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GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains

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Flowering of *Arabidopsis* is promoted by long days and delayed by short days. Mutations in the *GIGANTEA* (*GI*) gene delay flowering under long days but have little or no effect under short days. We have now isolated the *GI* gene and show that it encodes a novel, putative membrane protein. By comparing the sequence of the *Arabidopsis* gene with that of a likely rice orthologue and by sequencing mutant alleles, we identify regions of the GI protein that are likely to be important for its function. We show that *GI* expression is regulated by the circadian clock with a peak in transcript levels 8–10 h after dawn. The timing, height and duration of this peak are influenced by daylength. We analysed the interactions between *GI* and the *LHY, CCA1* and *ELF3* genes, previously shown to affect daylength responses; we show that the rhythmic pattern of *GI* expression is altered in the *elf3, CCA1-OX* and *lhy* genotypes, and that *CCA1* and *LHY* expression are reduced by *gi* mutations. Our results are consistent with the idea that *GI* plays an important role in regulating the expression of flowering time genes during the promotion of flowering by photoperiod.

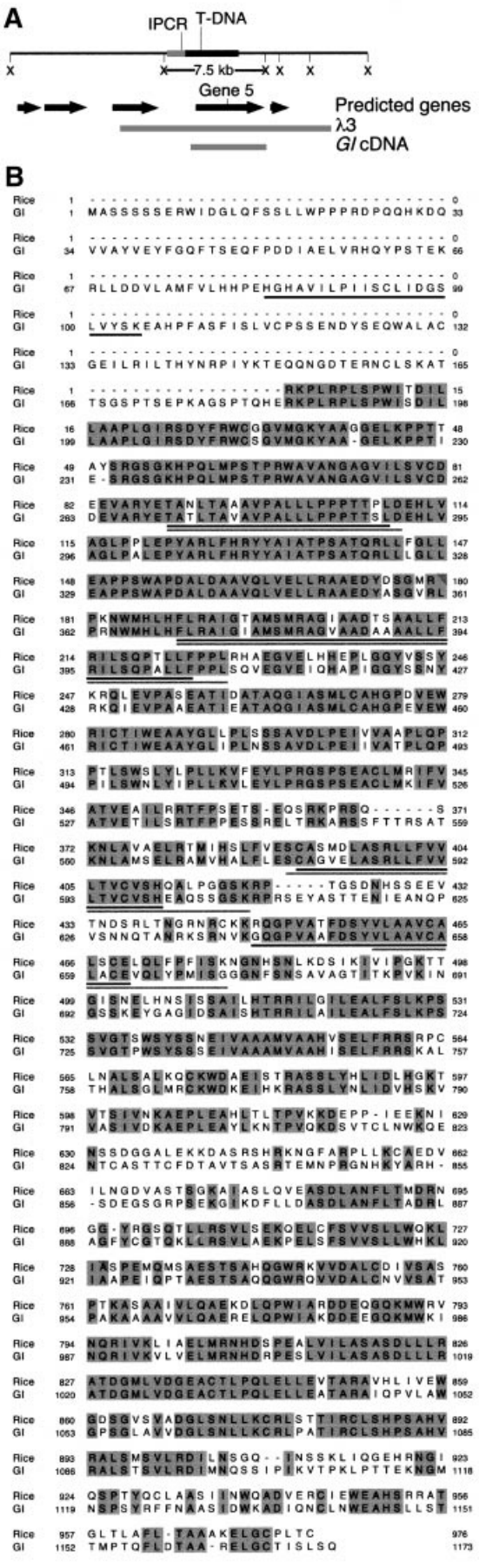
Keywords: *Arabidopsis*/flowering time/*GIGANTEA*/long day/photoperiod

Introduction

The reproduction and behaviour of many organisms are synchronized to favourable seasons of the year (Saunders, 1977). Many species recognize particular seasons by measuring daylength (photoperiod). Photoperiod controls a wide range of processes ranging from migratory restlessness and growth of sexual organs in birds to flowering in plants (Saunders, 1977). *Arabidopsis thaliana* L. Heynh (*Arabidopsis*) provides a model system in which to identify genes involved in photoperiodic flowering. *Arabidopsis* flowers rapidly in long days (LD, 16 h light, 8 h dark), but has delayed flowering in short days (SD, 8 h light, 16 h dark). Genetic screens have identified mutations that cause *Arabidopsis* to flower with reduced sensitivity to daylength. In genetic models of flowering in *Arabidopsis*, these genes have been placed in a single genetic pathway, often called the long-day flowering pathway, because it was first defined by a class of mutations that delayed flowering under long but not short days (Redei, 1962; Koornneef *et al*., 1991). Several other *Arabidopsis* flowering time genes are placed in a second genetic pathway, called the autonomous or constitutive flowering pathway, because they affect flowering time in both photoperiods (see recent reviews by Koornneef *et al*., 1998b; Levy and Dean, 1998; Piñeiro and Coupland, 1998).

Interactions between photoreceptors and the circadian clock are thought to allow plants to distinguish between different daylengths. The circadian clock is an endogenous timekeeper that controls many rhythmic processes (circadian rhythms) in organisms as they experience the 24 h cycle of day and night (Dunlap, 1999). Several genes in the long-day flowering pathway affect both circadian rhythms and flowering time in *Arabidopsis*. Plants carrying mutations in the *EARLY FLOWERING 3* (*ELF3*) gene have no detectable circadian rhythms in continuous light conditions, but do in darkness (Hicks *et al*., 1996). This suggests that *ELF3* may be responsible for light input signals to the clock. The *elf3* mutant also shows early, daylength-insensitive flowering indicating that *ELF3* functions to measure daylength and to repress flowering under short days (Zagotta *et al*., 1996). Late flowering plants resulted from overexpression of either of two related genes encoding proteins with a single Myb repeat (*LATE ELONGATED HYPOCOTYL*, *LHY*, Schaffer *et al*., 1998; and *CIRCADIAN CLOCK ASSOCIATED 1*, *CCA1*, Wang and Tobin, 1998). In both genotypes, circadian clock-controlled expression of several genes was disrupted. The *LHY* and *CCA1* genes were proposed to function either within the circadian oscillator or in output pathways from it. Finally, mutations in the *TIMING OF CAB 1* gene (*TOC1*, Somers *et al*., 1998), which is thought to be part of the clock, accelerate circadian rhythms in *Arabidopsis* and can lead to flowering with reduced sensitivity to photoperiod in the Landsberg *erecta* (Ler) ecotype of *Arabidopsis* by causing early flowering under short days.

Other genes that act within the long-day pathway and affect flowering time apparently do not affect circadian clock function. The *CONSTANS* flowering time gene (*CO*, Redei, 1962; Koornneef *et al*., 1991) has been placed in the long-day flowering pathway, because *co* mutations lead to late flowering in long days, but have little effect on flowering in short days. Hence the *CO* gene is thought to promote flowering in long days. The *CO* gene has been isolated and encodes a putative zinc finger transcription factor (Putterill *et al*., 1995). *CO* appears to promote flowering via up-regulating the activity of the *Arabidopsis* floral meristem identity gene *LEAFY* (Weigel *et al*., 1992; Simon *et al*., 1996; Nilsson *et al*., 1998). Six other mutants (*gi*, *fd*, *fe*, *fha*, *ft* and *fwa*) are late flowering, have little or no response to daylength and fall into the same epistatic



group as *co* in genetic tests (Koornneef *et al*., 1998a). These genes are thought to function mainly to promote flowering in long days (Koornneef *et al*., 1998a,b). One of the genes, *FHA*, encodes a flavin-containing blue light receptor involved in floral promotion (Koornneef *et al*., 1991; Lin *et al*., 1996; Guo *et al*., 1998).

The *Arabidopsis GIGANTEA* gene (*GI*) appears to act upstream of *CO*, as the late flowering phenotype of *gi* mutants is corrected by ectopic overexpression of the *CO* gene (I.Igeno, F.Robson and G.Coupland, unpublished data). The *gi* mutation has pleiotropic effects in addition to its effect on flowering time. For example, *gi* mutants have elongated hypocotyls (Araki and Komeda, 1993) and are resistant to the herbicide paraquat (Kurepa *et al*., 1998). Furthermore, *gi* mutant plants accumulate starch under some conditions, but the high starch levels are not the cause of the late flowering phenotype of *gi* mutants (Eimert *et al*., 1995; Hollis, 1999).

To analyse the molecular role of *GI* in *Arabidopsis* flowering, we have isolated and characterized the gene. Here we report that *GI* encodes a novel, putative membrane protein. We show that *GI* transcript levels are controlled by the circadian clock. The *ELF3*, *CCA1* and *LHY* genes are involved in its circadian regulation. Altered timing and increased levels of *GI* gene expression correlate with early daylength-insensitive flowering in the *elf3* mutant. Finally, we show that two genes influencing flowering in *Arabidopsis*, *LHY* and *CCA1* are expressed at lower levels in the *gi-3* mutant in long days.

Results

Identification of the sequence of the GI gene

Previously, we identified a new *gi* mutant allele (now *gi-11*) carrying a T-DNA tag closely linked to the *gi* mutation (Richardson *et al*., 1998). Southern hybridization analysis using a probe from the left border of the T-DNA confirmed that there was a single T-DNA insertion in the genome (data not shown). Inverse polymerase chain reaction (IPCR) was used to amplify a 1.3 kb fragment flanking the left border of the T-DNA (see Figure 1A). The DDBJ/EMBL/GenBank DNA sequence database was searched with the sequence derived from the IPCR fragment. This revealed that the IPCR fragment sequence was identical to sequence of an *Arabidopsis* cosmid clone CC17J13 (DDBJ/EMBL/GenBank accession No. Y12227, Terryn *et al*., 1997) that is located on chromosome 1. This cosmid was contained on a sequenced bacterial artificial chromosome (BAC), T22J18 (DDBJ/EMBL/GenBank

Fig. 1. Structure of the *GI* locus in mutant and wild-type plants and analysis of the GI protein. (**A**) Structure of the *GI* locus in the vicinity of the T-DNA insertion in *gi-11*. The black bar on the line represents the extent of the deletion caused by insertion of the T-DNA in *gi-11* and the grey bar represents the left border IPCR fragment. The positions of *Xba*I (X) restriction enzyme sites are shown. The position of the λ3 clone spanning the T-DNA insertion and the full-length *GI* cDNA are shown as grey bars below the line. Gene 5 and the other genes predicted in this region by Terryn *et al*. (1997) are shown as black arrows below the line. (**B**) Pileup of the predicted amino acid sequence of the GI protein (DDBJ/EMBL/GenBank accession No. AJ133786) and rice EST sequence (DDBJ/EMBL/GenBank accession No. AJ133787). Putative transmembrane domains predicted in both of the proteins by membrane topology prediction programs are underlined (black line, GI protein; grey line, rice protein).

accession No. AC003979). Our previous genetic mapping experiments showed that *GI* mapped 0.36 cM from the restriction fragment length polymorphism (RFLP) marker *TH1* (see Materials and methods), and *TH1* is located on the adjacent sequenced BAC F19G10 (DDBJ/EMBL/GenBank accession No. ATAF000657), ~50 kb from the cosmid sequence. Therefore, the CC17J13 sequence is present at a position consistent with high resolution mapping of *GI*.

The sequence of the IPCR fragment indicated that the T-DNA in *gi-11* was inserted 407 bp upstream of the predicted start codon of a novel gene, designated gene 5 (Terryn *et al*. 1997, Figure 1A). The IPCR fragment was used to screen a genomic library, and five overlapping λ clones from the region were identified. Southern hybridization analysis of wild-type and *gi-11* DNA indicated that the λ clones (e.g. λ3, Figure 1A) spanned the site of the T-DNA insertion. Comparison of the restriction maps of wild-type and *gi-11* genomic DNA in the region indicated that a deletion of 3–4 kb of plant DNA had occurred upon insertion of the T-DNA (data not shown). The deletion in *gi-11* removed the 5′ half of gene 5 and upstream sequence, but did not affect any of the other predicted genes in the region. This strongly suggested that gene 5 corresponded to *GI*. Our final confirmation of the sequence of the *GI* gene by identification of the mutations in six classical *gi* mutant alleles is described in detail below.

Isolation and analysis of the GI cDNA sequence

The *GI* gene was predicted to encode 13 exons which are spliced together to form an ~3.5 kb open reading frame (ORF) (Terryn *et al*., 1997). However, no *Arabidopsis* expressed sequence tags (ESTs) or cDNA clones corresponding to *GI* were identified in DDBJ/EMBL/GenBank database searches. Full-length *GI* cDNA clones were then isolated by RT–PCR and by screening a long insert *Arabidopsis* cDNA library (3–6 kb, Kieber *et al*., 1993). The DNA sequence of these cDNA clones indicated that the *GI* cDNA has a coding region of 3522 bp with a 5′-untranslated region of 318 and 217 bp of 3′-untranslated region. The structure of the gene 5/*GI* coding region predicted by Terryn *et al*. (1997) was correct, except that exon 6 was 18 bases longer in the *GI* cDNA. This had the effect of removing one amino acid and adding seven new amino acids to the predicted protein sequence.

The *GI* cDNA is predicted to encode a 1173 amino acid protein of 127 kDa (Figure 1B). Database searches using BLAST indicated that the GI protein has no significant homology to proteins of known function, but was similar to the predicted amino acid sequences of rice ESTs (Terryn *et al*., 1997). These rice ESTs all corresponded to a single gene. The longest EST clone (DDBJ/EMBL/GenBank accession No. C73052) contained an incomplete cDNA that was entirely sequenced (DDBJ/EMBL/GenBank accession No. AJ133787). The predicted protein product of this gene was 976 amino acids long and was 71% identical and 81% similar to the *Arabidopsis* GI protein (Figure 1B). Web-based membrane topology prediction programs (TopPred1, Von Heijne, 1992; PSORT and TMpred, Nakai and Kanehisa, 1992) predicted that the *Arabidopsis* GI protein contains up to 11 transmembrane domains, and that the rice protein contains up to six. Four of the most likely predicted domains were also conserved in the rice protein, suggesting that they might be functionally important, and a fifth, highly probable transmembrane domain in the *Arabidopsis* protein was not included in the region present within the incomplete rice sequence. The sequence analysis of the rice and *Arabidopsis* genes therefore predicts at least five transmembrane domains, all positioned within the N-terminal half of the protein (in the first 660 amino acids), indicating that this part of the protein may be inserted into a membrane. The PSORT program predicted that both the rice and *Arabidopsis* proteins are likely to be located in the plasma membrane.

Effect of gi mutations on the predicted GI protein and on flowering time

To confirm that gene 5 was *GI*, the corresponding gene from six classical *gi* mutant alleles (*gi-1* to *gi-6*) was sequenced. The region from bp 14 795 to bp 21 169 (Terryn *et al*., 1997) containing the gene was amplified by PCR from genomic DNA of the mutant alleles, in three overlapping fragments, which were sequenced directly. Alterations in the gene 5/*GI* region were identified in all six alleles. These were all predicted to alter the *GI* coding region (Figure 2A) providing confirmation that gene 5 was *GI*. Four of the mutations (*gi-2*, *gi-6*, *gi-3* and *gi-1*) are predicted to introduce premature stop codons into the *GI* sequence. The most severe of these is the *gi-2* allele which is predicted to encode a 144 amino acid protein rather than the 1173 amino acid GI protein. The remaining two mutations (*gi-4* and *gi-5*) are predicted to alter the C-terminus of the GI protein (Figure 2A). *gi-4* has a mutation in the 3′ splice acceptor site of intron 12 of the *GI* gene and *gi-5* has a point mutation and a single base deletion in exon 13 of the *GI* gene. This causes a frameshift which is predicted to both change the last eight amino acids and add 27 amino acids to the C-terminus of the GI protein (Figure 2A).

To determine if the expression of *GI* was altered in the *gi* mutants, tissue from mutant and wild-type plants grown in LD was harvested and *GI* transcript levels were analysed by Northern hybridization (Figure 2B). The samples were harvested 8 h after dawn (onset of illumination) which is close to peak *GI* expression in wild-type plants (see below). Three *gi* mutants (*gi-1* to *gi-3*) had lower levels of *GI* expression than wild-type plants, while three of the mutants (*gi-4* to *gi-6*) had *GI* expression levels similar to wild-type. No *GI* transcript was detected in the T-DNA allele, *gi-11*, which is consistent with deletion of the 5′ half of the *GI* gene and upstream region in this mutant.

To determine the functional importance of different regions of the GI protein, we analysed the effect of the six classical *gi* mutant alleles (*gi-1* to *gi-6*) on *Arabidopsis* flowering time. Two T-DNA mutant alleles (*gi-11* and *gi-12*) were also included in the experiment. The *gi-11* allele is highly likely to be a null mutation, because the 5′ half of the *GI* gene and part of the upstream region is deleted in *gi-11* and no *GI* transcript was detected by Northern hybridization analysis (Figure 2B). The *gi-12* allele is also likely to be a strong allele as it contains a T-DNA insertion in the *GI* coding region (M.Aukerman and R.Amasino, personal communication). The mutants and corresponding wild-type ecotypes were grown in LD and SD conditions and scored for the numbers of leaves visible at flowering (Figure 2C).

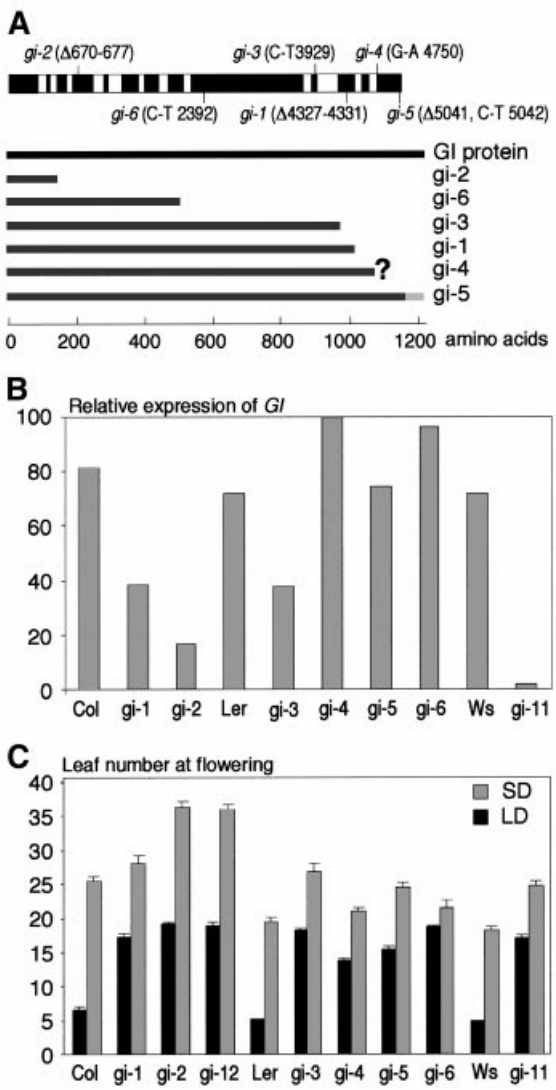


Fig. 2. The effect of *gi* mutations on the GI protein, *GI* transcript levels and flowering time. (**A**) Effect of the *gi* mutations on the GI protein. The position of the mutations in *gi-1* to *gi-6* on the transcribed region of the *GI* gene (top). The positions of the *gi* mutations correspond to the genomic sequence of the *GI* gene with position 1 at the A of the translation start codon. Exons in the transcribed region are in black and introns are in white. Sequence deletions are indicated by a triangle. The size and structure of the predicted *gi* mutant proteins are compared below. In *gi-4*, a mutation in the 3′ splice acceptor site of intron 12 is expected to cause aberrant splicing of the intron with unknown effects on the C-terminal end of the protein (?). In *gi-5*, the last eight amino acids of the predicted GI protein are altered and 27 amino acids are added to the C-terminus of the GI protein (light grey bar). (**B**) Northern hybridization analysis of *GI* expression. Plants were grown in LD conditions until the six leaf stage. Total RNA (10 μg) was extracted from aerial parts harvested 8 h after dawn (ZT 8) and analysed by Northern hybridization using a *GI* cDNA probe. Results are presented as a proportion of the highest value after normalization with respect to 25/26S rRNA levels. (**C**) Flowering time of wild-type and *gi* mutants under long day (LD) and short day (SD) conditions. The genotype tested is shown along the horizontal axis and the leaf number at flowering is plotted on the vertical axis. Data are presented as mean ± SE with *n* of 9–19 plants except for Ws in LD ($n = 2$) and *gi-3* in SD ($n = 2$).

The differences in flowering time between all six classical alleles and the T-DNA mutants were relatively slight in LD (Figure 2C). Interestingly, while the *gi-4* and *gi-5* mutations are likely to affect only the most extreme 3′ end of the *GI* gene, they resulted in long delays to flowering compared with Ler wild-type (Figure 2C). One explanation for the delayed flowering seen in *gi-4* and *gi-5* is that these mutations might have caused decreased stability of the *GI* transcript. However, we saw no difference in *GI* transcript levels in LD in *gi-4* and *gi-5* mutants compared with Ler, which suggests that the C-terminus of the GI protein is functionally important in flowering. The null T-DNA allele *gi-11* did not abolish flowering, indicating that even though *GI* promotes flowering, it is not essential for it to occur.

The *gi* mutations in the Ler and Ws ecotypes caused late flowering with greatly reduced daylength sensitivity compared with wild-type plants, as previously reported for *gi* mutants (Redei, 1962; Koornneef *et al*., 1991; Araki and Komeda, 1993). However, the severe *gi-2* and *gi-12* mutants in the Col ecotype responded quite strongly to daylength because they flowered much later in SD than in LD (Figure 2B). This is inconsistent with a previous report in which *gi-2* was found to flower more rapidly in SD than in LD (Araki and Komeda, 1993). In addition, in our experiment, the *gi* mutant plants all showed some response to SD because they flowered slightly later than wild-type in SD. This result is consistent with the main function of *GI* being to promote flowering in LD, but indicates that *GI* also has some role in promoting flowering in our SD conditions.

GI transcript levels are regulated by the circadian clock

The expression of some other flowering time genes such as *LHY* and *CCA1* is regulated by the circadian clock and appears rhythmically through light/dark cycles and in entrained plants shifted to continuous light (Schaffer *et al*., 1998; Wang and Tobin, 1998). To determine if *GI* transcript levels fluctuated within a cycle, tissue of wild-type ecotype Ler was collected every 2–4 h over a 24 h period in LD and analysed by Northern hybridization (Figure 3A). Sampling time was expressed in hours as zeitgeber time (ZT, Zerr *et al*., 1990) which is the number of hours after dawn (the onset of illumination). *GI* transcript levels cycled, with the highest level 10 h into the light (ZT 10) and the lowest level at dawn (ZT 0, Figure 3A). A second experiment gave similar results.

To determine if the rhythmic cycling of *GI* transcript levels was under the control of the circadian clock, plants entrained in LD were transferred to either continuous light (LL) or continuous dark (DD) and then assayed for *GI* expression every 4–8 h over a 48 h period. *GI* transcript levels continued to cycle in a similar phase under LL and DD, indicating that they were controlled by the circadian clock (Figure 3A). However, in DD, peak levels of *GI* expression decreased, while trough levels increased compared with LL. This caused an overall reduction in the amplitude of *GI* expression.

A preliminary experiment carried out over 24 h indicated that *GI* transcript levels cycled in SD, but peak levels in SD were earlier, higher and of a shorter duration (not detected at ZT 12) compared with LD. To compare *GI* expression in SD and LD in more detail, tissue was collected every 2 h over a 14 h period (ZT 0–ZT 14) in both conditions and analysed by Northern hybridization (Figure 3B). In SD, *GI* expression peaked ~8 h after dawn

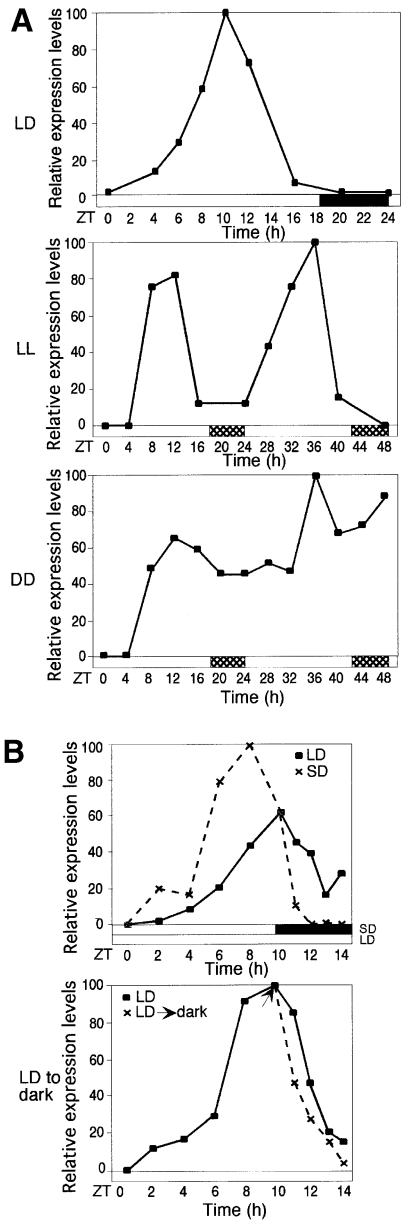


Fig. 3. Northern hybridization analysis of *GI* expression in different light regimes. Plants were grown until the six leaf stage. Total RNA (10 μg), was extracted from aerial parts of plants harvested at the times shown and analysed by Northern hybridization using a *GI* cDNA probe. Results are presented as a proportion of the highest value after normalization with respect to 25/26S rRNA levels. ZT 0 is at lights on. Horizontal bars under each graph represent the light (white) and dark (black) conditions provided. Hatched bars represent subjective night experienced in continuous light (LL) and continuous dark (DD) conditions. (**A**) Time course of *GI* expression in plants grown in LD (top), LL (middle) and DD (bottom). For the LL experiments, plants were entrained in LD and shifted to LL 24 h before tissue harvesting was initiated at ZT 0. (**B**) Effect of SD and the transition to darkness on *GI* expression. Plants were grown in SD and LD (top). In the bottom panel, plants were grown in LD and at ZT 10, half of the LD plants were shifted to darkness (indicated by the arrow).

and reached trough levels ~3 h later, which was ~1 h after the transition to darkness (Figure 3B). In LD, peak levels of *GI* expression were slightly lower and occurred 2 h later than in SD (~10 h after dawn) and reached trough levels later (~6 h later) before the transition to darkness (Figure 3A and B).

The rapid reduction in *GI* transcript levels in SD coincided with the onset of darkness at ZT 10. To test if *GI* transcript levels might be down-regulated directly by the transition to darkness, plants entrained in LD were prematurely transferred to the dark at ZT 10 and then assayed for *GI* expression. One hour after the transfer to darkness (ZT 11), *GI* transcript levels were approximately half the level of control LD plants (Figure 3B). A more extreme down-regulation of *GI* transcript levels by the transition to darkness was obtained in a second experiment. This indicates that the premature transition to darkness does reduce *GI* transcript levels in LD-entrained plants. However, in the reverse experiment, SD-grown plants were shifted into LD, but no immediate change in the pattern of *GI* expression between these plants and control SD plants was detected (data not shown). This suggests that the rapid reduction of *GI* transcript levels in SD-entrained plants is not controlled directly by the transition to darkness, because *GI* transcript levels did not increase immediately when SD plants were exposed to LD.

Early flowering Arabidopsis elf3 mutants misexpress GI

Plants carrying mutations in the *ELF3* gene show early daylength-insensitive flowering. To test whether the *elf3* mutation altered *GI* transcript levels, *elf3* and wild-type ecotype Col plants were grown in LD and SD and assayed for *GI* expression every 4 h over a 24 h period (Figure 4A and B). In *elf3* mutants, unlike wild-type plants, *GI* transcript was detected at all time points analysed including the beginning of the light period (ZT 0) and after the transition to darkness in SD (ZT 10). *GI* transcript levels were also higher in *elf3* mutants than in wild-type at all time points. *GI* transcript levels cycled in *elf3* mutants in LD and SD, but with much reduced amplitude. *GI* expression was also assayed in LD in 11 late-flowering mutants (*fca-1*, *fpa-1*, *fve-1*, *fy-1*, *co-2*, *fe-1*, *ft-1*, *fd-1*, *fha-1* and *fwa-1*, Koornneef *et al*., 1991; *ld-3*, Lee *et al*., 1994) and corresponding wild-type ecotypes, Ler, Col and Ws. No differences in *GI* expression between these mutants and wild-type plants were detected at the two time points analysed (ZT 0 and ZT 8, data not shown).

The *elf3* mutant has no detectable circadian rhythms in continuous light conditions, but the circadian clock does function in *elf3* mutants in darkness (Hicks *et al*., 1996). This result suggests that *ELF3* may be responsible for regulating light input signals to the clock. The *elf3* mutant and wild-type plants were grown in LD, transferred to LL for 24 h and then assayed for *GI* expression every 4–8 h over a 48 h period. The rhythmic pattern of *GI* expression was disrupted in *elf3* in LL (Figure 4C). *GI* transcript was detected at all the time points analysed and was present at higher levels in *elf3* mutants than in wild-type (Figure 4C). *GI* transcript levels appeared to fluctuate in *elf3* mutant plants, but further experiments are needed to test whether this is really a true cyclical pattern with greatly reduced amplitude. This result indicated that the circadian rhythm of *GI* expression in the light is regulated via *ELF3*.

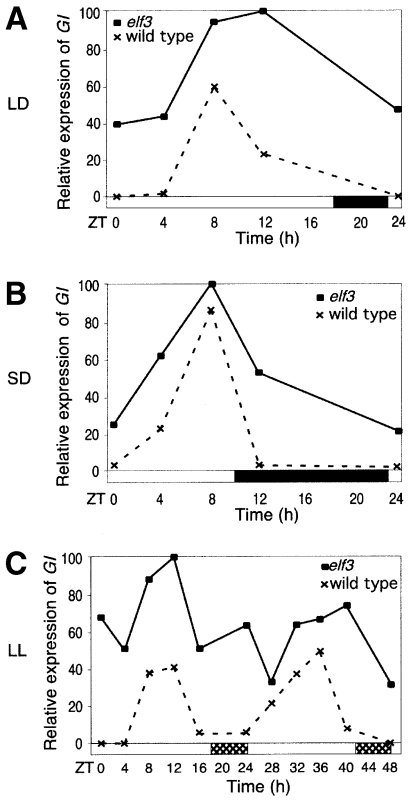


Fig. 4. Expression of *GI* in *elf3* mutants in LD, SD and LL. Wild-type and *elf3* mutant plants were grown in LD or SD conditions until the six leaf stage. Total RNA (10 μg) was extracted from aerial parts of plants at the times shown and analysed by Northern hybridization using a *GI* cDNA probe. Results are presented as a proportion of the highest value after normalization with respect to 25/26S rRNA levels. Horizontal bars under each graph represent the light (white) and dark (black) conditions provided. Hatched bars represent subjective night experienced in continuous light (LL) conditions. ZT 0 h is at lights on. (**A**) Time course of *GI* expression in LD. (**B**) Time course of *GI* expression in SD. (**C**) Time course of *GI* expression in LL. For the LL experiments, plants were entrained in LD and shifted to LL 24 h before tissue harvesting was initiated at ZT 0.

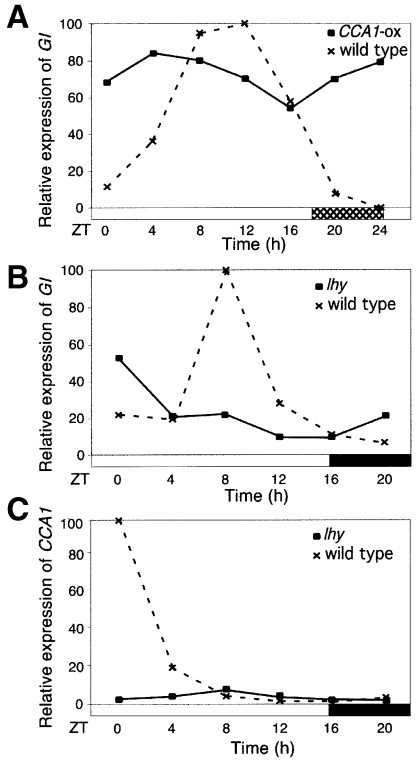


Fig. 5. Effect of the *CCA1-OX* transgene on *GI* expression and the *lhy* mutation on *GI* and *CCA1* expression. (**A**) Time course of *GI* expression in *CCA1-OX*. Wild-type and *CCA1-OX* plants were grown in LD conditions until the six leaf stage and shifted to LL 24 h before tissue harvesting was initiated at ZT 0. Hatched bars represent subjective night. Total RNA (10 μg) was extracted from aerial parts at the times shown. *GI* transcript levels were analysed by Northern hybridization using a *GI* cDNA probe. Results are presented as a proportion of the highest value after normalization with respect to 25/26S rRNA levels. (**B**) Time course of *GI* expression in *lhy*. (**C**) Time course of *CCA1* expression in *lhy*. Wild-type and *lhy* plants were grown in sterile conditions on GM medium in LD (16 h light, 8 h dark). Total RNA (10 μg) was extracted from whole 8-day-old plants at the times shown. *GI* and *CCA1* transcript levels were analysed by Northern hybridization. Results are presented as a proportion of the highest value after normalization with respect to ubiquitin 10 mRNA levels.

Effect of CCA1-OX and LHY on GI expression

To investigate further how the circadian clock controls *GI* expression, we analysed the effect of two circadian clock-controlled genes that affect flowering time on *GI* expression. *CCA1-OX* transgenic plants were grown in LD, transferred to LL for 24 h and then assayed for *GI* expression every 4 h over a 24 h period. In *CCA1-OX* plants in LL, the rhythmic pattern of *GI* expression was disrupted as *GI* transcript was detected at all the time points analysed (Figure 5A). This result indicated that the circadian rhythm of *GI* expression in LL is disrupted by constant expression of *CCA1*.

Overexpression of the *LHY* gene also disrupted circadian clock-controlled expression of several *Arabidopsis* genes, and of leaf movements (Schaffer *et al.*, 1998). *GI* gene expression in LD cycles is also affected in these *lhy* mutant plants, and does not show its characteristic peak in expression at ~ZT 10 (Figure 5B).

The *LHY* and *CCA1* genes are closely related in sequence, and overexpression of *CCA1* was shown previously to repress expression of both the endogenous *CCA1* gene and the *LHY* gene (Wang and Tobin, 1998). In Figure 5C, it is shown that the overexpression of *LHY* also represses *CCA1* expression, and therefore both of these genes will repress their own and each other's expression.

The gi-3 mutation lowers the expression of the LHY and CCA1 genes

The effect of the *gi-3* mutation on *LHY* expression was analysed in LD and SD by Northern hybridization analysis. The *gi-3* mutant (Ler ecotype) was selected for this analysis because Ler was the ecotype in which the wild-type pattern of *GI* expression was analysed. The *gi-3* mutation caused a 5- to 6-fold reduction in peak *LHY* transcript levels in LD compared with wild-type ecotype Ler in both conditions (LD, Figure 6A). A similar result was obtained in *gi-3* mutants grown in SD (data not shown). Although the level of *LHY* expression was reduced by the *gi-3* mutation, *LHY* transcript levels continued to cycle in LD and SD conditions in the same phase as observed previously in wild-type plants (Schaffer *et al.*, 1998). Interestingly, the *CCA1* gene was also down-

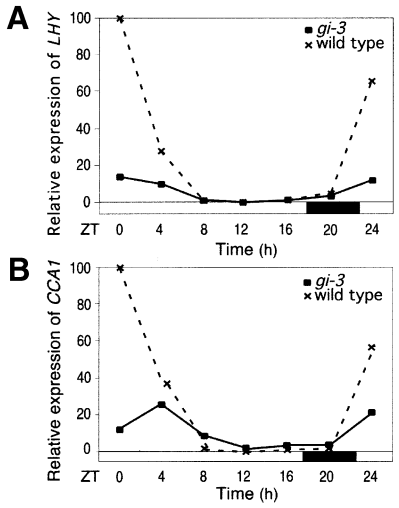


Fig. 6. Expression of *LHY* and *CCA1* in the *gi-3* mutant. (**A**) Time course of *LHY* expression in *gi-3*. (**B**) Time course of *CCA1* expression in *gi-3*. Wild type and *gi-3* mutant plants were grown in LD conditions until the six leaf stage. Total RNA (10 μg) was extracted from aerial parts of plants at the times indicated. Horizontal bars under each graph represent the light (white) and dark (black) conditions provided. ZT 0 h is at lights on. Northern hybridization results are presented as a proportion of the highest value after normalization with respect to 25/26S rRNA levels.

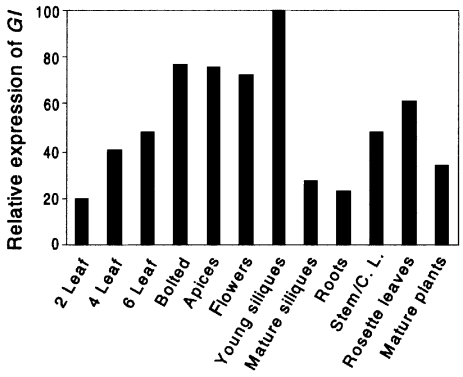


Fig. 7. Northern hybridization analysis of *GI* expression throughout development. Total RNA (10 μg) was extracted 8 h after dawn (ZT 8) from the aerial parts of LD grown plants at the stages shown (from two leaf stage to mature plant) and from specific organs of mature plants as indicated. Bolted plants had a primary inflorescence of ~4 cm. Mature plants had siliques which were fully expanded but still green. Apices were inflorescence apices with floral buds and flowers pre-anthesis. Flowers were at anthesis. *GI* transcript levels were analysed by Northern hybridization using a *GI* cDNA probe. Results are presented as a proportion of the highest value after normalization with respect to 25/26S rRNA levels. C.L., cauline leaves.

regulated in the *gi-3* mutant in LD (Figure 6B) and in SD (data not shown).

GI is expressed throughout development

GI expression during plant development was analysed by Northern hybridization. Wild-type ecotype Ler was grown in LD and samples were harvested 8 h into the light period when *GI* was known to be expressed. *GI* transcript was detected at all the stages of plant development tested, from seedlings at the two leaf stage to mature plants with developed siliques (Figure 7). *GI* transcript levels were analysed in individual organs and tissues from mature plants. *GI* was detected in all of the tissues tested, with the highest level of *GI* expression in inflorescence apices, young flowers and young siliques, and the lowest level in mature siliques and roots.

Discussion

GI sequence and spatial pattern of expression

The protein encoded by the *GI* flowering time gene does not show homology with genes of known function, but is predicted to be membrane localized. Membrane topology prediction programs found up to 11 membrane-spanning domains in the N-terminal half of the GI protein, and the protein most closely related to GI, a rice protein encoded by an EST, is predicted to share at least four of these domains with the GI protein. The possible conservation of these membrane-spanning domains through evolution suggests that they are likely to be important for GI function. The C-terminal end of the protein is not predicted to be localized within a membrane, but flowering time experiments with *gi* mutant alleles *gi-4* and *gi-5* suggest that it is required for GI function.

Previous experiments with the conditional *gi-2* allele suggested that *GI* acted 4–7 days after germination in LD (two-leaf to four-leaf stage plants) to promote flowering (Araki and Komeda, 1993). Detection of *GI* transcript in very young plants is consistent with a role early in plant development to promote flowering. Classical grafting studies of photoperiodic flowering in plants suggested that daylength was perceived in the leaves and a graft-transmissible signal moved to the shoot apex to activate or repress flowering. However, the tissues in which *GI* and other *Arabidopsis* flowering time genes are needed to promote flowering have not been defined. For example, *GI* transcript is detected throughout development and in all parts of mature plants, and the long day floral-promotive gene *CO* is detected in both leaves and shoot apices (Putterill *et al*., 1995; Simon *et al*., 1996).

GI expression in wild-type plants and photoperiodic flowering

Previous physiological experiments implicated the circadian clock in photoperiodic time measurement, and genetic experiments in *Arabidopsis* have also identified mutants that disrupt both circadian clock-controlled responses and photoperiodic responses (reviewed by Koornneef *et al*., 1998b). The *GI* gene has been implicated in the flowering photoperiodic response, because flowering time of *gi* mutants is delayed most severely under long days. The analysis of *GI* expression through day/night cycles, and in entrained plants shifted to LL or DD, further suggested that *GI* may be involved in controlling photoperiodic responses. First, *GI* expression was regulated by the circadian clock and its expression peaked during the light period in the light/dark cycles tested. Second, the pattern of *GI* expression differed under long and short days. *GI* expression responded to long days in three ways: the peak of expression was slightly lower and is shifted later, and the reduction in expression from peak levels was slower, thereby increasing the length of time that *GI* is expressed above trough levels. This difference may be due in part to entrainment of the oscillator to different daylengths (Millar and Kay, 1996).

GI is unique amongst genes acting in the long-day

pathway because it is the only one known to control photoperiodic flowering, to be circadian regulated and to have differing patterns of expression in LD and SD. Whether these changes in *GI* expression between LD and SD have a direct effect in controlling flowering time is so far unknown, but could be tested by manipulating *GI* expression in transgenic plants.

Interactions between GI and ELF3

The *elf3* mutation was shown previously to disrupt several circadian rhythms in plants in LL conditions. The *ELF3* gene is predicted to provide light input signals into the clock, because *elf3* mutations disrupt circadian rhythms in LL, but not in DD. As expected, circadian regulation of *GI* expression was disrupted by the *elf3* mutation in LL, and *GI* expression was observed at all time points at a level above trough levels of wild-type plants. This contrasts with the previous observation that *LHY* expression fell rapidly to trough levels in an *elf3* mutant in LL (Schaffer *et al*., 1998). Therefore, although both the *LHY* and *GI* genes are arrhythmic in an *elf3* background in LL, the level of expression of these genes responds very differently to a loss of *ELF3* function. This may be a consequence of the different phases in which these genes cycle, with *LHY* showing a peak in expression at dawn, but *GI* peaking in expression later during the light period.

In the *elf3* mutant, the waveform of expression of another circadian-regulated gene *LHCB2* (previously designated *CAB2*) was observed to be altered compared with wild-type in LD, particularly as the light period lengthened (Hicks *et al*., 1996). However, under short days, rhythmic *LHCB2* expression was similar in the mutant and wild-type (Hicks *et al*., 1996). The cyclical pattern of *GI* expression was again most affected by *elf3* under LD, but was also affected under our SD conditions. It is possible that the increased level of *GI* expression detected in an *elf3* mutant plays a role in the early flowering of these plants.

Interactions between GI, CCA1 and LHY

CCA1 and LHY are both predicted to encode Myb-related proteins and are thought to function in the circadian clock or in a clock output pathway, because *CCA1-OX* and *LHY-OX* disrupt circadian rhythms in LL and DD. *CCA1* and *LHY* are not simply redundant genes with identical functions because a *CCA1* knock-out allele reduces the period of a circadian cycle even in the presence of a wild-type *LHY* gene (Green and Tobin, 1999). As was shown previously for several other circadian-clock controlled genes, *CCA1-OX* and *LHY-OX* disrupted circadian expression of *GI*. However, the *gi-3* mutation also had an effect on *CCA1* and *LHY* expression, reducing the abundance of both transcripts. Therefore, it is not possible to propose a straightforward linear arrangement for the action of these genes with *GI* acting before or after *CCA1* or *LHY*, but rather they affect each other's expression.

It is not clear how *LHY* and *CCA1* levels relate to flowering time. Low levels of *LHY* and *CCA1* in *gi-3* are associated with late flowering, but reduced levels of *LHY* correlate with daylength-insensitive early flowering in *elf3* mutants, while high levels of *LHY* or *CCA1* in *lhy* mutants and *CCA1-ox* plants, respectively, are associated with late flowering. However, all of these genotypes in which *LHY* or *CCA1* expression is disrupted show altered responses to daylength, suggesting that rhythmic expression of these genes plays an important role in daylength responses.

Since *gi-3* affects the expression of *CCA1* and *LHY*, which are closely associated with the circadian clock in *Arabidopsis*, then *gi* mutations might also be expected to alter circadian rhythms in *Arabidopsis*. To test this, circadian regulation of *LHCB2* was assayed in F_2 populations segregating the T-DNA null mutant allele *gi-11* and the *LHCB2::LUC* reporter gene (D.Somers and S.Kay, personal communication). There was a gradual breakdown of circadian clock control of *LHCB2* cycling in approximately one-quarter of the F_2 plants in constant white light. However, initially, *LHCB2* cycled normally in these plants, suggesting that *GI* is not absolutely required for rhythmicity (D.Somers and S.Kay, personal communication).

Perspectives

The *GI*, *LHY*, *CCA1* and *ELF3* genes previously were proposed to act in the same genetic flowering time pathway (Koornneef *et al.*, 1998b). The expression analysis presented here strengthens these connections by showing that *gi-3* reduces the expression of *LHY* and *CCA1*, and that *CCA1-OX*, *lhy* and *elf3* affect *GI* expression. However, the complexity of these interactions suggests that this is unlikely to be a straightforward linear pathway because there are examples of genes affecting each other's expression. How direct these effects are is unknown. For example, it seems unlikely that the GI protein, which is probably located in a membrane, directly regulates the transcription of other genes. Clues to the identity of intermediates should be gained by identifying proteins that interact with the GI protein and by identifying genetic suppressors of *gi* mutations.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana L. Heynh wild-types were ecotypes Columbia (Col), Landsberg *erecta* (Ler, obtained from Lehle Seeds, Round Rock, TX) and Wassilewskija (Ws obtained from the *Arabidopsis* Biological Resources Centre, OH). The *gi* mutants used were *gi-1* and *gi-2* (Col ecotype, obtained from the *Arabidopsis* Biological Resources Centre, OH), *gi-3* to *gi-6* (Ler ecotype, obtained from Maarten Koornneef, Wageningen, The Netherlands), *gi-11* (T-DNA mutant allele in Ws ecotype; Richardson *et al*., 1998) and *gi-12* (T-DNA mutant allele in Col ecotype, a gift from M.Aukerman and R.Amasino, Wisconsin). The *elf3* mutant (Col ecotype) was obtained from the *Arabidopsis* Stock Centre and has been described previously (Zagotta *et al*., 1996). The *lhy* mutant (Ler ecotype) was described previously (Schaffer *et al*., 1998). Seed of transgenic plants overexpressing the *CCA1* gene (*CCA1-OX*, Col ecotype) was a gift from Elaine Tobin (UCLA, California) and has been described previously (Wang and Tobin, 1998). The late flowering mutants *fca-1, fpa-1, fve-1, fy-1, co-2, fe-1, ft-1, fd-1, fha-1, fwa-1* and *ld-3* were obtained from the *Arabidopsis* Biological Resources Centre (OH) and were described previously (Koornneef *et al.*, 1991; Lee *et al.*, 1994). Seeds were placed on moist filter paper at 4°C for 3 days, planted in soil, germinated in growth cabinets and scored for flowering time or harvested for Northern hybridization analysis as described below.

Measurement of flowering time

Flowering time analysis of *gi* alleles and wild-type was carried out on plants grown in Gallenkamp cabinets either in SD (10 h light/14 h dark) or in LD (10 h light + 6 h day extension/8 h dark) as described in Putterill *et al*. (1995). Flowering time was measured by counting the number of leaves when floral buds were visible at the centre of the rosette. Data from one experiment are presented as mean ± SE where n = 9–19 plants, except where indicated.

Isolation of plant genomic DNA sequences flanking the T-DNA insertion in gi-11

Previously, we identified a late flowering mutant (now *gi-11*), carrying a T-DNA insertion at a single site in the genome closely linked to the *gi* mutation (Richardson *et al*., 1998). In order to isolate DNA sequences flanking the T-DNA insertion, IPCR was carried out on *gi-11* plant genomic DNA. A 1.3 kb fragment was obtained from plant genomic DNA digested with *Bst*YI and, following ligation, amplified by nested PCR using primers gkb8 5′-agctggtacattgccgtag (6581–6560) and gkb9 5′-tttttgcttggactataatacc (6654–6675) followed by gkb7 5′-tagatgaaagactgagtgcgat (6581–6560) and gkb10 5′-ctacaaattgccttttcttatc (6737–6750). The co-ordinates of the PCR primers used correspond to the pGKB5 sequence which is available at http://nasc.nott.ac.uk:8300/Vol2ii/bouchez.html. The PCR product was purified with the High Pure™ PCR product purification kit (Boehringer Mannheim) and sequenced, confirming that the fragment contained T-DNA sequences as well as unknown DNA sequence.

Position of the TH1 CAPS marker relative to gi

The *TH1* CAPS marker is located on sequenced BAC F19G10 (DDBJ/EMBL/GenBank accession No. ATAF000657) and is defined by the following primers: F 5′-gttacttacaccacggttattc and R 5′-caccaactttgcttcctc. After cleavage with *Sau*3AI, this gives rise to a single fragment of 530 bp in Col and to two fragments of ~350 and 180 bp in Ler. Genetic mapping placed this ~0.36 cM from *gi-3*. This was determined by analysing the DNA of 699 *gi-3* homozygotes derived from the F_2 of a cross between *gi-3* (Ler) and Col. Only five of the 699 *gi-3* homozygotes carried a recombinant chromosome with a cross-over between *gi-3* and *TH1*.

Sequencing of gi mutant alleles

The *GI* gene was amplified by PCR from genomic DNA of six different *gi* mutant plants, *gi-1* to *gi-6*. Three overlapping genomic fragments of 2.15, 1.84 and 2.52 kb in size were amplified by three pairs of PCR primers respectively from each *gi* mutant. These were oli1 5′-ggtaatggcgcataaagg (14 795–14 812) and oli2 (R) 5′-tggttcaagagctggaag (16 924–16 941), oli3 5′-atgagactgctacgctgac (16 835–16 853) and oli4 (R) 5′-cccacttacatctcatcaac (18 673–18 692), and oli5 5′-gttcagacgttcaaagg (18 634–18 651) and oli6 (R) 5′-aactccaatcccaaaacc (21 152–21 169). The primer co-ordinates correspond to the DNA sequence of a cosmid containing the *GI* gene (DDBJ/EMBL/GenBank accession No. Y12227, Terryn *et al*., 1997). PCR products were purified with the High Pure™ PCR product purification kit (Boehringer Mannheim) and sequenced on one strand using a series of sequencing primers designed from the *GI* genomic sequence. The DNA sequence from the *gi* mutant alleles was compared with the wild-type sequence to identify the position of the mutations. DNA polymorphisms between the wild-type sequence (Col ecotype) and *gi-3* to *gi-6* (Ler ecotype) were excluded as they occurred in all four of these *gi* mutants. Approximately 0.46% of bases were polymorphic between Ler and Col. Once mutations were found, a second independently generated, PCR product was sequenced to verify that the DNA sequence alteration was real rather than a PCR-derived error.

Screening of phage and cosmid libraries

GI genomic clones were identified by screening 20 000 plaques of the CD4-8 *Arabidopsis* genomic λ library (Voytas *et al*., 1990). Partial *GI* cDNA clones were identified by screening 500 000 plaques of the CD4-7 *Arabidopsis* cDNA library (Newman *et al*., 1994). A full-length *GI* cDNA clone of 4077 bp was identified by screening 500 000 plaques of the CD4-16 *Arabidopsis* cDNA library (insert size range 3–6 kb, Kieber *et al*., 1993). This clone provided the sequence of the 5′-untranslated region of the *GI* gene.

Isolation of GI cDNA by RT–PCR

A *GI* cDNA was amplified in three overlapping fragments by RT–PCR. These were ligated together to form a full-length *GI* cDNA. First strand cDNA synthesis on 1 μg of total Ler RNA was primed using the dT_{17} adapter primer as described by Frohman *et al*. (1988). Three overlapping cDNA fragments of 0.9, 1.2 and 1.7 kb in size were amplified by three pairs of PCR primers designed from the predicted *GI* coding sequence, with some alterations for creation of restriction enzyme sites in the primers. These were oli26 5′-gttaagcttcggttcctggatggc (15 457–15 480, contains the *GI* translation start codon and *Hin*dIII site) and oli2 (R) 5′-tggttcaagagctggaag (16 924–16 941), oli28 5′-tggagagctcaagccgccaaccat (16 529–16 552) and oli30 (R) 5′-ctcttgctacctctagactgtgcttc (18 182–18 207, contains the *Xba*I site), and oli29 5′-cacagactagtggtagcaagagac (18 186 –18 209 contains the *Spe*I site) and oli7R 5′-gtgggtgctcgttattgg (20 519–20 536 contains the *GI* translation stop codon). The primer co-ordinates correspond to the DNA sequence of cosmid containing the *GI* gene (DDBJ/EMBL/GenBank accession No. Y12227, Terryn *et al*., 1997). The *Sac*I site (16 533, DDBJ/EMBL/GenBank accession No. Y12227, Terryn *et al*., 1997) was used to join fragments 1 and 2, and the *Xba*I (oli30) and *Spe*I (oli29) sites were used to join fragments 2 and 3. The PCR products were digested with restriction enzymes and ligated into pBluescript SK+ or pJIT60 (Guerineau and Mullineaux, 1993) and sequenced on one strand using universal primers and a series of sequencing primers designed from the *GI* genomic sequence. A PCR-derived error was discovered in the fragment amplified with oli29 and oli7 and corrected by site-directed mutagenesis with QuikChange™ site-directed mutagenesis kit (Stratagene).

Detection of mRNA by Northern hybridization analysis

Unless otherwise stated, plants were grown in Percival AR-32L cabinets at 22°C providing either continuous light (LL), short days (SD) of 10 h light/14 h dark, or long days (LD) of 18 h light/6 h dark. Light intensity of 150–170 μmol/m^2/s was provided by fluorescent tubes. Plants for the continuous dark (DD) experiment were entrained in LD of 18 h light/6 h dark in cabinets with light provided by metal halide lamps and then shifted to continuous darkness. The aerial parts of at least 10 plants, or at least three plants for specific organs, were pooled for RNA extractions (Whitelam *et al*., 1993). Total RNA (10 μg) was electrophoresed on agarose gels and transferred to Boehringer Mannheim positively charged nylon membrane or Hybond NX (Amersham) as described in Fourney *et al*. (1988). RNA was bound to the membrane using a UV Stratalinker (Stratagene). The *GI* probe used in Northern hybridization analysis was a 1817 bp cDNA fragment from the 3′ half of the *GI* cDNA (2235–4051 on *GI* cDNA, DDBJ/EMBL/GenBank accession No. AJ133786). This probe is specific for the *GI* transcript as it does not detect any transcript in *gi-11* mutant plants which carry a deletion of the 5′ end of the gene and promoter. The *LHY* probe used in Northern hybridization analysis was the full-length *LHY* cDNA (DDBJ/EMBL/GenBank accession No. AJ006404). A 605 bp *CCA1* fragment was generated by PCR using primer cca1f 5′-tccttcagagccagatagtc (3782–3801 on the *CCA1* gene, DDBJ/EMBL/GenBank accession No. U79156) and primer cca1r 5′-gtgccatcctcttgcctttc (4387–4386 on the *CCA1* gene, DDBJ/EMBL/GenBank accession No. U79156). For Northern hybridization analysis of the *lhy* mutant, a 1600 bp fragment from the 3′ end of the *GI* cDNA, a 2000 bp fragment of the *CCA1* cDNA (derived from plasmid CCA1-24, a gift from Elaine Tobin) and a 110 bp fragment from the 3′-untranslated region of the *UBQ10* cDNA (described in Schaffer *et al*., 1998, a gift from Elaine Tobin) were used as probes. The probes were radiolabelled by priming with random octamers (Gibco-BRL). The radiolabelled DNA was hybridized to the Northern blot membranes in hybridization buffer for 18 h at 65°C and then washed at moderate stringency using two washes of 0.5 or 1× SSC, 0.1% SDS at 65°C. After Northern hybridization, nylon membranes were exposed to a Fujifilm BAS-MP imaging plate at room temperature. The image was visualized using a Fujifilm FLA-2000 phosphorimager running Imagereader version 1.3E software. The expression levels were quantitated using the MacBAS version 2.5 program and background hybridization levels subtracted. Expression levels were normalized against the signal obtained by hybridizing the blot with an asparagus 25/26S rDNA probe. The normalized values were then expressed as a proportion of the highest value obtained and graphed.

The Northern analyses presented are the results of one experiment. The experiments presented in Figure 3 were replicated (except the DD panel). Similar results were obtained from the second experiment.

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