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Allelic polymorphism of GIGANTEA is responsible for naturally occurring variation in circadian period in Brassica rapa

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GIGANTEA (GI) was originally identified by a late-flowering mutant in Arabidopsis, but subsequently has been shown to act in circadian period determination, light inhibition of hypocotyl elongation, and responses to multiple abiotic stresses, including tolerance to high salt and cold (freezing) temperature. Genetic mapping and analysis of families of heterogeneous inbred lines showed that natural variation in GI is responsible for a major quantitative trait locus in circadian period in Brassica rapa. We confirmed this conclusion by transgenic rescue of an Arabidopsis gi-201 loss of function mutant. The two B. rapa GI alleles each fully rescued the delayed flowering of Arabidopsis gi-201 but showed differential rescue of perturbations in red light inhibition of hypocotyl elongation and altered cold and salt tolerance. The B. rapa R500 GI allele, which failed to rescue the hypocotyl and abiotic stress phenotypes, disrupted circadian period determination in Arabidopsis. Analysis of chimeric B. rapa GI alleles identified the causal nucleotide polymorphism, which results in an amino acid substitution (S264A) between the two GI proteins. This polymorphism underlies variation in circadian period, cold and salt tolerance, and red light inhibition of hypocotyl elongation. Loss-of-function mutations of B. rapa GI confer delayed flowering, perturbed circadian rhythms in leaf movement, and increased freezing and increased salt tolerance, consistent with effects of similar mutations in Arabidopsis. Collectively, these data suggest that allelic variation of GI—and possibly of clock genes in general—offers an attractive target for molecular breeding for enhanced stress tolerance and potentially for improved crop yield.

Significance

The plant circadian clock affects many aspects of growth and development and influences both fitness in natural settings and performance in cultivated conditions. We show that GIGANTEA (GI) underlies a major quantitative trait locus for circadian period in Brassica rapa by fine-mapping, analysis of heterogeneous inbred lines, and transgenic rescue of an Arabidopsis gi-201 loss-of-function mutant. Analysis of chimeric and mutated B. rapa GI alleles identified the causal nucleotide polymorphism responsible for the allelic variation in circadian period, cold and salt tolerance, and red light inhibition of hypocotyl elongation. Allelic variation of GI and of clock genes in general offers targets for marker-assisted (molecular) breeding for enhanced stress tolerance and potentially for improved crop yield.
**Fig. 1.** Fine mapping and genetic definition of the PERA9a circadian period QTL. (A) Circadian period QTL on chromosome 9 (PERA9a), redefined by composite interval mapping using the data of Lou et al. (14). (B) Fine mapping of the PERA9a QTL using new molecular markers flanking the QTL. (C) Three overlapping BAC clones (17) spanning the PERA9a QTL with molecular markers used for fine mapping. (D) Period analysis of HIL RIL256, R500 homozygous for R500 and RIL256 IMB211 homozygous for IMB211 in the region of the PERA9a QTL. Box plots show median with the bottom and top of the box indicating 25% and 75%, respectively; whiskers indicate the maximum and minimum values.

PERA9a was detected both at 12° and at 25 °C and at each temperature explained ~10% of the variation in period length (14). To confirm the presence of PERA9a by genetic means, we took advantage of residual heterozygosity in the GI region of chromosome A9 in RIL256 (Fig. S1). We allowed RIL256 to self-fertilize and identified lines homozygous for IMB211 (RIL256 IMB211) or for R500 (RIL256 R500) in the GI region of A9 in an otherwise uniform genetic background. An additional recombination during the development of RIL256 R500 reduced the region of R500 DNA to ~10 cM covering the PERA9a QTL (Fig. S1A). The period of RIL256 R500 was significantly shorter than that of RIL256 IMB211 (Fig. 1D and Table S1), which is consistent with the effects of the QTL (14).

If GI were indeed the gene responsible for the PERA9a QTL, it should be polymorphic in either sequence or expression level between the RIL parents. We detected many single nucleotide polymorphisms (SNPs) in the transcribed portions of the two parental GI alleles (Fig. 2A). Most SNPs were predicted to be functionally silent, either falling in introns or failing to change the predicted amino acid sequence. However, three SNPs at nucleotide positions 1,274; 2,208; and 2,475 (numbered relative to the A of the initiator AUG in a multiple sequence alignment) were predicted to result in the amino acid polymorphisms S264A, I541T, and D610V, respectively, where the amino acid found in IMB211 is listed before and that found in R500 is listed after the amino acid number. To test the functionality of the two alleles, we performed transgenic rescue experiments in the Arabidopsis gi-201 loss-of-function background, introducing full genomic copies of each B. rapa GI allele driven by their endogenous promoters. In our hands at 22 °C, gi-201 does not affect circadian period (Fig. 2B, Fig. S2, and Table S1), consistent with earlier observations with gi-2, another strong loss-of-function allele (22). gi-201 mutants are late flowering (Fig. 3A and Fig. S3A) and exhibit a long hypocotyl in red and blue light (Fig. 3B and Fig. S3B). Both the IMB211 (BrGI<sub>IMB211</sub>) and R500 (BrGI<sub>R500</sub>) GI alleles fully rescued the late flowering defect of Arabidopsis gi-201 (Fig. 3A and Fig. S3A), indicating that both alleles are expressed and functional, at least for flowering time. In contrast, BrGI<sub>IMB211</sub>, but not BrGI<sub>R500</sub>, fully rescued the long hypocotyl in red light (Fig. 3B), indicating that BrGI<sub>R500</sub> is defective in this trait. Both BrGI<sub>IMB211</sub> and BrGI<sub>R500</sub> rescued the long hypocotyl in blue light (Fig. S3B), although the rescue by BrGI<sub>R500</sub> was only partial, suggesting that it is partially but not fully functional for this trait. BrGI<sub>IMB211</sub> had no effect on circadian period of gi-201 but, strikingly, introduction of BrGI<sub>R500</sub> resulted in a significant period shortening (Fig. 2B, Fig. S2A, and Table S1), consistent with the shorter period of R500 relative to IMB211. Both BrGI alleles show similar expression patterns in the B. rapa RIL parents (Fig. S4A) and in Arabidopsis gi-201 (Fig. S4B), indicating that the differential effect on circadian period does not result from differential expression of the two alleles.

As noted above, three SNPs were predicted to change the amino acid sequence of the GI protein. To determine whether one or more of those changes was responsible for the functional differences between the two alleles, we constructed chimeric alleles, BrGI<sub>IMB211 R500</sub> and BrGI<sub>R500 IMB211</sub>, in which a SacI–NdeI restriction fragment containing four SNPs at nucleotides 1,210; 1,213; 1,274; and 1,295 (Fig. 2A) was exchanged.
When tested in transgenic plants, BrGI\textsuperscript{R500} shortened period, but BrGI\textsuperscript{R500}(IMB211) did not (Fig. 2B, Fig. S2A and B, and Table S1). Conversely, although BrGI\textsuperscript{IMB211}(R500) did not shorten period, BrGI\textsuperscript{IMB211}(R500) shortened period to the same extent as BrGI\textsuperscript{R500} (Fig. 2B, Fig. S2A and B, and Table S1). Thus, we conclude that the differential effects of these two alleles on period length are conferred by the polymorphisms in this SacI–NdeI restriction fragment. Of the four SNPs in this region, only the one at nucleotide 1,274 was predicted to change the amino acid sequence.

We therefore used site-directed mutagenesis to make two new GI alleles, BrGI\textsuperscript{IMB211(S264A)} in which nucleotide 1,274 of BrGI\textsuperscript{IMB211} was changed from T to G, changing amino acid 264 from S to A, and BrGI\textsuperscript{R500(A264S)} in which nucleotide 1,274 of BrGI\textsuperscript{R500} was changed from G to T, changing amino acid 264 from A to S. These alleles were introduced into Arabidopsis gi-201. Although BrGI\textsuperscript{R500} shortened period, BrGI\textsuperscript{R500(A264S)} did not (Fig. 2B, Fig. S2A and C and Table S1). Conversely, although BrGI\textsuperscript{IMB211(S264A)} did not shorten period, BrGI\textsuperscript{IMB211(S264A)} shortened period to the same extent as BrGI\textsuperscript{R500} (Fig. 2B, Fig. S2A and C, and Table S1). All of the BrGI alleles were similarly expressed in Arabidopsis gi-201 seedlings (Fig. S4B and C), indicating that the different effects on circadian period do not result from differential expression of the transgenes. Thus, we conclude that the polymorphism at nucleotide 1,274 (T1274G, encoding S264A, with the IMB211 allele listed first) is responsible for the differential effects of these two alleles on period length and that it is the presence of R500 information at nucleotide 1,274, encoding an A residue, that shortens period.

We also tested the phenotypic consequences of introduction of these chimeric and point mutant BrGI alleles on flowering time and hypocotyl length. All six BrGI alleles rescued the late flowering time of Arabidopsis gi-201 equally (Fig. 3A and Fig. S3A). As indicated above, BrGI\textsuperscript{IMB211} rescued the long hypocotyl in red light (Fig. 3B). Both BrGI\textsuperscript{R500(IMB211)} and BrGI\textsuperscript{R500(A264S)} rescued the long hypocotyl in red light (Fig. 3B). In blue light, a similar pattern was observed, although in this case BrGI\textsuperscript{R500}, BrGI\textsuperscript{IMB211(R500)}, and BrGI\textsuperscript{IMB211(S264A)} each retained partial function and partially but not fully rescued the long hypocotyl phenotype of gi-201 (Fig. S3B). Thus, we conclude that the polymorphism at nucleotide 1,274 is responsible for the differential effects of these alleles on hypocotyl length, and the presence of IMB211 information at nucleotide 1,274 is necessary to fully rescue the long hypocotyl in blue and red light phenotypes of gi-201.

Our transgenic rescue experiments established the importance of BrGI in circadian period and flowering time determination in Arabidopsis. To test whether GI functions similarly in B. rapa, we identified a set of TILLING mutations in B. rapa R-o-18, which is closely related to R500 (23). Two putative null alleles, gi-1 and gi-3, were predicted to be strong loss-of-function alleles due to missense mutations (gi-1, G1223A; W258Stop and gi-3, G1737A; W375Stop) and were analyzed after two back-crosses to the R-o-18 parent. B. rapa lines homozygous for either gi-1 or -3 flower late (Fig. 4A and B), consistent with loss of GI function. Circadian period was unaffected at 18 °C (Fig. 4C–E), but at 22 °C, most seedlings were arrhythmic, and those that showed circadian rhythms in leaf movement (31% of B. rapa gi-1 and 42% of gi-3 mutant seedlings) showed long period and increased relative amplitude error (RAE), a measure of the strength of a circadian rhythm (Fig. 4E and Table S1). Thus, mutational disruption of GI weakens rhythms in B. rapa leaf movement at high temperature. This finding is consistent with Arabidopsis, where GI plays a critical role in maintaining rhythmicity in leaf movement at higher temperatures (24). However, the role of GI in rhythmicity may not be limited to high temperature. In Arabidopsis, GI is important for maintenance of rhythmicity of CAB2:LUC expression at both high and low temperatures (24).
 freezing tolerance (Fig. 3C). However, transformation of gi-201 with BrGI$^{R500}$ or with either of the two BrGI$^{R500}$ alleles carrying IMB211 information at nucleotide 1,274 (BrGI$^{IMB211}$ or BrGI$^{IMB211(S264A)}$) significantly reduced freezing tolerance, rescuing the gi-201 phenotype of increased freezing tolerance (Fig. 3C). Thus, we conclude that B. rapa GI plays a role in freezing tolerance and that the BrGI$^{IMB211}$ allele is functional in this role, whereas the BrGI$^{R500}$ allele has at least partially lost this function, as has the Arabidopsis gi-201 allele. To confirm that GI also serves as a determinant of freezing tolerance in B. rapa, we tested B. rapa gi-1 and -3 mutants and found that loss of GI function enhanced freezing tolerance, compared with the cognate wild type, R-o-18 (Fig. 4F).

Our data also suggest a role for GI in nitrogen accumulation. gi-201 accumulates less N than does Col-0 (Fig. S3C). BrGI$^{R500}$ and BrGI$^{R500}$ rescue the N content of gi-201, but BrGI$^{R500}$ and BrGI$^{R500}$ do not. This finding suggests that IMB211 information at nucleotide 1,274 is necessary for this rescue. However, both point mutants (BrGI$^{IMB211(S264A)}$ and BrGI$^{IMB211(S264A)}$) rescue the N content of gi-201, which is inconsistent with the difference between the two alleles being solely attributable to that single position. Thus, regulation of leaf N content by allelic state at GI is not strongly supported by our data; further investigation will be required to resolve the discrepancy among rescue lines.

In contrast, we found no difference among our panel of Arabidopsis gi-201 mutants carrying the B. rapa GI alleles for delta C13 (Fig. S3D) and rosette size (Fig. S3E). Thus, these alleles of GI do not affect water use efficiency or plant size.

In Arabidopsis, GI has been shown to function as a negative regulator of resistance to salt stress, and gi loss-of-function mutants show increased salt resistance (28). Accordingly, we tested our panel of Arabidopsis gi-201 mutants carrying the B. rapa GI alleles for salt tolerance as measured by fresh weight of aerial tissues following growth in the presence or absence of NaCl. Growth of wild-type Col-0 seedlings or of glabrous-1 (gl-1) was reduced by $\sim$1/3 at 15 mM NaCl, but gi-201 was unaffected. Transformation of gi-201 with BrGI$^{R500}$ or with BrGI$^{R500}$ had no effect on the increased salt tolerance of gi-201 (Fig. 3D and Fig. S5). However, transformation of gi-201 with BrGI$^{R500}$ or with BrGI$^{R500}$ significantly reduced salt tolerance, rescuing the gi-201 phenotype of increased salt tolerance (Fig. 3D and Fig. S5). Thus, we conclude that B. rapa GI plays a role in salt tolerance and that the BrGI$^{IMB211}$ allele is fully functional in this role, whereas the BrGI$^{R500}$ allele is impaired in this function, as is the Arabidopsis gi-201 allele. To confirm that GI also serves as a determinant of resistance to salt stress in B. rapa, we tested B. rapa gi-1 and -3 mutants and found that loss of GI function enhanced salt stress resistance compared with the cognate wild type, R-o-18 (Fig. 4G).

Discussion
GI was first identified as a supervital mutant of Arabidopsis (29), with a prolonged phase of vegetative growth that increased reproductive capacity (30, 31). GI, in addition to this role in photoperiodic flowering, also is required for phytochrome signaling (20, 32, 33) and for proper clock function (18, 19), although the roles of GI in photoperiodic flowering, photomorphogenesis, and the clock can be dissociated (20, 33, 34).

Our results with the B. rapa GI alleles extend conclusions drawn from Arabidopsis to Brassica. Both BrGI$^{R500}$ and BrGI$^{IMB211}$ fully rescue the photoperiodic flowering defect of Arabidopsis gi-201 mutants. gi-201 is a strong loss-of-function allele, and full rescue of the gi-201 late-flowering phenotype demonstrates that both Brassica alleles are equally functional in terms of flowering time in Arabidopsis. However, the two B. rapa GI alleles are not equally functional in terms of hypocotyl elongation, freezing tolerance, and salt tolerance. In each case, BrGI$^{IMB211}$ fully rescued the gi-201 phenotype, whereas BrGI$^{R500}$
failed to rescue. Thus, the flowering timing function is distinct from the hypocotyl elongation, freezing tolerance, and salt tolerance functions. Amino acid 264 (A in R500 vs. S in IMB211) is therefore important for these latter three functions. Amino acid 264 is also important for circadian period determination, because introduction of BrGI_{R500} and BrGI_{IMB211} alleles carrying R500 information at amino acid 264, but not BrGI_{IMBE211} (and BrGI_{R5000} alleles carrying IMB211 information at amino acid 264), shortens circadian period.

GI functions typically involve protein–protein interactions. For example, in flowering timing, GI interacts with the F-box protein FLAVIN BINDING KELCH REPEAT F-BOX PROTEIN1 (FKF1), and in the afternoon this GI–FKF1 complex degrades CYCLING DOF FACTORS (CDFs) bound at the CONSTANS (CO) promoter, relieving transcriptional repression and allowing the accumulation of CO mRNA in the light (35, 36). CO protein is stabilized in the light, accumulates, and induces expression of FT, which induces floral identity genes. GI also binds to the FT promoter and interacts directly with the FT repressors, SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO 1 (TEM1), and TEM2, to directly induce FT (37). Relevant to the nuclear roles of GI in the regulation of CO and FT expression is a protein–protein interaction with ELF4 that confers sub-nuclear localization and restricts chromatin access of GI (38). Finally, GI protein stability is controlled by interaction with the E3 ubiquitin ligase EARLY FLOWERING3 (ELF3) and the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHGENIC1 (COP1) (39). At least some of these functions are retained in B. rapa, because B. rapa gi loss-of-function mutants are late flowering. Both BrGI alleles fully rescue the flowering delay of Arabidopsis gi-201, suggesting that both BrGI proteins retain all these interactions.

In its role in circadian period determination, in the light GI binds to and stabilizes ZEITLUPE (ZTL), an F-box protein closely related to FKF1. The conformational shift in ZTL that follows the cessation of blue light signaling after dusk releases GI, freeing ZTL to interact with and thereby target critical clock transcriptional repressors, TOC1 and PRR5, for ubiquitination and proteasomal degradation (40, 41). Because ZTL is a cytoplasmic protein, the ZTL–GI interaction also retains GI in the cytosol, thereby limiting the nuclear accumulation of GI and antagonizing its roles in flowering timing and regulation of hypocotyl length (42).

The effects on circadian period of BrGI_{R500} and BrGI_{IMB211} and their derivatives when transformed into Arabidopsis gi-201 demonstrate an important role for amino acid 264 in circadian period definition. First, we note that BrGI_{R500} shortens circadian period length and acts as a gain-of-function mutant relative to BrGI_{IMBE211}. The amino acid change of S264 in BrGI_{IMB211} to A in BrGI_{R500} is similar to that observed in the Arabidopsis gi-200 short period allele (S932A) (20), although the two mutations lie in different regions of the coding sequence. Moreover, the Arabidopsis Col-0 amino acid at the position corresponding to B. rapa GI 264 is A, and this residue is conserved in four Arabidopsis accessions, barley and wheat (12). This finding suggests that the phenotypic consequences of differing information at amino acid 264 cannot be determined solely by the amino acid at that residue but, rather, must be considered in the context of the entire protein sequence.

One possible molecular explanation for the effect of the BrGI_{R500} phenotype of short period could be an increased affinity of BrGI_{R500} for ZTL, which would stabilize ZTL, increase ZTL accumulation, and thereby shorten period (43). This explanation would be consistent with the observed effects on red light inhibition of hypocotyl elongation, because the increased affinity of BrGI_{R500} for ZTL would limit nuclear accumulation and thereby antagonize the ability of BrGI_{R500} to rescue red light inhibition of hypocotyl elongation. However, both B. rapa GI alleles fully rescue the flowering timing phenotype of gi-201.

Therefore, if differential affinities for ZTL explain the period differences of the B. rapa GI alleles, these effects must not limit nuclear accumulation of GI to the point where flowering timing is compromised.

The role of GI in red and blue light suppression of hypocotyl elongation is incompletely defined. GI has been suggested to regulate CRY-mediated blue light signaling (44). The long hypocotyl in red light phenotype of gi-2 is suppressed in spinach-4 (spn-4) gi-2 double mutants, and, consistent with this genetically defined interaction, SPY and GI proteins interact (22).

More recently, GI has emerged as a key player in a number of stress responses, notably to drought, cold, and salinity (25, 27, 28). Loss of GI function in Arabidopsis results in increased tolerance to both freezing and salt stress in Arabidopsis, and our analysis of B. rapa GI loss-of-function mutants shows a similar enhancement of freezing and salt resistance in B. rapa, suggesting that the roles of GI in resistance to these two stresses is conserved between species. Among these stresses, the role of GI in salt tolerance is best understood. In Arabidopsis, GI sequesters SALT OVERLY SENSITIVE2 (SOS2), a protein kinase that serves as a positive regulator of salt tolerance (28). Release of SOS2 from GI in response to elevated salt permits formation of the SOS2/SOS3 protein kinase complex that associates with and phosphorylates SOS1, activating its Na\(^+\)/H\(^+\) antiport activity and enhancing salt tolerance (45). Differential rescue of the salt tolerance phenotype of Arabidopsis gi-201 by BrGI_{IMB211} and BrGI_{R500} allows the prediction of the Arabidopsis model is that BrGI_{IMBE211} has greater affinity for SOS2 than does BrGI_{R5000}.

It is important to note that most of our experiments tested the function of BrGI alleles in Arabidopsis. Extrapolating the complex network of GI functions elucidated in Arabidopsis into B. rapa and determining the mechanistic basis of the functional differences observed between the two BrGI alleles will likely be complicated by the triplication of the B. rapa genome since its separation from Arabidopsis (46). In the B. rapa reference Chifu genome, subsequent gene loss has eliminated two of the duplicated GI copies, leaving a single GI locus (47). Similarly, there are single copies of SOS2 and of TEM2 (47). However, other potential GI interactors are present in two (CDF1, CDF2, CDF3, COP1, ELF3, FT, SPY, SVP, and TEM1) or three (ELF4) copies (47), and some or all of these copies have likely diverged from their Arabidopsis homologs as well as from each other through subfunctionalization. Of interest in the context of circadian period determination, B. rapa has lost all copies of both ZTL and FKF1 (48). In B. rapa, the functions of ZTL and FKF1 are presumably carried out by a third family member, LOV KELCH PROTEIN2 (LKP2), which exhibits partial functional redundancy with ZTL and FKF1 in Arabidopsis (49). In B. rapa, LKP2 is present in three linked copies resulting from a complex tandem gene triplication (48). It will be of interest to determine whether subfunctionalization among these three copies of LKP2 has resulted in the specialization of one or more copies for flowering timing or circadian functions.

Materials and Methods

Plant Materials. All constructs were transformed by floral dip into the Arabidopsis gi-201 mutant (20) carrying the proCCAT:Luc transgene (50). Seeds were sterilized in 20% (vol/vol) bleach and placed on half-strength Murashige and Skoog (MS) medium with 0.8% agar and 2% (wt/vol) sucrose, then stratified for 3 d at 4 °C in the dark.

Fine Mapping the GI QTL. To confirm the circadian period QTL on chromosome 9, simple sequence repeat (SSR) markers were developed based on sequenced BAC clones (17). In total, 13 SSR markers were used to fine-map the A9 QTL region (Table S2). Heterogeneous inbred lines (HILs) were generated from an F4 RIL, RIL256, heterozygous in the A9 QTL region. We then genotyped 144 plants of the F5 generation of RIL256 homozygous for either IMB211 (RIL256_IMB211) or R500 (RIL256_R500) in the QTL region.


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