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ORIGINAL PAPER

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Characterisation of a barley (*Hordeum vulgare* L.) homologue of the *Arabidopsis* flowering time regulator *GIGANTEA*

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Abstract Barley cDNA and genomic clones homologous to the *Arabidopsis* flowering time regulator *GIGANTEA* were isolated. Genetic mapping showed that *GIGANTEA* is present as a single copy gene in barley (3HS) and rice (1S), while two copies are present in maize (3S and 8S) at locations consistent with previous comparative mapping studies. Comparison of the barley peptide with rice and *Arabidopsis* gave 94% and 79% similarity, respectively. Northern and semi-quantitative RT-PCR analysis of the barley gene (*HvGI*) showed the presence of a single mRNA species, with a peak of expression between 6 h and 9 h after dawn in short days (8 h light) and a peak 15 h after dawn in long days (16 h light). This behaviour is similar to that seen in *Arabidopsis* and rice, showing that sequence and expression pattern were well conserved. A lack of correspondence with the map positions of QTL affecting flowering time (heading date) suggests that variation at *HvGI* does not provide a major source of adaptive variation in photoperiod response.

Introduction

The regulation of flowering time in plants is necessary to optimise pollination, seed set and dispersal. Mechanisms that control flowering in response to environmental stimuli such as day length (photoperiod) and periods of low temperature (vernalization) are important adaptive factors and have major impacts on agriculture. Much of the work towards understanding the genetic control of flowering time has used the model plant *Arabidopsis thaliana* which, like barley (*Hordeum vulgare*), is a quantitative long-day (LD) plant that flowers earlier under long days than short days. Genetic analysis has identified photoperiod, vernalization, autonomous and gibberellic acid pathways that interact to regulate flowering time in *Arabidopsis* (see Searle and Coupland 2004; Hayama and Coupland 2004; Boss et al. 2004 for recent reviews).

Induced mutations show that the *Arabidopsis GIGANTEA* gene (referred to as *AtGI* here) has an important role in the photoperiod pathway, promoting flowering in response to long days. *AtGI* expression is regulated by the circadian clock, and the gene encodes a nuclear protein of 1,173 amino acids (127 kDa), whose exact function is unknown (Fowler et al. 1999; Park et al. 1999). Mutations of *AtGI* are late flowering, probably because of a reduction in the mRNA levels of *CONSTANS* (*CO*) (Suàrez-López et al. 2001). *AtGI* is likely to act upstream of *CO* in the photoperiod pathway because the phenotype of *gi* mutants can be corrected by over-expression of *CO* in transgenic plants (Suárez-López et al. 2001).

Studies in rice have shown that *Hd1*, a major photoperiod response gene in rice, is a *CO* homologue (Yano et al. 2000). Rice is a quantitative short-day (SD) plant, and current data suggest that rice differs from *Arabidopsis* in the way *CO* interacts with downstream targets such as *FLOWERING LOCUS T* (*FT*) (*Hd3a* in rice, Kojima et al. 2002). Under LD conditions *Arabidopsis FT* expression is promoted by *CO*, while rice *FT* expression is inhibited by *Hd1* (Hayama et al. 2003; Hayama and Coupland 2004). Over-expression of *AtGI* in transgenic *Arabidopsis* plants promotes flowering (Fowler et al. 1999), while in rice (*Oryza sativa*) over-expression of the *GIGANTEA* gene (*OsGI*) inhibits flowering (Hayama et al. 2003), consistent with a conserved role in the regulation of *CO* and *Hd1*, respectively. These data suggest that LD and SD plants differ by modification of a conserved photoperiod pathway.

Although *GI* genes have conserved roles in *Arabidopsis* and rice flowering process, it is unclear whether variation in these genes provides adaptive variation in

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flowering behaviour. As a first step towards assessing this in temperate cereals, we characterized a *GI* homologue in terms of sequence, expression patterns, and genetic map location in relation to known major genes and QTL affecting flowering time. Comparative mapping tested the relationship among barley, rice and maize *GI* homologues.

Materials and methods

Identification of *HvGI* clones

Barley *GI* homologues were first identified by BLAST searches (Altschul et al. 1990) of EST databases, using facilities at the NCBI Web site(http://www.ncbi.nlm.nih.gov/BLAST/). Searches were made using the complete peptide from the *Arabidopsis* cultivar 'Columbia' (AJ133786) and a rice partial cDNA sequence (AJ133787). Two partial cDNA clones were identified (MCG015.C04, BE413084 from 'Barke' and HVSMEh0092F05f, BE196448 from 'Morex'). Clones were obtained from the Institut für Pflanzengenetik und Kulturpflanzenforshung, Gatersleben, Germany, and Clemson University Genomics Institute, USA, respectively, and fully sequenced using ABI Prism Big Dye terminator reagents (PerkinElmer) and an ABI 3700 sequence analyser according to the supplier's instructions. Sequences were analysed using the Staden group of computer programs (Gleeson and Staden 1991), BLAST and GCG programs (Wisconsin Package, version 10.1, Genetics Computer Group, Madison, Wis., USA). Rice cDNA sequence (AJ133787) used to screen filters of a barley BAC library (Yu et al. 2000) was purchased from the Clemson University Genomics Institute (http://www.genome.clemson.edu). BAC DNA was prepared and tested by PCR and Southern hybridisation to confirm the presence of *GI* sequence. BACs were then subcloned and screened as described in Griffiths et al. (2003).

Southern analyses followed Laurie et al. (1993). Blots hybridised with the probe derived from *OsGI* were washed at a final stringency of 2× SSC or 0.2× SSC at 65°C for rice and 2× SSC at 65°C for barley and maize.

Isolation of full-length cDNA and genomic sequences

Total RNA was isolated from leaf tissue of 10-day-old barley seedlings, using a Qiagen RNeasy kit according to the supplier's instructions. The genotype used was 'ITDH91', a doubled haploid line from an 'Igri' × 'Triumph' cross (Laurie et al. 1995) lacking vernalization requirement but responsive to long photoperiod. First-strand cDNA synthesis was performed with a poly-dT oligo, using SuperScript reagents (Invitrogen). cDNA was used as a template for PCR, using *Pfu* DNA polymerase (Stratagene) and the primers Hv-F (5' ATGTCAGCGTCAAATGG) and Hv-R (5' TACAGCACAAGCAGTTC). An identical amplification was carried out using BAC DNA as a template. Both fragments were cloned using the pCR4-Blunt-TOPO kit (Invitrogen), following the supplier's instructions and sequenced. Northern blotting and hybridisation according to Sambrook et al. (1990) used RNA extracted from leaf tissue of 10-day-old seedlings, using TriReagent (Sigma, St. Louis, Mo.) following the supplier's instructions.

Analysis of *HvGI* expression, using semi-quantitative RT-PCR

Barley plants of line 'ITDH91' were grown in a controlled environment room under a daily lighting regime of 16 h light/8 h dark (long-day, LD) or 8 h light/16 h dark (short day, SD). Leaf tissue was harvested from 10-day-old seedlings over a 24-h period starting at 'lights-on' and subsequently every 3 h. Semi-quantitative PCR was carried out using first-strand cDNA as template and the primers GI-F (5' CAATTGCCACACCAAGTGCTA) and GI-R (5' TGATGAATTCAGAGGTAACAAACC) which amplified a 497-bp fragment from exons 8 to 10. Amplification was for 28 cycles of 96°C for 1 min, 58°C for 1 min and 72 °C for 1 min. A fragment of *GAPDH* (accession M36650) was amplified as a control, using GAPDH-F (5' CAGAAACCCCGAGGAGATTCCAT) and GAPDH-R (5' TGGCTGGCTTGGCAAGTCTAACAGTCAG) primers. PCR products were resolved on agarose gels, using standard procedures (Sambrook et al. 1990). The abundance of the barley *GIGANTEA* gene (*HvGI*) and *GAPDH* products was determined at each time point, using a scanning fluorimeter (Typhoon, Amersham-Pharmacia, Uppsala, Sweden). Estimated levels of expression for *HvGI* were normalised against those obtained for *GAPDH*, expressed as a proportion of the highest *HvGI* value obtained and plotted for each time point.

Genetic mapping

The following populations were used: Rice, 'IR20' × '63-83' (123 F_2 plants, Quarrie et al. 1997); barley, 'Captain' × *H. spontaneum* (120 F_2 plants, Wang et al. 1992; Laurie et al. 1993); maize 'Tx232' × 'CM27' (41 recombinant inbred lines [(RIL) Burr et al. 1988]; and 'DTP' × 'B73' (176 F_2 plants, S.A. Quarrie, unpublished). Genetic maps were produced using MAPMAKER software (Lander et al. 1987).

Results and discussion

Identification of the *HvGI* gene

Two barley partial cDNA (MCG015.C04 from 'Barke' and HVSMEh0092F05f from 'Morex') were identified by BLAST searches, using the *AtGI* and *OsGI* sequences described by Fowler et al. (1999). The barley sequences were highly homologous to *OsGI* and *AtGI* but comprised only part of the gene and were shorter than the

longest rice EST. The rice sequence was predicted to lack about 150 amino acids from the N terminus, and this was supported by analysis of rice genomic sequence (clone P0666G04, AP003047). To obtain a full-length barley gene, a subset of BAC clones from 'Morex' (Yu et al. 2000) representing approximately 1.5 barley genomes was screened using rice sequence (AJ133787) as a hybridisation probe. A single positive clone (HV_Mba-159E1) was detected. Direct sequencing of the BAC and sequencing of subclones confirmed that it contained the complete *HvGI* sequence. Hv-F and Hv-R primers were then used to amplify the full-length gene. *Pfu* polymerase was used to amplify genomic (AY740524) and cDNA (AY740523) clones, using the barley BAC and first-strand cDNA derived from barley leaf RNA as templates.

Comparison of genomic and cDNA sequences revealed 14 exons and 13 introns spanning 5,837 bp. The intron/exon structure was identical between barley and rice (AP003047) and was similar to *Arabidopsis* (AF076686), except that *Arabidopsis* exon 1 was divided into two exons in *OsGI* and *HvGI*. The predicted peptide was 1,156 amino acids in length and comparison of predicted peptide sequences (Fig. 1) showed that *HvGI* had 94% and 79% similarity and 88% and 66% identity with *OsGI* and *AtGI*, respectively. The nucleotide sequence of the coding region of the barley gene showed 97–98% identity with three wheat *GI* homologues (AF543844, AY679114 and AY679115). Comparison of the barley cDNA and genomic sequences revealed a single SNP in exon 14 (G in 'Morex' at position 7414 in the genomic sequence, C in 'ITDH91' and 'Barke'), which did not result in a changed amino acid.

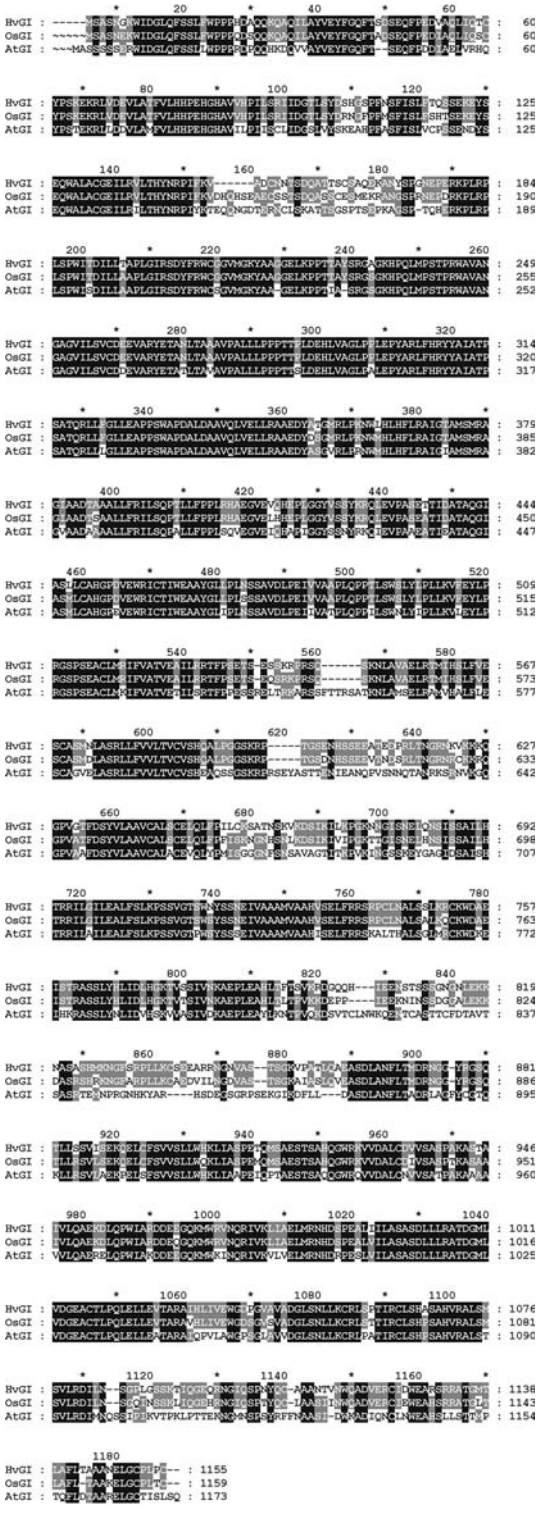
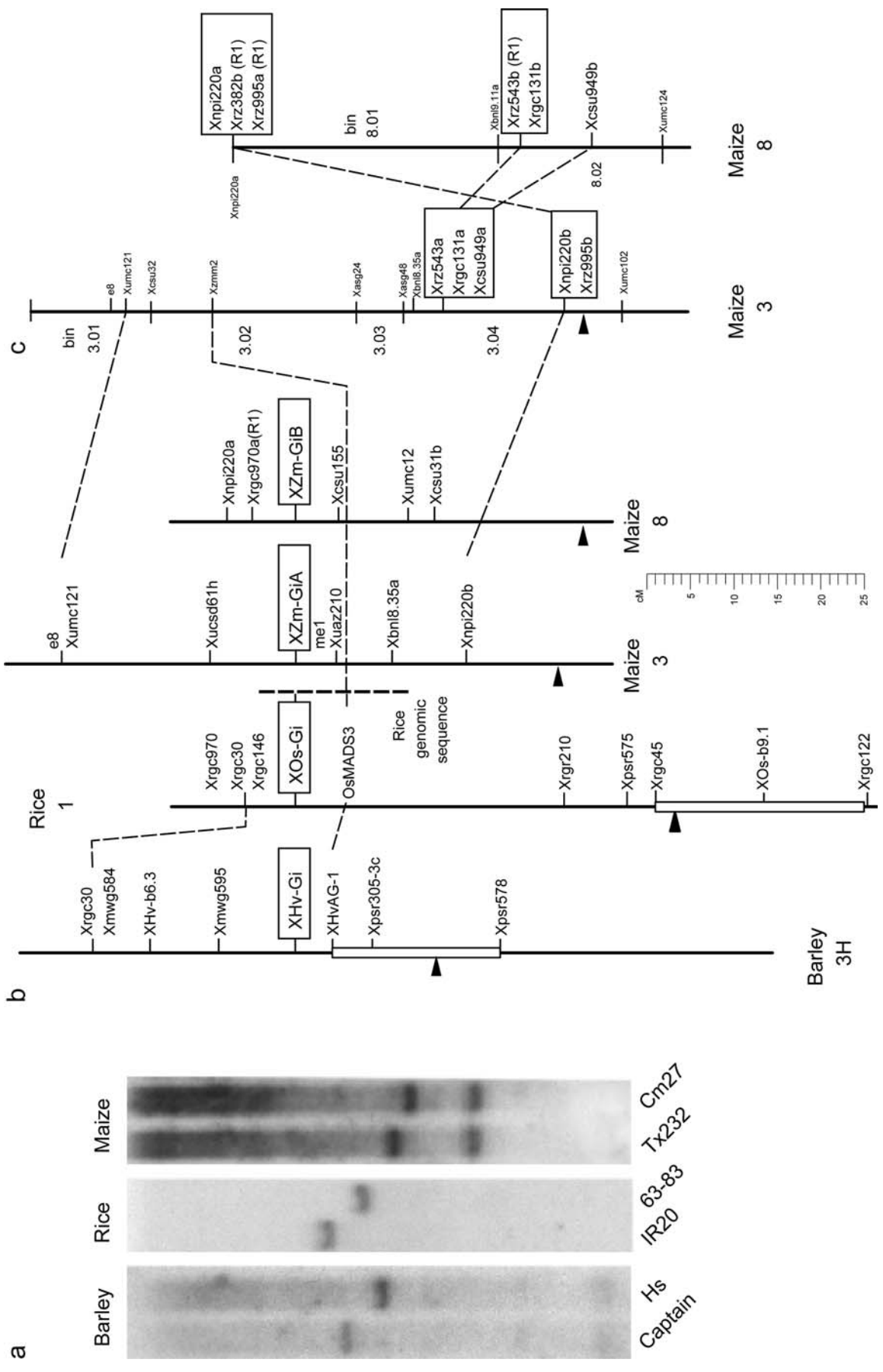


Fig. 1 Comparison of protein sequences from the *GIGANTEA* (*GI*) genes of barley [*Hordeum vulgare* (*HvGI*), 'Morex'], rice [*Oryza sativa* (*OsGI*), 'Nipponbare'] and *Arabidopsis thaliana* [(*AtGI*), 'Columbia']. Sequences accessions are AY740524, AP003047 and AJ133786, respectively

Genetic mapping

cDNA clones from rice and barley were used as probes for Southern hybridisation analyses of rice, barley and maize genomic DNA digested with *Eco*RI, *Eco*RV, *Dra*I or *Hin*dIII. In rice and barley, a single strongly hybridising band was detected in most digests, indicating a single copy of the *GI* gene. This was consistent with analysis of rice genomic sequence. In maize, two strong hybridisation bands were seen consistently, indicating the presence of two *GI* sequences (Fig. 2).

RFLP among the parents of rice, barley and maize genetic mapping populations allowed the genetic mapping of GI homologues. The rice 'IR20' × '63-83' population located *OsGI* to the short arm of chromosome 1 (rice 1S, Fig. 2). This was confirmed by analysis of rice 1S genomic sequence where a single *GI* homologue was found (AP003047). In barley, an *Eco*RI polymorphism in the 'Captain' × *H. spontaneum* F_2 population placed *HvGI* on the short arm of barley chromosome 3H (Fig. 2). In maize a *Hin*dIII RFLP in the 'Tx232' × 'Cm27' RIL population (Fig. 1) located one of the two copies to the short arm of maize chromosome 3 (*ZmGIa*, Fig. 2). No other polymorphism was found in this population. Analysis of the 'DTP' × 'B73' F_2 population identified an RFLP in *Dra*I digests that mapped to the short arm of maize chromosome 8 (*ZmGIb* Fig. 2).


a
Barley
Rice
Maize
Hs
Captain
63-83
IR20
Tx232
Cm27
b
Barley
3H
Rice
1
Maize
3
Maize
8
Xrgc30
Xmwg584
XHv-b6.3
Xmwg595
XHv-Gi
XHvAG-1
Xpsr305-3c
Xpsr578
Xrgc970
Xrgc30
Xrgc146
XOs-Gi
OsMADS3
Rice genomic sequence
Xrgr210
Xpsr575
Xrgc45
XOs-b9.1
Xrgc122
e8
Xumc121
Xucsd61h
XZm-GiA
me1
Xuaz210
Xbnl8.35a
Xnpi220b
Xnpi220a
Xrgc970a(R1)
XZm-GiB
Xcsu155
Xumc12
Xcsu31b
cM
5
10
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3.01
3.02
3.03
3.04
e8
Xumc121
Xcsu32
Xzmm2
Xasg24
Xasg48
Xbnl8.35a
Xrz543a
Xrgc131a
Xcsu949a
Xnpi220b
Xrz995b
Xumc102
Maize
3
Xnpi220a
Xrz382b (R1)
Xrz995a (R1)
bin
8.01
8.02
Xbnl9.11a
Xrz543b (R1)
Xrgc131b
Xcsu949b
Xumc124
Maize
8

Fig. 2 Genetic mapping of *GI* sequences in barley, rice and maize. **a** Southern analysis of *GI*-like sequences in rice, maize and barley. RFLPs among the parent cultivars of the genetic mapping populations used in this study are shown. *Hs H. spontaneum*. **b** Genetic map locations of *GI* sequences in barley (*HvGI*), rice (*OsGI*) and maize [*Zea mays* (*ZmGIa* and *ZmGIb*)]. Other shared markers are joined by *dotted lines*. *HvAG-1* (AF486648), *Os-MADS3* (L37528 and AP003105) and *ZMM2* (L81162) are orthologous *AGAMOUS*-like genes. The *rice genomic sequence* represents overlapping genomic clones (http://rgp.dna.affrc.go.jp/); *OsGI* is on PAC P0489A01 (accession AP003047). The centiMorgan (*cM*) scale applies to all maps shown. *Arrowheads* show approximate positions of centromeres. **c** To facilitate comparison, segments of the 'Tx303' × 'CO159' maps of maize from Davis et al. (1999) are shown which include markers from rice chromosome 1 (*R1*)

The map locations of *GI*-like genes in rice, barley and maize are consistent with previous work showing that rice 1S is collinear with the short arms of wheat and barley group 3 chromosomes (Moore et al. 1995; Van Deynze et al. 1995) and chromosomes 3 and 8 of maize (Ahn and Tanksley 1993; Moore et al. 1995; Van Deynze et al. 1995; Wilson et al. 1999; http://www.gramene.org).

Transcriptional analysis of *HvGI*

Northern analysis of total RNA from 'ITDH91' barley leaves, using the complete *HvGI* cDNA as a hybridisation probe, revealed a single transcript which was in the expected size range (approximately 3.6 kb, data not shown). Expression analysis of *GI* in *Arabidopsis* has shown transcript abundance oscillates in a circadian fashion that differs in SD and LD conditions (Fowler et al. 1999). To test the behaviour of *HvGI*, we used a semi-quantitative RT-PCR assay, using RNA from 10-day-old seedlings grown in SD (8 h light) and LD (16 h light) conditions. The line used ('ITDH91') is responsive to photoperiod and should therefore contain functional photoperiod pathway genes. The abundance of *HvGI* transcript was compared to a control sequence (*GAPDH*), and this showed that under SD conditions, *HvGI* transcript was at its lowest level at the start of illumination and peaked 6–9h later, decreasing rapidly in the dark. A similar pattern was seen under LD conditions, but the peak of expression was higher and later (15 h post-illumination) and extended for a greater duration than under SD (Fig. 3). This difference, with expression peaks at the end of the light period in SD and LD conditions, is consistent with previous work in *Arabidopsis* (Fowler et al. 1999) and rice (Hayama et al. 2003), showing that the overall patterns of transcriptional regulation are conserved.

Genetic mapping of *HvGI* in relation to known flowering time genes

Literature and database searches showed that although sequence and expression pattern were well conserved, there was no correspondence between the genetic positions of the cereal *GI* homologues and known loci with major effects on flowering time, particularly photoperiod response. Genetic mapping shows that *HvGI* is unrelated to the known photoperiod genes of barley (*Ppd-H1* on 2HS and *Ppd-H2* on 1HL, Laurie et al. 1995). No major flowering time gene has been reported on the short arm of barley chromosome 3H (Laurie et al. 1995; http://www.graingenes.org; http://www.barleyworld.org), in the equivalent regions of the wheat group 3 chromosomes (Snape et al. 2001; http://www.graingenes.org) or in the corresponding regions of maize chromosomes 3 and 8 (http://www.maizegdb.org). Similarly, the region of rice chromosome 1 containing *OsGI* included none of the Heading date loci for which a genetic map location has been determined (Yano et al. 1997, 2001).

Studies in rice (Hayama et al. 2003) indicate a conserved role for cereal *GI*-like genes, and the lack of association of *GI*-like genes with major flowering time loci therefore suggests two explanations. First, the number of crosses examined may simply be insufficient to reveal genetic variation. Second, it is possible that mutations have not been selected to provide variation in this trait. *AtGI* is regulated by the circadian clock, but *gi* mutants also affect circadian rhythms (Fowler et al. 1999; Park et al. 1999), *PhyB* genes red light

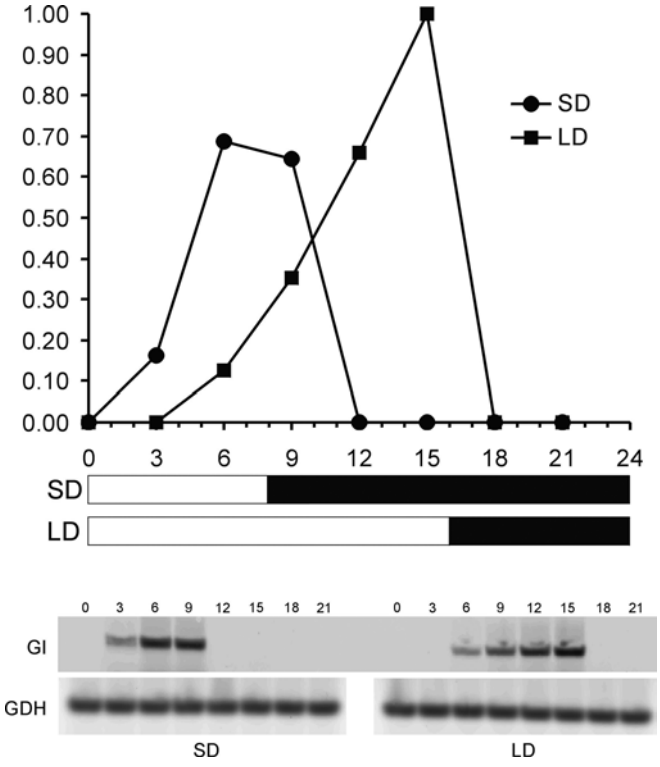

Fig. 3 Expression profiles of *HvGI*. **a** Levels of *HvGI* transcript relative to *GAPDH* transcript estimated at time points over a 24-h period in short-day (SD, 8 h light) and long-day (LD, 16 h light) conditions. Periods of darkness are shown by *shading within the underlying bars*. **b** *HvGI* and control (*GAPDH*) RT-PCR amplification products resolved on agarose gels. Time points (in hours) are shown *above each lane*

signalling (Huq et al. 2000) and have elevated levels of leaf starch (Eimert et al. 1995). *AtGI* also interacts with *SPINDLY*, a negative regulator of gibberellin signalling (Tseng et al. 2004), and is implicated in circadian regulation of transpiration (Sothern et al. 2002). Therefore, one possibility is that *gi* mutations in crops can affect flowering time but have not become established because of additional effects that reduce their agronomic performance. To resolve this, further work is needed to understand the biological function of cereal *GI* genes and the effect of mutations. A linkage disequilibrium approach could also be used to associate sequence variation with flowering time characteristics, an approach recently used to investigate yield QTL in barley (Kraakman et al. 2004) and flowering time in *Arabidopsis* (Olsen et al. 2004).

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