanced the ability of the plants to survive after 1 week of exposure to high (150 mM) NaCl (Fig. 5a). Both GK4 lines, which retained greater *GI* expression than that of the GK1 lines, showed increased interveinal chlorosis (Figs. 5b, S4). In *GI*-knockdown

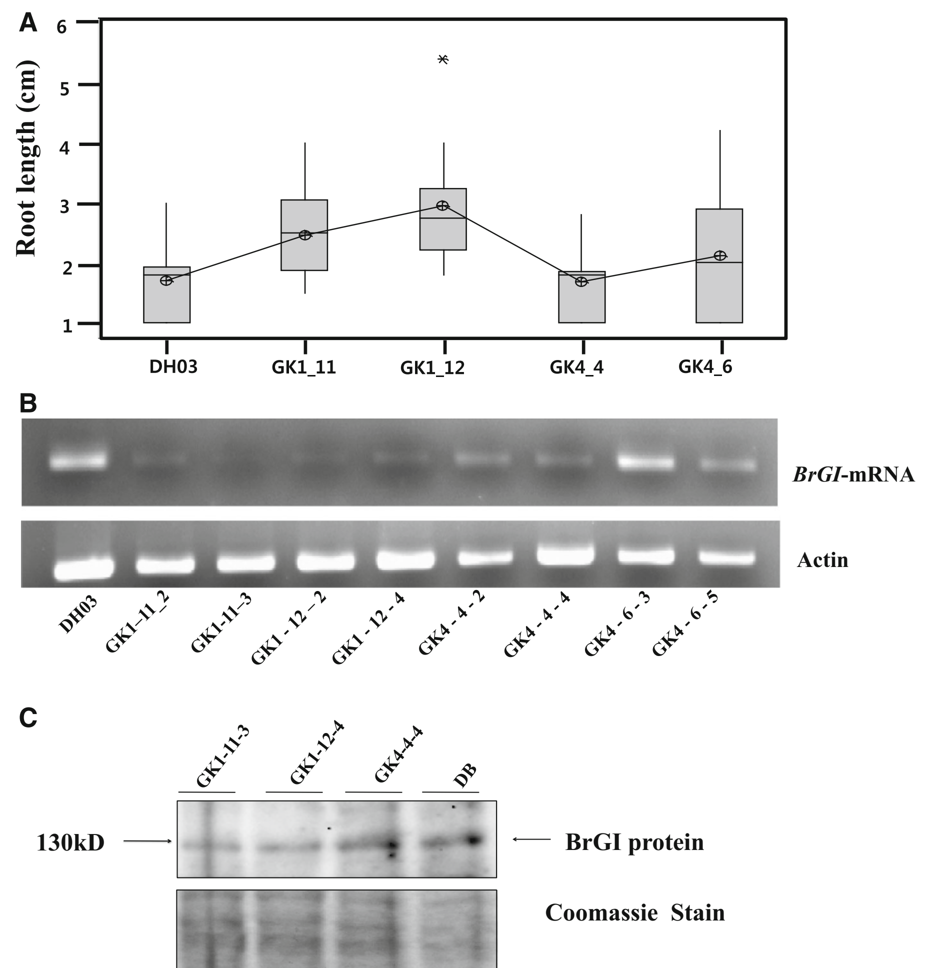

Fig. 4 Knockdown of *GI* expression in *B. rapa* by RNAi enhances salt tolerance. **a** Root length (mean ± SEM, $n = 15$) of *B. rapa* transgenic lines, GK1-11, 1-12, 4-4, and 4-6, and an inbred line, DH03, grown on ½ MS salts, 2 % sucrose, and 1 % agar supplied with 150 mM NaCl. *Asterisk* indicates value that are significantly different ($p < 0.05$), as determined by ANOVA followed by Tukey's test (Table S4). **b** *BrGI* mRNA abundance in transgenic RNAi lines and wild-type DH03 measured by semiquantitative PCR. **c** GIGANTEA protein abundance in 2-week-old transgenic *B. rapa* and non-transformed DH03 lines measured by Western blot analysis using an antibody directed against Arabidopsis GI

RNAi lines, chlorosis was less severe than that in the wild-type, DH03 (Fig. 6a). The tolerance of each genotype to salt was quantified in terms of fresh weight, chlorophyll content and chlorophyll fluorescence. Each metric was expressed as the ratio of the value in salt-stressed seedlings to that in unstressed seedlings. As a positive control, we showed that a loss-of-function *gi-1* mutant obtained by TILLING in *B. rapa* R-o-18 increased salt tolerance, as determined by photosynthetic efficiency (Fv/Fm) (Fig. 6b), consistent with the results of Xie et al. (2015). In response to salt stress, fresh weight and photosynthetic efficiency (Fv/Fm) were significantly greater in the GK1-11 and GK1-12 lines than in the DH03, GK4-4 and GK4-6 lines.

Discussion

The considerable influence of the circadian clock on plant life is mediated, at least in part, by widespread regulation of gene expression; roughly one-third of the plant transcriptome shows circadian oscillations in transcript abundance (Covington et al. 2008), and an even larger proportion exhibits robust oscillation under diurnal cycles of light and temperature (Michael et al. 2008). It is becoming increasingly clear that this widespread circadian clock control enhances the growth and fitness of plants (Edwards et al. 2011; Hsu and Harmer 2014; Kim et al. 2008), and modulation of circadian clock function has been suggested as a strategy to ameliorate the adverse consequences of climate change (Kim et al. 2013b; Sanchez et al. 2011; Seo et al. 2012).

GI is encoded by a single gene in Arabidopsis (Huq et al. 2000), is plant specific, and has been found in all plants (Hayama et al. 2002; Dunford et al. 2005; Zhao et al. 2005; Curtis et al. 2002). *GI* was first characterized with respect to its functions in the elicitation of photoperiod-dependent flowering and in circadian clock maintenance (Koornneef et al. 1991; Fowler et al. 1999; Park et al. 1999; Mizoguchi et al. 2005). More recently, it has been discovered that *GI* also plays important roles in the response to multiple abiotic stresses, including tolerance to high salt and low (freezing) temperature (Cao et al. 2005; Kim et al. 2013b; Riboni et al. 2013; Xie et al. 2015).

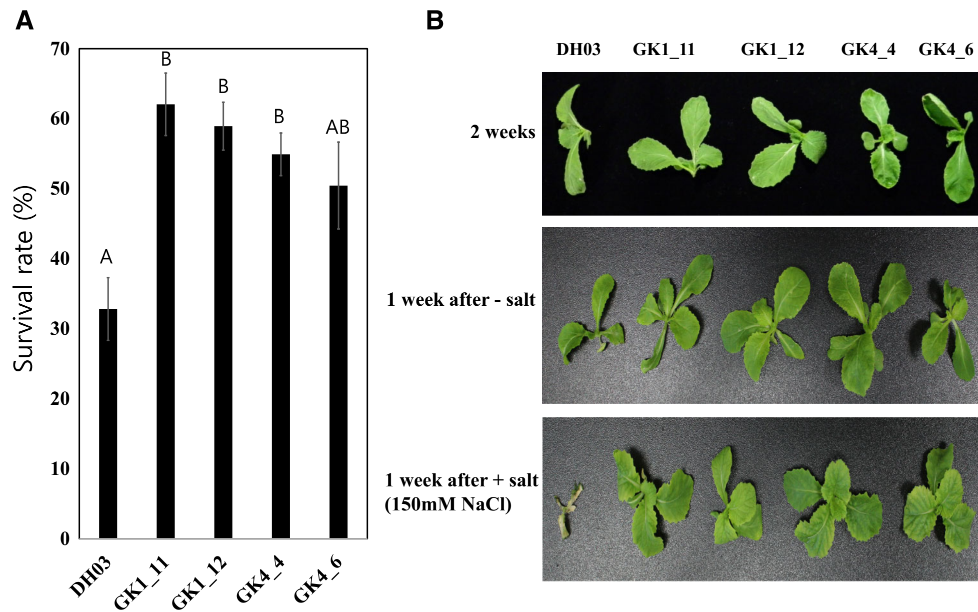

Fig. 5 Responses to salt stress of *B. rapa* grown in hydroponic culture. Seeds were sown in sponge cubes and supplemented with nutrient solution for 2 weeks. After two to three true leaves had developed, the nutrient solution was replaced with fresh nutrient solution amended to include 150 mM NaCl every 2 days for 2 weeks. **a** Survival rate after salt treatment (%, presented as mean ± SEM of three biological replicates, each containing 14 seedlings per genotype). Different letters indicate values that are significantly different ($p < 0.05$), as determined by ANOVA followed by Tukey's test (Table S5). **b** Images of plants from one representative experiment

Since its divergence from Arabidopsis, the *B. rapa* genome has undergone whole genome triplication followed by considerable fractionation (gene loss) (Parkin et al. 2005; Wang et al. 2011). Although circadian clock genes have been retained preferentially in the *B. rapa* genome (Lou et al. 2012), only a single copy of *GI* persists (Wang et al. 2011). The circadian cycling of *BrGI* mRNA abundance is similar to that of Arabidopsis *GI* (Fig. 1; Fowler et al. 1999; Park et al. 1999; Xie et al. 2015). *BrGI* also exhibits functional conservation with Arabidopsis *GI*, and expression of *BrGI* in the Arabidopsis late-flowering *gi-201* mutant restores flowering time to that of the wild-type (Xie et al. 2015). In Arabidopsis, GI functions at least in part through direct interactions that affect the functions of other proteins (Sawa et al. 2007; Kim et al. 2007c, 2013c; Tseng et al. 2004). Consistent with this functional conservation, we show that BrGI retains a number of protein–protein interactions defined for Arabidopsis GI. GI physically and functionally interacts with ZTL, FKF1, and LKP2, which serve as blue light receptors that mediate light input to the clock (Kim et al. 2007c; Tseng et al. 2004). GI establishes and sustains oscillations of ZTL and maintains the TOC1 and PRR5 rhythms necessary for proper clock function through the GI–ZTL interaction, which mediates the regulated proteasomal degradation of TOC1 and PRR5 (Kim et al. 2007c). Yeast two-hybrid assays have also identified additional GI-interacting proteins, such as ADL3, a plant dynamin-like protein, and the COP1 E3 ubiquitin ligase, which serves as a repressor of light signal transduction (Abe et al. 2008; Yu et al. 2008). BrGI physically interacted with COP1 as well as with the ZTL/FKF1/LKP2 LOV-domain-based protein group (Fig. 2; Zoltowski and Imaizumi 2014). These observations strongly suggest that the BrGI protein is a functional *Brassica rapa* ortholog of GI that contributes to regulation of circadian rhythms and promotes photoperiodic flowering.

Recent studies have examined the role of *GI* in the *B. rapa* oilseed variety, R-o-18, but similar resources are not available for multiple varieties and for other morphotypes. We wished to expand our genetic analysis of *GI* into additional genetic backgrounds. Accordingly, using our transformation protocol, we perturbed *GI* expression through RNAi in a Chinese cabbage morphotype of *B. rapa* (Fig. 4c) and found that reduction of *GI* expression enhanced salt tolerance. These results are consistent with those of others (Kim et al. 2013b; Xie et al. 2015), but extend the results obtained from the oilseed and rapid cycling morphotypes to Chinese cabbage.

One important emphasis of current agricultural research is translation of our improved understanding of molecular mechanisms obtained from studies of model plants to diverse crops of agricultural significance. RNAi strategies have the potential to improve the resistance of crop plants against biotic stress, as well as increase knowledge of target genes (Koch and Kogel 2014). In this study, we implemented an RNAi strategy to manipulate expression of *BrGI* with the aim of improving salt tolerance in Chinese cabbage. We showed that

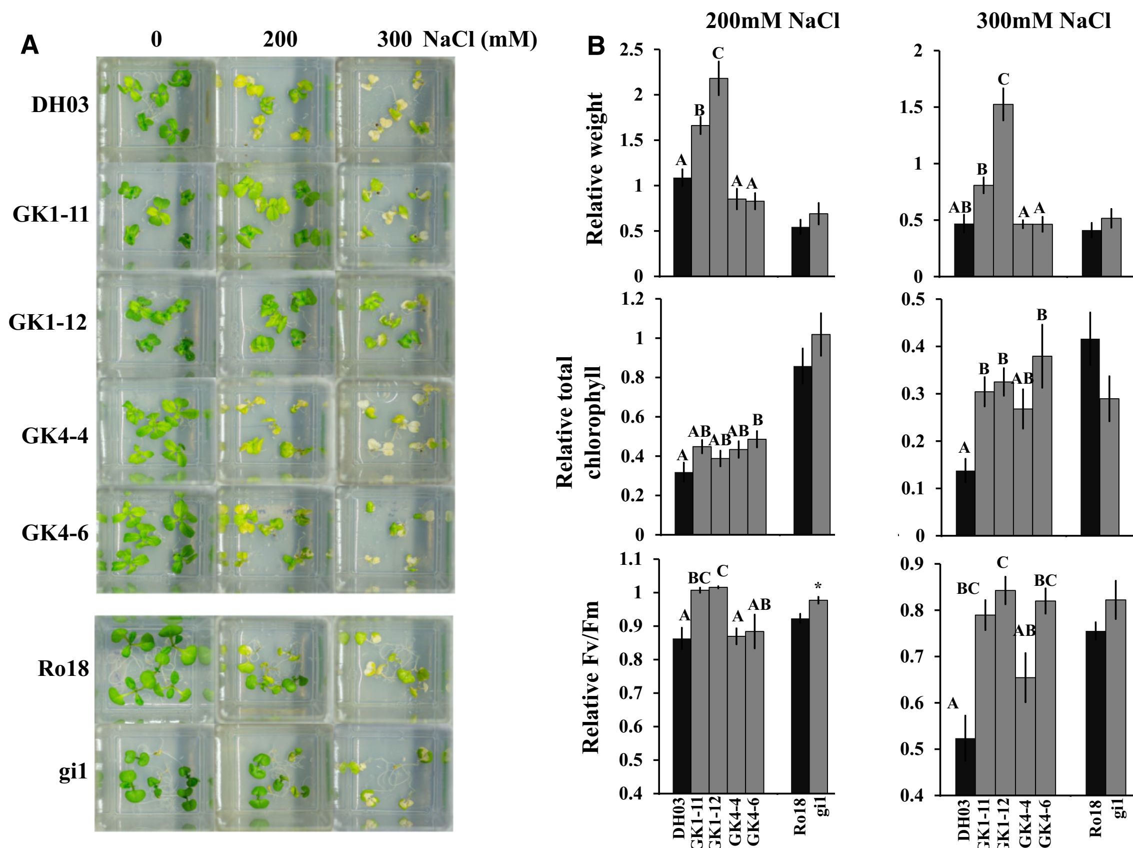

Fig. 6 Effects of reduced GI expression on salt tolerance of *B. rapa* grown on solid medium. **a** Plants of the indicated genotypes were grown for 1 week on ½ strength MS medium plus 2 % sucrose and then transferred to fresh medium amended to include 0, 200, or 300 mM NaCl and grown for a further 1 week. **b** Fresh weight, chlorophyll content, and chlorophyll fluorescence of plants from A. Fresh weights are presented as mean ± SEM (from three independent replicates) fresh weights of seedlings ($n = 10$) grown with NaCl/fresh weights of seedlings ($n = 10$) grown without NaCl. Total chlorophyll content is presented as the mean ± SEM total chlorophyll concentration of seedlings ($n = 10$) grown with the indicated NaCl/total chlorophyll concentration of seedlings ($n = 10$) grown without NaCl. Fv/Fm data are presented as the mean ± SEM Fv/Fm of seedlings ($n = 10$) grown with NaCl/Fv/Fm of seedlings ($n = 10$) grown without NaCl. Different letters indicate values that are significantly different as determined by ANOVA followed by Tukey's test (Table S6). Isogenic wild-type *B. rapa* R-o-18 and loss-of-function GI mutations were statistically compared using Student's *t* test. $^{*}p < 0.05$

decreased *BrGI* mRNA and protein levels in *B. rapa* enhanced salt tolerance compared with the wild-type (Figs. 5, 6). Critically, the partial downregulation of *GI* expression achieved via RNAi was sufficient to increase salt tolerance, yet was insufficient to delay flowering, which would likely have serious adverse consequences for crop yield. These results confirm *GI* as an attractive target for further efforts to improve a number of stress responses in agriculturally significant crops through transgenic manipulation. Moreover, standing natural variation in *GI* present among diverse cultivars (this study and Xie et al. 2015) also suggests *GI* to be a tractable target for conventional molecular breeding to enhance abiotic stress tolerance in both *B. rapa* and other crops.

Author contribution statement JAK and WYK conceived and designed research. JAK, HJ, and VH conducted experiments. JKH, YHL, and JYK contributed new analytical tools. MJJ, JK, and DJY contributed plant materials and mutant seeds. JAK and CRM wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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