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The homeologous *Zea mays gigantea* genes: characterization of expression and novel mutant alleles

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Abstract

The two homeologous *Zea mays gigantea* (*gi*) genes, *gi1* and *gi2*, arose from the last genome duplication event in the maize lineage. Homologs of these genes in other species are required for correct circadian rhythms and proper regulation of growth and development. Here we characterized the expression of these two maize *gi* genes. Although *gi1* and *gi2* shared comparable 24-hour rhythmic expression profiles, *gi1* levels were consistently higher than *gi2*. Furthermore, short day photoperiods repressed *gi2* expression. The transcriptional unit for *gi1* is established based on 5'-RACE analysis. Two independent mutant alleles for *gi1* are described that are caused by transposons of the *Mutator* (*Mu*) class inserted into the 5'-end of the gene. The type of *Mu* element and position of the transposon in *gi1* was different for each *gi1* allele. Mutant plants had a marked reduction in *gi1* expression and carried transcripts interrupted by the *Mu* element. Together, these results provide a deeper understanding of the *gi* genes in maize. In addition, the novel *gi1* mutant alleles described here will be valuable tools to study *gi1* function in maize, as well as the role of circadian clock regulation in maize metabolism, growth, and development.

Keywords: *gigantea*, circadian clock, photoperiod, *Mutator*, maize

Introduction

The *gigantea* (*gi*) mutant was originally identified as a "supervital" mutant in *Arabidopsis thaliana* that exhibited more robust growth than normal plants (Redei, 1962). Subsequent intensive study of *gi* mutants demonstrated that *GI* acts in key regulatory pathways governing multiple aspects of Arabidopsis growth and development (Rubio and Deng, 2007), such as the circadian clock (Park et al, 1999), flowering time (Araki and Komeda; Fowler et al, 1999), and responses to light cues (Huq et al, 2000; Martin-Tryon et al, 2007; Oliverio et al, 2007). Arabidopsis *gi* mutants flower extremely late, exhibit elongated plant architecture, and have circadian rhythms with long or short periods depending on the allele (Fowler et al, 1999; Huq et al, 2000; Martin-Tryon et al, 2007; Mizoguchi et al, 2005; Oliverio et al, 2007; Park et al, 1999). GI is a novel nuclear-localized protein (Park, 1999; Fowler, 1999; Huq, 2000) that appears to act as a scaffold protein to promote formation of protein complexes that act in the COP1-26S proteasome system (Kim et al, 2007; Sawa et al, 2007; Yu et al, 2008). Also, *GI* is implicated in carbohydrate metabolism (Dalchau et al, 2011), cold stress responses (Cao et al, 2005), phloem development (Edwards et al, 2010), and regulating signaling for both gibberellin (Tseng et al, 2004) and abscisic acid (Penfield and Hall, 2009). All sequenced vascular plant genomes have homologs of *GI*, which encode highly conserved proteins, and these are typically encoded by a single copy gene (Dunford et al, 2005; Hecht et al, 2007; Hong et al, 2010; Izawa et al, 2011; Liang et al, 2010; Serikawa et al, 2008). The maize genome contains two *gigantea* (*gi*) homeologs (originally designated as *gigantea of zea mays 1a/b* (Miller et al, 2008), now designated *gi1* and *gi2* for clarity. These *gi* homeologs arose from the tetraploidy event that occurred approximately 5-12 million years ago (Gaut and Doebley, 1997; Swigonova et al, 2004). Subsequent genome evolution created two subgenomes that have experienced different levels of gene loss or fractionation (Schnable et al, 2011). *gi2* is present on the less fractionated maize1 subgenome, whereas *gi1* is on the more fractionated maize2 subgenome (Schnable and Freeling, 2011). The expression of these *gi* homeologs is circadian-regulated (Khan et al, 2010), and diurnal expression of each is greater in leaves than immature ears (Hayes et al, 2010). Aside from this limited description, these genes are largely uncharacterized.

The two maize *gi* genes are studied here with respect to expression pattern, structure of transcriptional start sites (TSS), and novel mutant alleles. The results show that peak *gi1* expression is largely unchanged between long day (LD) and short day (SD) photoperiods, while SD photoperiods suppress *gi2* expression. The transcriptional unit of *gi1* has two major TSS. Two *Mutator* (*Mu*) element insertions that interrupt the *gi1* gene are described, as well as the negative effect of these transposon insertions on gi1 expression. Together, these results provide a deeper understanding of this pair of gi genes from maize. In

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addition, the *gi1* mutant alleles described here will be valuable tools to study *gi1* function in maize, as well as the role of circadian clock regulation in maize metabolism, growth, and development.

Materials and Methods

Plant materials and growth conditions

Dr Sarah Hake at the Plant Gene Expression Center (Albany, CA) generously provided maize inbred A632. Dr Robert Meeley at Pioneer Hi-Bred International, Inc (Johnson, IA) screened for *Mu* insertions in gi1 and provided putative mutant seed through Cooperative Research And Development agreement number 58-3K95-7-1225-M. Each allele was backcrossed to the parental A632 inbred background. All mutant plants were genotyped with the proper combination of Mu9242 primer and gene-specific primer to confirm the presence of the transposon (Supplementary Table1). Both insertions were stable in backcrossing as indicated by the expected segregation ratios for the transposon in each generation. Growth conditions for LD photoperiods were 16 hour days in a greenhouse during the summer with ambient sunlight until evening, at which time an equal mix of light from Ecolux® Lucalox® High Pressure Sodium ED18 and Multi-Vapor® Quartz Metal Halide ED37 lamps (General Electric, gelighting.com) was provided to achieve 16 hours of lighting. Plants for SD conditions were grown in a Conviron growth chamber with 12 hours of light for day provided by cool white fluorescent bulbs at a fluence rate of 300 µmol m^{-2} s^{-1}, followed by 12 hours of darkness for night. The temperature for days was 26°C and for nights it was 22°C for both photoperiods.

Real-time PCR (qPCR) for gene expression analysis

Leaf tissue samples for qPCR were collected at 4-hour intervals beginning at dawn for a total of 24 hours from V8 stage plants grown in the indicated photoperiod. Tissue was harvested from leaf tips of 10-15 plants, without taking midvein tissue, with 2.5 or 4.0 mm Uni-Core punch (www.tedpella.com). Leaf tips were chosen because this is where circadian clock and light signaling genes are primarily expressed (Khan et al, 2010; Li et al, 2010) within a proximal-distal gradient of expression along the maize leaf (Evert et al, 1996; Li et al, 2010). The first two punches were taken 8 cm from the leaf tip, and subsequent punches were excised away from the leaf tip down toward the leaf base. At each time point, two punches were taken from each plant and these were pooled in a 1.5 ml centrifuge tube. A green LED light (www.photonlights.com) was used for collection during dark periods. Tissue samples were collected in 1.5 ml microcentrifuge tubes and immediately frozen in liquid nitrogen.

Total RNA was extracted from frozen tissue after pulverization under liquid nitrogen to a fine powder with 3.2 mm stainless steel beads (www.nextadvance.com) and a MixerMill 301 (www.retsch.com). Total RNA was purified with Plant RNA Reagent according to the manufacturer's recommendations (www.invitrogen.com). Contaminating genomic DNA in 5 µg of total RNA was removed with the TURBO DNAfree Kit (www.ambion.com). First-strand cDNA was generated from 1.6 µg of DNase treated total RNA and 125 µM each of oligo(dT) and random hexamer with the Maxima Universal First Strand cDNA Synthesis Kit (www.fermentas.com) according to the manufacturer's recommendations.

After dilution by 5-fold, 2 µl of the cDNA mixture was used as template in two technical replicate qPCR reactions that consisted of 1x Ex Taq buffer (www.clontech.com), 1x EvaGreen dye (Biotium.com), 0.2 mM dNTP mix (www.neb.com), 5% vol/vol DMSO, 0.05 mg/ml BSA (www.neb.com), 0.01% vol/vol Tween-20 and 0.3 µM of each primer. Thermocycling conditions in a CFX96 Real-Time PCR Detection System (www.bio-rad.com) were a step of 95°C for 3 min, then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds (detection of EvaGreen occurred here), followed by a melt curve starting at 60°C ramping up to 95°C in 0.5°C increments that were 10 seconds each. C_T values were calculated with the regression function in the Bio-Rad CFX Manager Software (www.bio-rad.com) from reactions exhibiting a single melting peak at the melting temperature of the specific qPCR product. The primers in Supplementary Table1 were used to detect *gi1* and *gi2* transcript. These primers amplify regions of the 3'-untranslated region of the corresponding transcript, which are far from the *Mu* elements in the mutant alleles, so that PCR efficiency was unaffected by the presence of the transposon in the *gi1* mutant alleles. The gene GRMZM5G816228 was used for normalization because its transcript is broadly expressed in leaves at moderate levels and its expression does not have a circadian rhythm (Khan et al, 2010). All primers were synthesized by Eurofins MWG Operon (www.operon.com). The expression level for each experimental gene was calculated using the formula $2^{(C_T GRMZM5G816228 - C_T experimental)}$, where C_T is the average threshold cycle for the two technical replicates.

Sequencing of the gi1 gene and transcripts

The *gi1* gene corresponds to gene model GRMZM2G107101 and *gi2* to gene model GRMZM5G844173 from the B73 filtered gene set 5b.60 for RefGen_v2 (www.maizesequence.org). The identity and precise genomic location of each *Mu* element was determined by sequencing cloned PCR products amplified with primers flanking the left and right borders of the transposon insertion (Supplementary Table1). Phusion High-Fidelity DNA Polymerase (www.finnzymes.com) or Terra™ PCR Direct Polymerase Mix (www.clontech.com) was used according to the manufacturer's recommended protocol to amplify sequences from genomic DNA prepared with

a modified Cetyl Trimethyl Ammonium Bromide extraction method (Lukowitz et al, 2000). PCR products were cloned into the pCR2.1-TOPO vector with the TOPO TA Cloning Kit according to the manufacturer's recommendations (www.invitrogen.com). Cloned products were sequenced with primers M13F and M13R (Supplementary Table1) with the BigDye Terminator v3.1 Cycle Sequencing Kit (www.appliedbiosystems.com). The complete transposon sequence obtained from the *gi1-m1* allele matched that of *Mu3* (GenBank sequence: U19613.1); therefore, this mutant was designated *gi1-m1::Mu3*. The GenBank accession number for the complete sequence of Mu3 in the *gi1-m1::Mu3* allele is JX843286. The *Mu* element of the *gi1-m2* allele matched *Mu1* (GenBank sequence: EF532824.1); therefore, this mutant was designated *gi1-m2::Mu1*. The GenBank accession number for the complete *Mu1* sequence in the *gi1-m2::Mu1* allele is JX843285.

Analysis of transcripts from mutant alleles involved preparation of total RNA from leaf tissue from plants of the indicated genotype. Leaf tissue was collected 12 hours after dawn from V8 stage plants grown in LD photoperiods. Total RNA was extracted and DNase-treated as described above. *gi1*-specific first-strand cDNA was generated from 1 µg of DNase-treated total RNA with primer gi1+482R (Supplementary Table1) using the Maxima Universal First Strand cDNA Synthesis Kit (www.fermentas.com). *Mu* allele specific sequences were amplified from this first-strand cDNA pool with a gene-specific primer (gi1-384F) and a *Mu*-specific primer (Mu9242) (Supplementary Table1). The PCR product amplified from *gi1-m1::Mu3* cDNA was a doublet of ~1000 base pairs. Amplification from *gi1-m2::Mu1* cDNA produced a single PCR product of ~300 bp. The PCR fragments were cloned and sequenced as above. The sequence of two predominant cDNA products from *gi1-m1::Mu3* correspond to GenBank sequence JX843288 and JX843289. The sequence of the single product from *gi1-m2::Mu1* is GenBank sequence JX843287.

Mapping of gi1 transcriptional start sites

5'-RACE to amplify *gi1*-specific cDNA ends used the GeneRacer Kit (www.invitrogen.com) according to manufacturer's recommendations, except Maxima Reverse Transcriptase (www.fermentas.com) and primer gi1-88raceR (Supplementary Table1) were used for first-strand cDNA synthesis. The template was total RNA isolated from leaf tissue collected 8 hours after dawn from V8 stage plants grown in LD photoperiods. Total RNA extraction and DNase-treatment was performed as above. The amplified 5'-RACE products were gel-purified with the QIAquick Gel Extraction Kit (www.qiagen.com) and cloned into the pCR2.1-TOPO vector with the TOPO TA Cloning Kit (www.invitrogen.com) according to manufacturer's recommendations. A total of 40 independent clones for each genotype were sequenced with the T7, M13F, and M13R primers as described above. Sequences were aligned to the *gi1* genomic region with the NCBI Standard nucleotide BLAST server (http://blast.ncbi.nlm.nih.gov).

Results

Expression behavior of the maize gi homeologs

gi1 and *gi2* are homeologous genes located in different parts of the maize genome that have undergone contrasting rates of fractionation. To determine whether the circadian clock equivalently regulates these two genes, the expression of *gi1* and *gi2* was

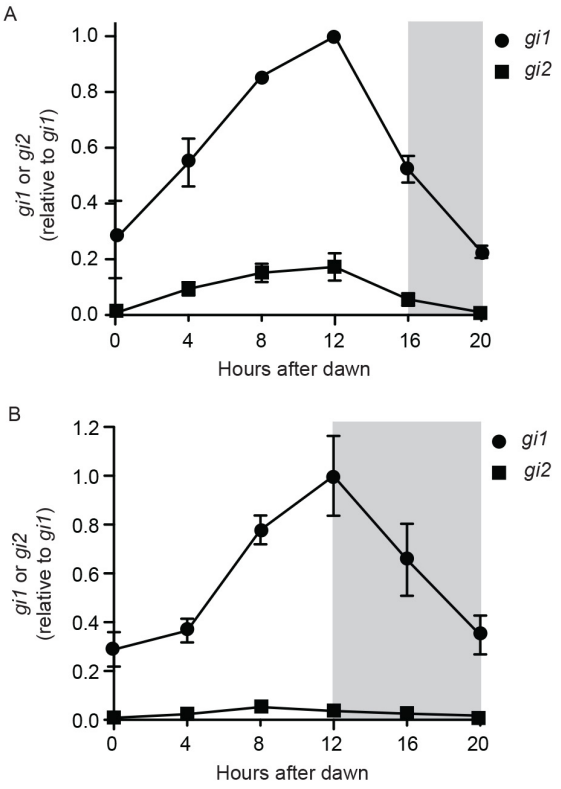

Figure 1 - *gi1* and *gi2* expression are regulated differently, particularly with respect to photoperiod. Relative expression of *gi1* (circles) and *gi2* (squares) in A632 inbred plants grown in either long day (LD) (A) or short day (SD) (B) photoperiods. Time points are the average of two independent biological replicates and error bars are standard error of the mean. In each experiment, time points are from pooled leaf samples (leaf numbers 7 and 8) from five to ten V8 stage A632 plants. Gene expression was determined by qPCR and expression of GRMZM5G816228 was used as a normalization control (see Materials and Methods). The expression level for each experimental gene was calculated using the formula $2^{(C_T GRMZM5G816228 - C_T experimental)}$, where C_T is the average threshold cycle for two technical replicates. The relative expression value for each gene is relative to the highest value for each experiment, which was the *gi1* sample from 12 hours, as this consistently had the highest expression value in each experiment. Grey areas indicate periods of darkness within the photoperiod.

examined with qPCR over a 24-hour period in LD and SD photoperiods. Both *gi1* and *gi2* had rhythmic expression that responded differently to photoperiod length. Circadian clock-driven expression was clearly evident for both *gi1* and *gi2*, as described previously (Hayes et al., 2010; Khan et al, 2010; Miller et al, 2008). Peak transcript levels from either gene appeared between 8 hours and 12 hours after dawn in either LD or SD photoperiods (Figure 1A). In both conditions, *gi1* transcript achieved higher levels than *gi2* transcript in LD, as observed previously (Hayes et al, 2010).

Unexpectedly, the prevailing photoperiod substantially influenced the magnitude of the difference between *gi1* and *gi2* expression. *gi1* transcript achieved peak expression levels in LD that were 6-fold higher than *gi2*, whereas *gi1* transcript levels were 20-fold higher than *gi2* levels in SD (Figure 1B). The cause of the photoperiod difference was that SD appeared to suppress *gi2* expression relative to *gi1*. Indeed, the difference between *gi1* and *gi2* expression was ~3-fold greater in SD than in LD. Thus, *gi2* expression was sensitive to photoperiod and SD downregulated its expression.

gi1 has two major transcriptional start sites

Miler et al, previously described the probable transcript sequence for *gi1* based on *in silico* analysis of public sequence datasets (Miller et al, 2008). To confirm this work, the transcriptional start sites (TSS) for *gi1* in the A632 inbred were determined experimentally with a combination of 5'-rapid amplification of cDNA ends (RACE) and DNA sequencing of the cloned fragments. The 5'-RACE protocol captured two different regions of *gi1* that serve as the major TSS in leaf at the time of peak *gi1* expression (12 hours after dawn) (Figure 2). The most frequently occurring TSS (26/47 clones) was centered around -413 to -420 nucleotides relative to start codon in the transcript, with the most common positions being -418 (15/47 clones) and -420 (8/47 clones). The *in silico* predicted TSS location was just downstream from this location (Figure 2) (Miller et al, 2008). The second site for initiation of transcription spanned the region of -366 to -384, with the most common site being -371 (5/47). Therefore, the pool of *gi1* transcripts exhibited two distinct TSS.

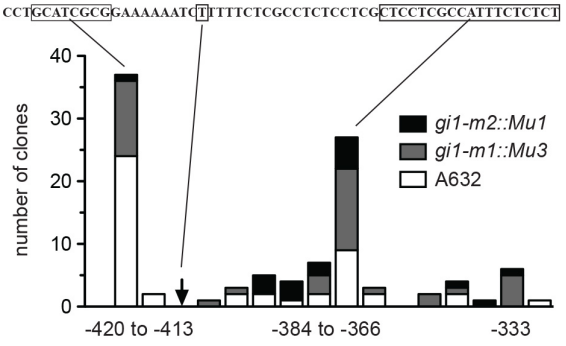

Figure 2 - *gi1* has two major transcriptional start sites (TSS). The TSS positions in *gi1* were determined with 5'-RACE from transcripts present at 12 hours after dawn. The positions of TSS are shown along the X-axis as the number of nucleotides upstream of the initiation codon and above as the boxed areas of the segment of *gi1* gene sequence. The TSS from Miller et al is marked with the arrow and the boxed nucleotide (Miller et al, 2008). TSS were mapped for transcripts isolated from leaves of A632 inbred (white bars), *gi1-m1::Mu3* (grey bars), and *gi1-m2::Mu1* (black bars) plants. The Y-axis is the number of clones with that TSS found in a population of 40 independent clones (for each genotype).

Identification of gi1 transposon insertion alleles

To study *gi* function in maize, mutant alleles were necessary; therefore, we took a reverse genetics approach to identify potential *gi1* and *gi2* mutant alleles. Two independent *Mu* transposon insertion alleles were identified in *gi1* from the TUSC collection (Meeley and Briggs, 1995). The first mutant *gi1* allele is caused by a *Mu3* element and, therefore, was named *gi1-m1::Mu3*. The transposon in this allele was located 2,495 base pairs downstream from the most common TSS of *gi1*, which corresponds to exon 7 of the transcript (Figure 3A). Exon 7 is within the coding sequence, so that the *Mu3* element was likely to interfere with correct translation of any mRNA derived from the mutant locus (Figure 3A). The second mutant *gi1* allele contained a *Mu1* element and, therefore, was named *gi1-m2::Mu1*. The *Mu* element in this case was within exon 2 at 1,453 base pairs downstream from the beginning of *gi1*. The second exon comprises part of the 5'-untranslated region (5'UTR) in the mRNA (Figure 3A). Based on the position of this *Mu* element, the *gi1* mRNAs generated from transcription of the mutant locus presumably retain the potential to encode a full-length protein.

The gi1-m1::Mu3 and gi1-m2::Mu1 alleles reduce gi1 expression

Transcript levels from *gi1* were evaluated in each gi1 mutant background to assess whether the *Mu* elements in *gi1-m1::Mu3* and *gi1-m2::Mu1* change normal *gi1* gene expression. Compared to maize inbred line A632 grown in LD photoperiods, the *gi1-m1::Mu3* insertion lowered *gi1* transcript accumulation by 4-fold. Similarly, the *gi1-m2::Mu1* allele reduced maximal *gi1* expression by 2.5-fold. (Figure 3B, C). Therefore, the *gi1-m1::Mu3* and *gi1-m2::Mu1* alleles significantly reduced gi1 expression relative to maize inbred A632 regardless of their insertion site (exon 7 compared to 5'UTR) within *gi1*.

In addition to the severely reduced *gi1* expression in the *gi1-m1::Mu3* and *gi1-m2::Mu1* alleles, the transcripts remaining in these mutants were aberrant. The effect of the gi alleles on the initiation of transcription was determined by identifying the TSS used in each mutant background. In the *gi1-m1::Mu3* background, the preference for TSS shifted from the primary site at -413 to -420 (12/39 clones) to the secondary location of -366 to -384 (26/39 clones). The same bias toward

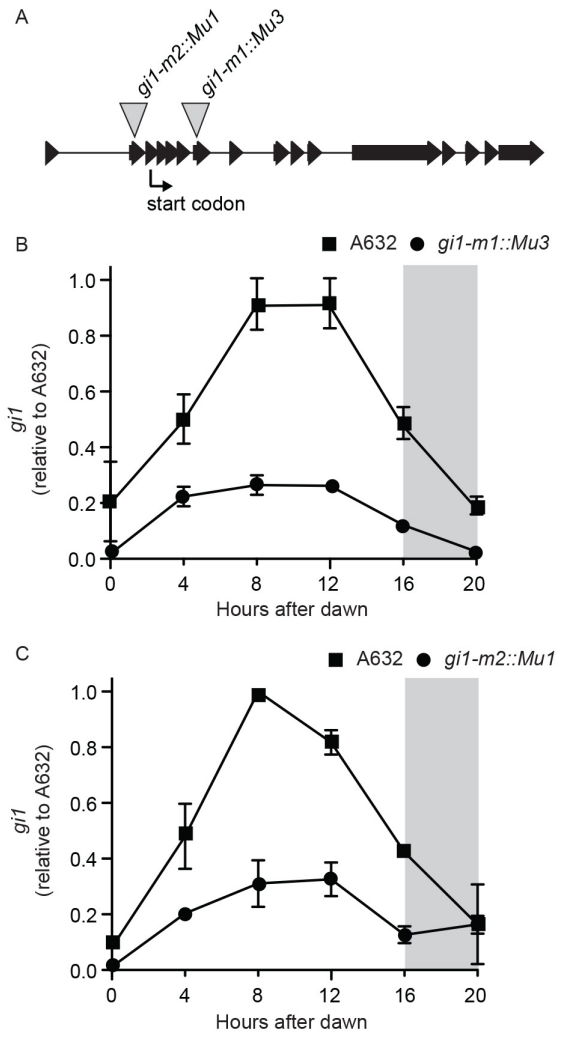

Figure 3 - *gi1-m1::Mu3* and *gi1-m2::Mu1* mutant alleles severely reduce *gi1* expression. A) The location of *Mu* insertions in the *gi1* gene. Grey inverted triangles indicate the corresponding insertion. Thick, black horizontal arrows represent exons and the thin, black horizontal arrow below indicates the position of the initiation codon. B and C) Relative expression of *gi1* in leaf samples taken from *gi1-m1::Mu3* (B, circles), *gi1-m2::Mu1* (C, circles), and A632 inbred (B and C, squares) plants grown under LD conditions. Time points are the average of two independent biological replicates and error bars are standard error of the mean. In each experiment, time points are from pooled leaf samples (leaf numbers 7 and 8) from five to ten V8 stage plants. Gene expression was determined by qPCR and expression of GRMZM5G816228 was used as a normalization control (see Materials and Methods). The expression level for each experimental gene was calculated using the formula $2^{(C_T GRMZM5G816228 - C_T experimental)}$, where C_T is the average threshold cycle for two technical replicates. The relative expression value for each gene is relative the highest value for A632 in each experiment. Grey areas indicate periods of darkness within the photoperiod.

this second TSS was stronger in the *gi1-m2::Mu1* mutant (Figure 2).

To further define the structure of the transcripts remaining in each *gi1* mutant allele, gene- and *Mu* element-specific primers (Supplementary Table1) were used to amplify the region of the *gi1* transcript upstream of the *Mu* insertion in cDNA samples prepared from mutant leaves. Sequencing of the DNA products revealed that the transcripts were interrupted by Mu insertion in both alleles (Supplementary Figure 1A-C). Transcripts from *gi1-m1::Mu3* also harbored unspliced introns upstream of the insertion (Supplementary Figure 1B, C). In one case, a dramatic missplicing event was present (Supplementary Figure 1B), which resulted from the use of a unique splice donor within exon 2 and acceptor site within intron 3. This event removed the last 103 nucleotides of exon 2 and left behind the 12 nucleotides at the 3'-end of intron 3. Therefore, splicing errors were common in this mutant background and the normal transcript sequence was interrupted by *Mu* transposon sequence. Along with the lower overall expression, the accumulation of aberrant transcripts indicated that *gi1* function was likely substantially blunted by each mutant allele.

Beyond the expression level for *gi1*, it was important to assess whether the mutant alleles altered the rhythmic pattern of *gi1* expression. Neither mutant allele showed a disruption in the rhythmic waveform of *gi1* expression in LD photoperiods (Figure 3B, C). Therefore, it is likely that the maize circadian clock remained functional in the *gi1* mutant background.

Discussion

To understand the function of the homeologous gi genes in maize, this study described the expression profiles of these genes with respect to their 24-hour accumulation pattern and the effect of photoperiod on expression level. In addition, the structure of the gi1 transcript was defined. Two novel *gi1* mutant alleles were described, as well as the effect of each on the expression of *gi1*. These mutants will be important tools for defining the role of *gi1* and *gi2* in the maize circadian clock, as well as their contribution to regulation of key processes like seed germination, phytohormone signaling, vegetative phase change, and flowering time.

Circadian regulation of the *gi* paralogs shared the same rhythmic expression pattern over the course of a 24-hour day/night interval, but each gene displayed contrasting expression levels and responses to photoperiod. *gi1* and *gi2* had maximal expression 12 hours after dawn. Late day expression is common for genes of the *GI* family across multiple plant species, including *Arabidopsis thaliana*, *Pisum sativum*, *Oryza sativa*, and *Brachypodium distachyon* (Dunford et al, 2005; Fowler et al, 1999; Hayama et al, 2002; Hayama et al, 2003; Hong et al, 2010; Izawa et al, 2011; Serikawa et al, 2008). The relative expression of *gi1* was generally higher than that of *gi2*, but the prevail-

ing photoperiod had a strong effect on the absolute magnitude of the difference. The relative expression of *gi2* appeared to be suppressed by SD conditions, whereas photoperiod had little or no effect on the relative expression of *gi1*. Similarly, peak *gi1* expression is unchanged by photoperiod in the temperate B73 maize inbred (Meng et al, 2011). This observation indicates that *gi2* may have a photoperiod-specific function. For example, *gi2* may be involved in either accelerating or delaying flowering time in LD conditions, given that *GI* family genes participate in photoperiod-dependent regulation of flowering time in other species (Araki and Komeda, 1993; Dunford et al, 2005; Hayama et al, 2003; Hecht et al, 2007; Izawa et al, 2011). *gi1* is important for regulation of flowering time. A recent study of the flowering behavior of the *gi1-m1::Mu3* and *gi1-m2::Mu1* mutants shows that *gi1* is a repressor of flowering time in LD photoperiods (Bendix et al, 2013). Since *gi1* gene expression is insensitive to photoperiod, activity of the protein may be controlled post-transcriptionally, which has been described for Arabidopsis *GI* (Yu et al, 2008).

The more highly expressed *gi1* is on the highly fractionated maize2 subgenome, while the lower expressed *gi2* is on the less fractionated maize1 subgenome (Schnable et al, 2011). Interestingly, the expression behavior of *gi1* and *gi2* is opposite to the general trend for maize homeologs between the two subgenomes. In their analysis, Schnable et al observed that genes on maize1 subgenome are generally more highly expressed than those on the maize2 subgenome (Schnable et al, 2011), as well as being disproportionately represented among genes identified based on mutant phenotype (Schnable and Freeling, 2011). Hayes et al described the 24-hour expression profiles of two other sets of homeologous circadian clock genes: *zmcca1/zmlhy* and *zmtoc1a/zmtoc1b* (Hayes et al, 2010). *zmcca1* was more highly expressed than *zmlhy* and, similarly, *zmtoc1a* was more highly expressed than *zmtoc1b*. Identification of the subgenome locations for these genes revealed that, like *gi1* and *gi2*, the more highly expressed paralogs (*zmcca1*; *zmtoc1a*) were on maize2 subgenome and the others (*zmlhy*; *zmtoc1b*) were on the maize1 subgenome. Collectively, these observations indicate the expression of duplicated maize circadian clock genes may be opposite to the trend for genes as a whole, with the homeologs on maize2 subgenome being more highly expressed. Although this is an intriguing result, the functional implications of the inverse expression with respect to subgenome localization for *gi1* and *gi2*, as well as for other clock genes, remain to be determined through study of single and double mutants in these genes.

Two independent mutant alleles for *gi1* were identified, each caused by insertion of a *Mu* element into the 5'-end of the gene. The *gi1-m1::Mu3* allele harbors a complete *Mu3* element within exon7, which is a location deep within the coding sequence of the transcript. The *Mu* element in the *gi1-m1::Mu3* background caused severe reductions in *gi1* gene expression, particularly late in the daytime when *gi1* transcript normally reached peak levels. However, the rhythmic expression pattern of the transcript was nearly normal in the mutant background, which indicated that mutation within *gi1* did not completely disrupt its circadian rhythm. The residual transcripts in the *gi1-m1::Mu3* mutant were aberrant. Mutant transcripts contained the *Mu3* sequence, as well as unspliced and improperly spliced introns; therefore, the *gi1-m1::Mu3* allele likely represents a strong loss-of-function allele.

On the other hand, the characteristics of the *gi1-m2::Mu1* mutation indicated this was likely a weaker allele than the *gi1-m1::Mu3* allele. In the *gi1-m2::Mu1* allele, a fulllength *Mu1* element was inserted into exon 2, which is the region of the gene that contributes to the 5'-untranslated region of the transcript. The presence of this *Mu1* element reduced *gi1* expression to the same degree as in *gi1-m1::Mu3* mutant plants. However, correctly spliced transcripts were present in this mutant, although the *Mu1* element was incorporated into these transcripts. Since the *Mu* element was ~200 base pairs upstream of the translation start codon, these transcripts retained the potential to encode the complete GIGANTEA1 protein. For these reasons, the *gi1-m2::Mu1* allele likely represents a weaker loss-of-function allele. Taken all together, the observations presented here provide a deeper understanding of the expression behavior of the *gi* paralogs in maize. In addition, the *gi1* mutant alleles described here will be valuable tools to study *gi1* function in maize, as well as the role of circadian clock regulation in maize metabolism, growth, and development.

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S_figure 1

A *gi1-m2::Mu1* clone 1 - JX843287

CTCCTCGCCATTTCTCTCTCTTCCCCTCCGCTTCGCCCACCGCCCGCTCCGAAATCT
GCGCGCCGACGCCGCCGGCTGCCCGTGGAGAGCCTCGAATTAGAGCGCGTTCCGTCG
AGAGGTTTTCAAGCAACTATGTTTGAGTTTGGTTGATATGATACTCATTCACGTTGG
GGATGCAGACCCCCCCTCTGTGGG*GAGAVAATTGCCATTATGGACGAAGAGGGAAGG*
GGATTCGACGAAATGGAGGCGATGGCGTTGGCTTCTCT

B *gi1-m1::Mu3* clone 1 - JX843288

CTCCTCGCCATTTCTCTCTCTTCCCCTCCGCTTCGCCCACCGCCCGCTCCGAAATCT
GCGCGCCGACGCCGCCGGCTGCCCGTGGAGAGCCTCGAATTAGAGCGCGTTCCGTCG
AGAGGTTTTCAAGCAACTATGTTTGAGTTTGGTTGATATGATACTCATTCACGTTGG
GGATGCAGACCCCCCCTCTGTGGGGGTTGATTGAAGAAGTTAACTGTACACTTAGGA
GAACTTGTGTCCTACTGAAAGTTTCTTGGTTTCGAG*TTTCTTGGGC*AGGCTCTTGAG
CTACCAAGC*ATG*TCAGATTCAAATGTGAAGTGGATTGATGGGCTCCAGTTCACATCA
TTGTACTGGCCTCCACCCCTCGATGCGGAGCAGAAGCAG*GTGTCTTTCTTGCTTATT*
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*TTGGCCATTCTTGTTTGAAG*GCACAAATTTTGGCATATGTCGAGTACTTTGGCCAGT
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ATCCGGAGCATGGTCATGCAGTTGCACATCCAATTCTTTCACGCATCATAGATGGGA
CGCTGTGCTATGATAGACATGGTCCCCCATTCAGTTCCTTCATCTCTTTATTCAGCC
ATAATTCTGAGCAAGAGTACTCAGAGCAGTGGGCCTTGGCCTGTG*GGAGATAATTGC*
CATTATAGAAGAAGAGAGAAGGGGATTCGACGAAATAGAGGCGATGGCGTTGGCTTC
TCT

C *gi1-m1::Mu3* clone 2 - JX843289

CTCCTCGCCATTTCTCTCTCTTCCCCTCCGCTTCGCCCACCGCCCGCTCCGAAATCT
GCGCGCCGACGCCGCCGGCTGCCCGTGGAGAGCCTCGAATTAGAGCGCGTTCCGTCG
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TTGAATGTCTTACTAAGAGTAGGGCTTTGGTGGCATAGATGGATGAACTGATTGTCA
ACTTCAACAAGTTGTTGATTACAG*GTGCTCTTATTGGTTATTGGATCGCTGTGTGTA*
*ATTTGTTTCTTTCCCTTTGAGAGCCTTTCTAGTTTCTTGG*GCAGGCTCTTGAGCTAC
CAAGC*ATG*TCAGATTCAAATGTGAAGTGGATTGATGGGCTCCAGTTCACATCATTGT
ACTGGCCTCCACCCCTCGATGCGGAGTAGAAGCAGGCACAAATTTTGGCATATGTCG
AGTACTTTGGCCAGTTTACAGCTGACACCGATCAATTCCCTGAAGACATAGCTCAGC
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TCTCTTTATTCAGCCATAATTCTGAGCAAGAGTACTCAGAGCAGTGGGCCTTGGCCT
GTG*GGAGATAATTGCCATTATAGAAGAAGAGAGAAGGGGATTCGACGAAATAGAGGC*
GTTGGCGTTGGCTTCTCT