

Photoperiod insensitive *Ppd-A1a* mutations in tetraploid wheat (*Triticum durum* Desf.)

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Abstract Variation in photoperiod response plays an important role in adapting crops to agricultural environments. In hexaploid wheat, mutations conferring photoperiod insensitivity (flowering after a similar time in short or long days) have been mapped on the 2B (*Ppd-B1*) and 2D (*Ppd-D1*) chromosomes in colinear positions to the 2H *Ppd-H1* gene of barley. No A genome mutation is known. On the D genome, photoperiod insensitivity is likely to be caused by deletion of a regulatory region that causes misexpression of a member of the pseudo-response regulator (*PRR*) gene family and activation of the photoperiod pathway irrespective of day length. Photoperiod insensitivity in tetraploid (durum) wheat is less characterized. We compared pairs of near-isogenic lines that differ in photoperiod response and showed that photoperiod insensitivity is associated with two independent deletions of the A genome *PRR* gene that cause altered expression. This is associated with induction of the floral regulator *FT*. The A genome deletions and the previously described D genome deletion of hexaploid wheat remove a common region, suggesting a shared mechanism for photoperiod insensitivity. The identification of the A genome mutations will allow characterization of durum wheat germplasm and the construction of

genotypes with novel combinations of photoperiod insensitive alleles.

Introduction

The timing of flowering during the year is an important component of adaptation that affects cereal yield and grain quality. Previous studies of hexaploid wheat showed that early flowering conferred by “photoperiod insensitive” mutations on the B or D genomes is advantageous in environments with hotter drier summer conditions as it allows the life cycle to be completed before the onset of damaging stresses (Law 1987; Kato and Yokoyama 1992; Law and Worland 1997; Worland and Snape 2001).

Previously described *Photoperiod* loci in barley (*Ppd-H1*) and wheat (*Ppd-B1* and *Ppd-D1*) have been mapped to colinear positions on the respective group 2 chromosomes (Laurie 1997; Börner et al. 1998). *Ppd-H1* was identified as a member of the pseudo-response regulator (*PRR*) gene family by Turner et al. (2005). Beales et al. (2007) described the cloning and sequencing of orthologous *PRR* genes from the A, B and D genomes of hexaploid wheat. The recessive *ppd-H1* mutation in barley, which confers late flowering in long days, is likely to be a loss of function allele. In wheat, the semi-dominant photoperiod insensitive allele on chromosome 2D (*Ppd-D1a*), which confers early flowering in short or long days, was associated with a deletion of 2,089 bp upstream of the coding region and altered expression of the gene, specifically a loss of its normal circadian regulation. The early flowering phenotype of the *Ppd-D1a* mutation is likely to be caused by this alteration and the resultant induction of the key floral regulator *FLOWERING LOCUS T (FT)* in short or long days (Beales et al. 2007).

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The *Ppd-B1* locus is well defined genetically in hexaploid wheat (Worland and Snape 2001) but for chromosome 2A the situation is much less clear. The existence of a photoperiod locus on 2A has been postulated from the behaviour of chromosome substitution lines (Law et al. 1978; Scarth and Law 1984) but no genetic locus has been defined in mapping populations. A *PRR* gene homologous to *Ppd-H1* is present on 2A but sequencing revealed no mutation likely to cause photoperiod insensitivity (Beales et al. 2007). Furthermore, analysis of a ‘Mercia’ single chromosome substitution line thought to carry a photoperiod insensitive allele on chromosome 2A from ‘C591’ in fact carried a *Ppd-B1a* allele (Mohler et al. 2004). Therefore, evidence for a photoperiod insensitive allele on chromosome 2A of hexaploid wheat is currently lacking.

Photoperiod insensitivity exists in tetraploid (AB genome) wheat (Motzo and Giunta 2007) but its genetic basis is poorly understood compared to the hexaploid. However, a QTL that is a candidate for a *Ppd* locus has recently been mapped on chromosome 2A of tetraploid wheat by Maccaferri et al. (2008). Clearly any photoperiod insensitivity in durum wheat must result from a mutation different to that on 2D (*Ppd-D1a*) and in this paper we explored *PRR* gene variation in five pairs of near-isogenic tetraploid wheat lines developed by Clarke et al. (1998) that differ in photoperiod sensitivity. We show that photoperiod insensitivity is associated with mutation of the *PRR* gene on chromosome 2A.

Materials and methods

Plant materials

Seeds of five pairs of near isogenic tetraploid spring wheat lines (‘GS-100’/‘GS-101’, ‘GS-102’/‘GS-103’, ‘GS-104’/‘GS-105’, ‘GS-106’/‘GS-107’ and ‘GS-108’/‘GS-109’) were kindly provided by Dr. J. M. Clarke, Semiarid Prairie Agricultural Research Centre, Agriculture and Agri-Food Canada, Swift Current, SK S9H 3XS, Canada. Each pair was derived by crossing two parents with contrasting flowering times and selecting segregating families to F₈, at which point fixed lines with different flowering times were extracted following selfing (Clarke et al. 1998). Each near-isogenic pair should therefore be equivalent to a recombinant inbred line that varies only for the region or regions affecting flowering time.

Four plants of each line were grown in summer in a photoperiod glasshouse with a fixed day length of 10 h natural light and ambient temperature. This confirmed the early and late flowering phenotypes (Table S1). F₂ populations of ‘GS-100’ × ‘GS-101’ and ‘GS-105’ × ‘GS-104’ were grown in a photoperiod glasshouse with a fixed day length

of 10 h natural light and ambient temperature in the following year with a planting date of the 18th March. F₂ plants were scored for the date of ear emergence (ear emerged half way from the flag leaf on the leading tiller), anthesis (day on which dehisced anthers were first found on the leading tiller) and the number of spikelets on the leading tiller.

DNA extraction and sequencing

DNA was extracted from the parental lines using the phenol/chloroform method of Sharp et al. (1988). Oligonucleotide primers used to amplify and sequence A and B genome *PRR* alleles from hexaploid wheat (Beales et al. 2007) were used to amplify the tetraploid wheat sequences. Overlapping segments were amplified by PCR and directly sequenced. Sequences were assembled using VectorNTI (Invitrogen, UK) and analysed using GCG10 (Wisconsin Package V10.1, Genetics Computer Group, Madison, Wisconsin) and by BLAST using programs at the NCBI web site (<http://www.NCBI.org>). The sequences illustrated were aligned using the pileup command in GCG and adjusted manually to identify homologous regions. GenBank sequence accessions are; *Ppd-A1* ‘GS-100’ EU117146, ‘GS-101’ EU117147, ‘GS-104’ EU117148, ‘GS-105’ EU117149. *Ppd-B1* ‘GS-100’ EU118678, ‘GS-101’ EU118679, ‘GS-104’ EU118680, ‘GS-105’ EU118681.

DNA from F₁ plants was extracted using a DNEasy Mini Kit (catalogue 69106; Qiagen Ltd, UK) according to the manufacturer’s specifications. For F₂ plants, leaf tissue was collected from 2-week-old seedlings, lyophilized and DNA extracted by the Chao and Summers protocol given at http://maswheat.ucdavis.edu/protocols/general_protocols/DNA_extraction_003.htm. All DNA samples were diluted to 20 ng/μl for PCR assays.

DNA of ‘Kofa’ and ‘Svevo’ was kindly provided by Dr. M. Maccaferri, Department of Agroenvironmental Science and Technology, University of Bologna, 40127 Bologna, Italy.

Assays for the 1,027 bp ‘GS-100’ type and 1,117 bp ‘GS-105’ type deletions

In the F₂ populations the ‘GS-100’ type deletion was detected using two forward primers and a common reverse primer in a single 20 μl PCR reaction comprising 1 × Buffer with Green Dye (Promega Corp., USA), 0.2 mM dNTPs, 2 mM MgCl₂, 0.5 μM durum_Ag5del_F1 (gtatgcgattcgctgaagt), 0.5 μM durum_Ag5del_F2 (cgtcaccatgactctgtt), 0.5 μM durum_Ag5del_R1 (gagcaagggattgagactgc), 0.35 units Taq Polymerase (Promega) and 20 ng DNA template. The PCR profile was 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 40 s. The durum_Ag5del_F2 primer site is within the deletion

and amplifies a 245 bp product from plants with the intact sequence. The durum_Ag5del_F1 primer site is upstream of the deletion and gives a 173 bp product from the deleted allele. The assay for the ‘GS-105’ type deletion was identical except that the R1 reverse primer was replaced by the durum_Ag5del_R2 primer (ctggctccaagaggaaacac). The durum_Ag5del_F2 primer site lies within the deletion and amplifies a 452 bp product from the intact sequence. Durum_Ag5del_F1 is upstream of the deletion and gives a 290 bp product from the deleted allele. Amplification products were separated on 1% agarose gels in TAE buffer and visualized under UV light with ethidium bromide (Fig. 1a). Sequencing confirmed that the PCR amplicons were specific to the A genome. As the F1 primer site is present in the intact allele it could theoretically give a 1,198 or 1,407 bp product in the ‘GS-100’ or ‘GS-105’ assays, respectively. This was never observed in practise, probably because the smaller products amplified more efficiently.

Subsequently, it was found that a single reaction using forward primers Ag5del_F1 (gtatgcgattcgctgaagt), Ag5del_F2 (cgtcaccatgcactctgtt) and Ag5del_R2 (ctggctccaagaggaaacac) in the reaction conditions described above

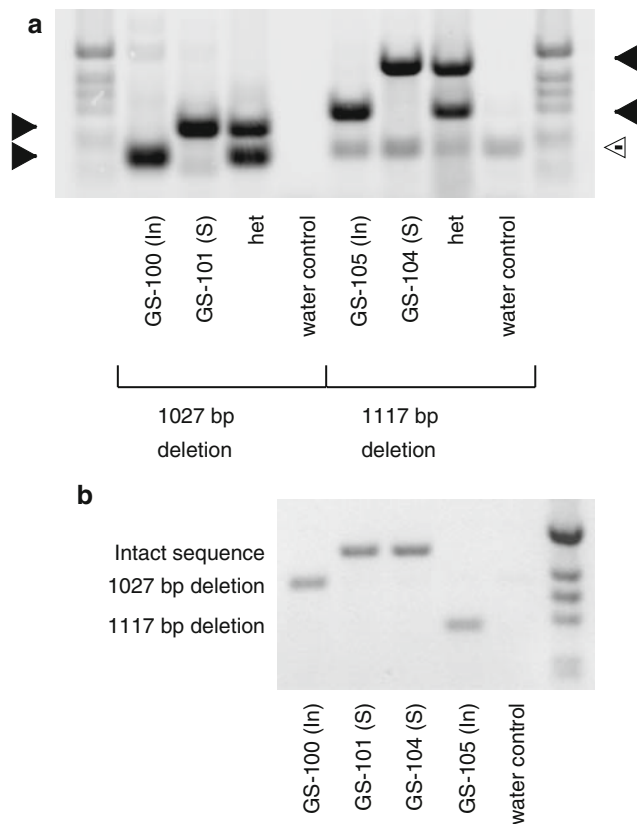


Fig. 1 Colour inverted images of PCR assays for the deletions characterizing the ‘GS-100’ and ‘GS-105’ type A genome photoperiod insensitive alleles. **a** Individual assays used to characterize the F₂ populations. The 1,117 bp assay also gives a non-specific band (open arrow) that is smaller than the main amplicons. *Het* indicates heterozygotes. **b** Combined assay for detecting either deletion

detected both deletions efficiently. A 452 bp product was amplified from the intact sequence, a 380 bp product was amplified when the ‘GS-100’ deletion was present and a 290 bp product was amplified when the ‘GS-105’ type deletion was present (Fig. 1b).

Expression analysis

RNA was extracted from seedlings grown for 20 days in a controlled environment room under short day conditions (9 h light; 69 $\mu\text{mol m}^{-2} \text{s}^{-1}$ average light intensity at bench level; 22°C, 18°C dark). Plants were compared from three biological replicates using a single RNA extraction for two plants per time course sample in each replicate. The time course was one 24-h period. RNA was extracted using Trizol reagent according to the manufacturer’s protocols (Invitrogen Corp., 1600 Faraday Ave., Carlsbad, CA 92008, USA; <http://www.invitrogen.com>). DNA was removed by digestion with DNaseI prior to reverse transcription. cDNA was synthesised using Superscript II (Invitrogen) using the manufacturer’s protocols with 5 μg of total RNA as template and a mixture of OligodT(12–18) (250 ng) and random hexamers (150 ng) as primers. One-fortieth by volume of the final cDNA aliquot was then subjected to real-time PCR as described below. In the case of 18s rRNA analysis, cDNA samples were diluted 1:100 and 1/40 of this dilution was analysed.

Primers for each gene and the respective reaction conditions are listed in Table S2. Where possible, primers amplifying across intron positions were selected to detect any genomic contamination. Specificity of genome-specific assays was verified by sequencing amplicons. RNA was quantified using a Chromo4 real-time PCR instrument (Bio-Rad Laboratories, Inc., 1000 Alfred Nobel Drive, Hercules, CA 94547, USA) with SybrGreen I (Molecular Probes, Inc., 4849 Pitchford Ave., Eugene, OR, USA) as a fluorogen. Reactions included 10 μl 2 \times Sigma Jumpstart SybrGreen Mastermix (Sigma-Aldrich Co. Ltd, Fancy Rd., Poole, Dorset BH12 4QH; <http://www.sigmaaldrich.com>), 5 picomoles of each primer, and an aliquot of cDNA in a total of 20 μl . Reaction conditions were [95°C 10 min; (95°C 15 s, 54°C 15 s, 72°C 40 s) \times 40 cycles], followed by a melting curve with 0.2°C steps between 60 and 95°C, and a final polymerization of 72°C for 10 min. Optical read temperatures were set such that primer dimers and non-specific products were melted (as determined by melting curve analysis) when reads were taken.

Fluorescence data was collected and analysed using Opticon Monitor v3 software (Bio-Rad) with baseline subtraction. The fluorescence threshold was set as close to 0.1 absolute units as possible whilst ensuring it was within the exponential phase of all reactions. The C_t value (the cycle value at which each sample reached the fluorescence

threshold) was extracted for each sample and imported into Microsoft Excel[®]. Delta C_t values were generated by subtracting the minimum C_t within each assay plate from each sample in turn. Relative expression levels were calculated by expressing the efficiency value of the PCR reaction in question, E , to the power of minus delta C_t . E values were determined by calculating the average rate of fluorescence increase during the exponential phase across all samples in the plate showing exponential amplification (Ramakers et al. 2003). Target gene expression was normalized against 18s rRNA levels within samples.

Genetic mapping using SSR markers

The parental lines were screened for polymorphism using 24 simple sequence repeat (SSR) markers covering the short arm of chromosome 2A (barc124, 212, 231; gwm71.1, 95, 122, 296, 359, 425, 448, 497, 512, 614, 636; psp3153; wmc149, 177, 382, 407, 453, 474, 598, 602, 728). Reaction conditions for each assay were as described on the Graingenes database (<http://wheat.pw.usda.gov>).

Results

PRR gene sequence polymorphisms

Amplification of the A genome *PRR* gene segments revealed two size polymorphisms upstream of the coding

region. Sequencing the polymorphic PCR products showed that the photoperiod insensitive lines ‘GS-100’ and ‘GS-103’ had identical 1,027 bp deletions while the photoperiod insensitive lines ‘GS-105’, ‘GS-106’ and ‘GS-108’ had identical 1,117 bp deletions that included a region of 886 bp common to the ‘GS-100’ type deletion. This region was also common to the deletion previously found in the hexaploid wheat *Ppd-D1a* allele (Fig. 2). The photoperiod sensitive ‘GS-101’, ‘GS-102’, ‘GS-104’, ‘GS-107’ and ‘GS-109’ lines had intact sequences that were the same as previously sequenced hexaploid wheat alleles.

Additional upstream regions and the entire coding region of the A genome *PRR* gene were then sequenced from ‘GS-100’, ‘GS-101’, ‘GS-104’ and ‘GS-105’ (Fig. 2). This showed that the ‘GS-100’, ‘GS-101’ and ‘GS-104’ sequences were identical apart from the 1,027 bp deletion in the photoperiod insensitive ‘GS-100’ allele (Table 1). The photoperiod insensitive ‘GS-105’ allele had a distinct haplotype with 18 single nucleotide polymorphisms (SNPs) and three insertion/deletion (indel) polymorphisms compared to the other tetraploid wheat sequences (Table 1). Three of these SNPs altered predicted amino-acids (red arrows in Fig. S1 in electronic supplementary material). In two cases (polymorphisms 15 and 29 in Table 1, Fig. S1) this is unlikely to cause insensitivity because ‘GS-105’ had the same amino-acid as photoperiod sensitive hexaploid wheat alleles. The third change gave a Gln to Arg change in the central, less conserved region of the protein (polymorphism 23 in Table 1, Fig. S1) that was unique to ‘GS-105’.

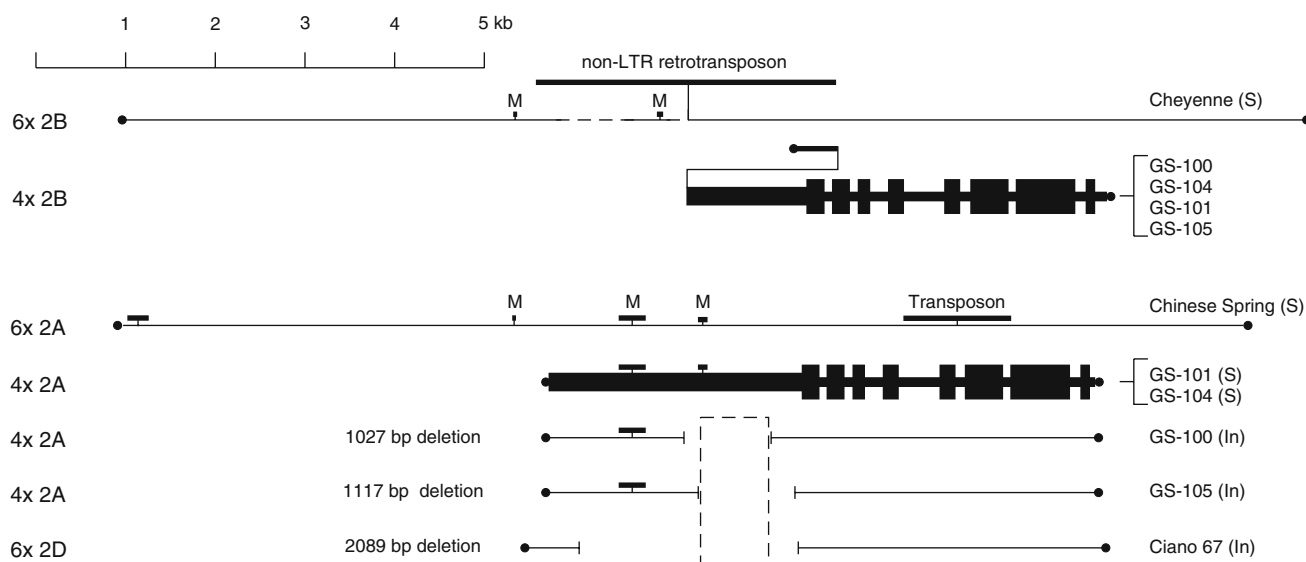


Fig. 2 Sequence features of *Pseudo-Response Regulator* (*PRR*) genes from chromosomes 2A and 2B of tetraploid (4×) wheat. Sequences from the photoperiod sensitive (S) ‘GS-101’ parent and others that were identical are shown as rectangles (tall rectangles are exons, medium rectangles the upstream region and small rectangles are introns and 3′ untranslated regions). Dots show the limits of the sequence and horizontal dotted lines in the 2B sequence of hexaploid (6×) wheat

‘Cheyenne’ show gaps in the alignment with the 2A sequence remaining after the removal of various transposable elements (M indicates MITE) whose sizes and insertion positions are shown. Other alleles are shown as black lines. Vertical black lines show the limits of deletions in the photoperiod insensitive (In) alleles and the dotted rectangle shows the common region deleted in the tetraploid wheat alleles and a previously sequenced D genome allele from ‘Ciano 67’ (DQ885767)

Table 1 *Pseudo-Response Regulator* sequence polymorphisms in hexaploid (6×) and tetraploid (4×) wheat alleles

Polymorphism	Chromosome 2A Position relative to Chinese Spring (DQ885753)	6×		4×			
		Chinese Spring	C591	GS-100	GS-101	GS-104	GS-105
1	4759 SNP	G	G	A	A	A	G
2	5356 SNP	C	C	C	C	C	T
3	5368 SNP	G	G	G	G	G	A
4	5910 indel	T	T	T	T	T	–
5	6028 SNP	A	A	A	A	A	T
6	6140–7169 deletion	No	No	Yes	No	No	No
7	6141 SNP	A	A		A	A	G
8	6201 indel	T	T		T	T	–
9	6236 SNP	A	A		A	A	G
10	6256 SNP	G	G		G	G	A
11	6280–7399 deletion	No	No	No	No	No	Yes
12	7169 SNP	C	C	G	G	G	
13	7255 SNP	C	C	T	T	T	
14	7724 SNP; intron 1	G	G	G	G	G	C
15	8094 SNP; exon 3 Predicted amino-acid	C Asp	C Asp	G Glu	G Glu	G Glu	C Asp
16	8198 SNP; intron 3	G	G	C	C	C	G
17	8313 SNP; intron 3	T	T	C	C	C	T
18	8540 indel; intron 4	T	T	GT	GT	GT	–
19	8574 indel; intron 4	C	–	–	–	–	–
20	8610 SNP; intron 4	A	A	C	C	C	A
21	TE in intron 5	Yes	No	No	No	No	No
22	10823 SNP; exon 6 Predicted amino-acid	A Asp	A Asp	G Gly	G Gly	G Gly	G Gly
23	10850 SNP; exon 6 Predicted amino-acid	A Gln	A Gln	A Gln	A Gln	A Gln	G Arg
24	10904 SNP; intron 6	A	A	A	A	A	G
25	10906 SNP; intron 6	T	T	T	T	T	A
26	10910 SNP; intron 6	A	A	A	A	A	C
27	10921 SNP; intron 6	C	C	C	C	C	G
28	11006 SNP; exon 7 Predicted amino-acid	C Arg	C Arg	G Gly	G Gly	G Gly	G Gly
29	11045 SNP; exon 7 Predicted amino-acid	A Ser	A Ser	G Gly	G Gly	G Gly	A Ser
Chromosome 2B Position relative to Chinese Spring (DQ885757)	6×	4×					
	Chinese Spring	Cheyenne	GS-100	GS-101	GS-104	GS-105	
10822 SNP; exon 3 Predicted amino-acid	A Asn	G Asp	G Asp	G Asp	G Asp	G Asp	G Asp

Two further SNPs causing amino acid changes separated the A genome hexaploid and tetraploid wheats (polymorphisms 22 and 28 in Table 1, blue arrows in Fig. S1). In both cases the tetraploid A genome had the same amino acid as the B and D genome proteins suggesting these are conservative changes. The only common feature of the photoperiod insensitive tetraploid wheats was the loss of the

886 bp region shared by the two upstream deletions in the 2A gene (Fig. 2).

‘GS-105’, ‘GS-106’ and ‘GS-108’ derive from different photoperiod insensitive parents (Table S2) but sequencing of the 2A *PRR* gene showed that they had an identical allele. The ‘GS-103’ sequence was the same as ‘GS-100’ as expected from their common photoperiod insensitive parent (Table S2).

No sequence variation was found between the tetraploid wheat 2B gene sequences from ‘GS-100’, ‘GS-101’, ‘GS-104’ or ‘GS-105’ or between these and the photoperiod sensitive ‘Cheyenne’ type hexaploid wheat sequence previously described by Beales et al. (2007) (Table 1).

Analysis of flowering time variation

Four plants of each parental line were grown in a photoperiod glasshouse with 10 h natural light. Early and late flowering plants in each pair differed in flowering time by 29–36 days (Table S1). Seeds from confirmed early or late flowering plants were used to generate sufficient seedlings for DNA extraction and gene expression analyses. Separate plants grown in natural long days were crossed to generate F₂ populations.

F₁ plants were tested using PCR assays for the 2A deletions (see materials and methods) and confirmed heterozygotes were used to generate F₂ populations. Two populations were selected, one segregating for the 1,027 bp deletion (‘GS-100’ × ‘GS-101’) and one for the 1,117 bp deletion (‘GS-105’ × ‘GS-104’). For each population, 140 F₂ plants and five plants of each parental line were grown in a photoperiod glasshouse with a fixed day length of 10 h natural light. Ear emergence time, anthesis date and spikelet number on the leading tiller were recorded. At the seedling stage, an equal small amount of leaf tissue was taken from each F₂ plant for DNA extraction and genotyping. One plant was discarded from each population because of poor growth.

The numbers of early and late flowering plants in the ‘GS-100’ × ‘GS-101’ (108 : 31) and ‘GS-105’ × ‘GS-104’ (97 : 42) populations were not significantly different from a 3:1 ratio (χ^2 1 *df* = 0.54 and 2.01, respectively) showing that a single locus was the major determinant of variation. PCR assays showed that the presence of the deletion in the 2A *PRR* gene was completely correlated with early flowering in both crosses, consistent with this being the causal basis of photoperiod insensitivity (Fig. 3). Plants homozygous for the deletion were significantly earlier flowering than heterozygotes (Fig. 3; Table S3) as has been shown for the 2B and 2D loci in hexaploid wheat (Tanio and Kato 2007). Similar results were obtained from analyses of anthesis date (Table S3). Early flowering plants had two to five fewer fertile spikelets on the leading tiller (Table S3) which is also consistent with previous findings in hexaploid wheat (Scarath et al. 1985).

The *Ppd-A1* locus has not previously been mapped in wheat. To determine if it could be located in the near-isogenic lines we screened the parents of the F₂ populations with 24 SSR markers for the short arm of chromosome 2A. One marker (*wmc177*) was polymorphic and could be scored in both crosses. Comparison with the phenotype

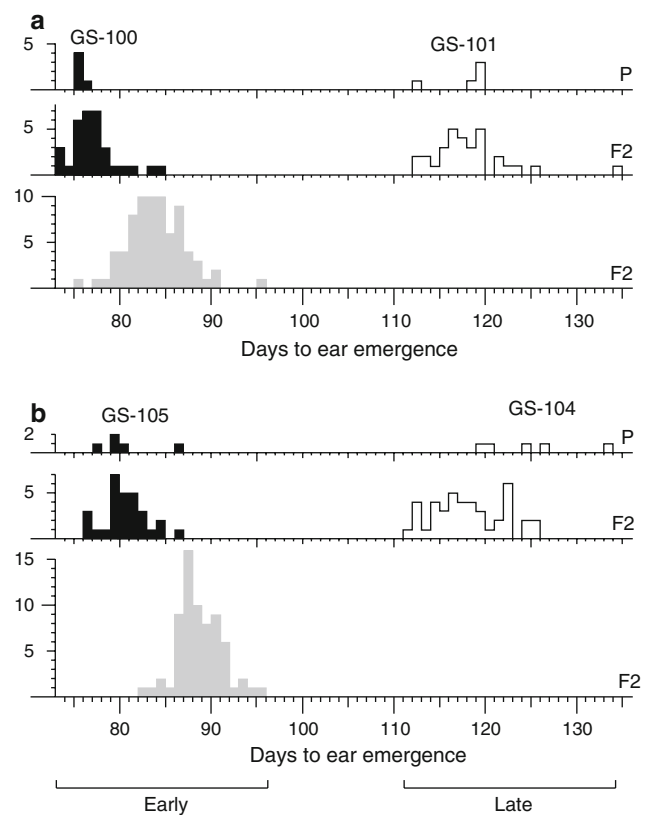


Fig. 3 Days from sowing to ear emergence for parental lines and F₂ plants grown in 10 h natural light. **a** ‘GS-100’ [photoperiod insensitive (*In*)] × ‘GS-101’ [photoperiod sensitive (*S*)]. **b** ‘GS-105’ (*In*) × ‘GS-104’ (*S*). Plants of the parental (*P*) lines are shown on the *top* rows. In each F₂ population plants homozygous for the 2A deletion are shown in *black* and plants homozygous for the intact sequence are shown in *white* on the *middle* rows. Heterozygotes are shown in *grey* on the *bottom* rows

scores and genotype scores from the deletion assays placed *wmc177* 2.2 and 2.8 cM from *Ppd-A1* in the ‘GS-100’ × ‘GS-101’ and ‘GS-105’ × ‘GS-104’ crosses, respectively. This places *Ppd-A1* in the central region of the 2A short arm (Somers et al. 2004), consistent with the map position of the *Ppd-D1* locus on chromosome 2D (Börner et al. 2002).

As in the preliminary test of the parental lines, ‘GS-105’ plants flowered about five days later than ‘GS-100’ plants in the second experiment (Fig. 3, Tables S1, S3). This could be due to a difference between the *Ppd-A1a* alleles or to additional background genetic variation. Analysis of ‘Kofa’ and ‘Svevo’, the parents of the population described by Maccaferri et al. (2008) showed that ‘Kofa’ carries the ‘GS-100’ type allele and ‘Svevo’ carries the ‘GS-105’ type allele. No major flowering time gene segregates in the ‘Kofa’ × ‘Svevo’ cross but a QTL was found with a peak in the 8.6 cM *Xwmc177–Xcfa2201* interval with ‘Svevo’ providing the later flowering allele (Maccaferri et al. 2008). This is consistent with our results and suggest that the two

deletions confer different degrees of photoperiod insensitivity. However, the presence of a linked minor gene affecting flowering time is also a possibility.

Previous studies of hexaploid wheat showed that in short days the photoperiod sensitive genotypes flowered at least 60, and typically more than 100, days after the photoperiod insensitive genotypes (Worland et al. 1994; Tanio and Kato 2007). A feature of both tetraploid wheat populations studied here was a smaller difference of 30–40 days between the parents and between the early and late homozygotes in the F₂. The smaller difference between the early and late flowering classes in the tetraploid wheats could be due to differences in experimental procedure (10 h rather than 8 h light), but an alternative possibility was that the late flowering tetraploid parents carried a weakly insensitive *Ppd-A1* allele or that all the tetraploid lines we studied carried a weakly insensitive *Ppd-B1* allele. As no sequence variation diagnostic for photoperiod insensitivity on chromosome 2B was identified by Beales et al. (2007) the lack of polymorphism in the tetraploid wheat 2B genes does not exclude the latter possibility.

Gene expression studies

To assess the behaviour of the chromosome 2A alleles and the possibility of a weak photoperiod insensitive allele on chromosome 2B the expression profiles of the *PRR* genes and other photoperiod pathway components previously studied by Beales et al. (2007) were sampled at 3-h intervals over a 24-h period in seedlings of ‘GS-100’, ‘-101’, ‘-104’ and ‘-105’ grown for 20 days in short day (9 h light) conditions. Previous studies in Arabidopsis and rice showed that *PRR* genes normally show a marked cycle of expression, peaking during the day and falling to very low levels during the dark (Matsushika et al. 2000; Murakami et al. 2003).

Analysis of the 2A *PRR* transcript showed that the photoperiod sensitive lines showed a peak of expression at 6 h and a subsequent drop to very low levels during the dark period (Fig. 4a, f), consistent with the behaviour of wild type wheat genes in our previous study (Beales et al. 2007). The photoperiod insensitive lines, in contrast, showed a loss of this normal pattern and expressed the gene throughout the day, resulting in significantly higher levels of expression in the dark and early light periods (Fig. 4a, f).

We then analysed the expression of wheat homologues of the core photoperiod pathway genes *GIGANTEA* (*TaGI*), *CONSTANS* (*TaCO1*) and *FLOWERING LOCUS T* (*TaFT1*) as described in Beales et al. (2007). The expression peak of *TaGI* was not affected (Fig. 4c, h), while *TaCO1* expression showed reduced amplitude of the main peak in the ‘GS-100’/‘GS-101’ comparison (Fig. 4d) and a significant increase at two points in the light period in the

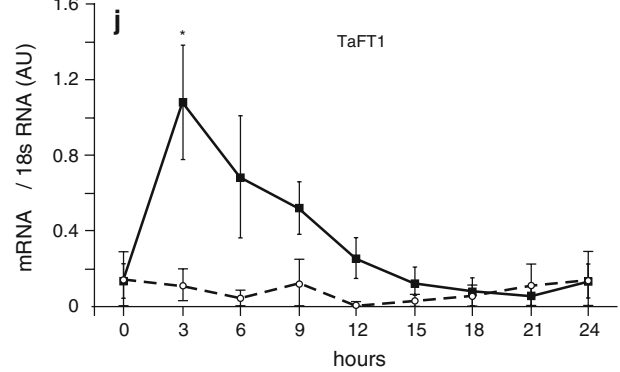
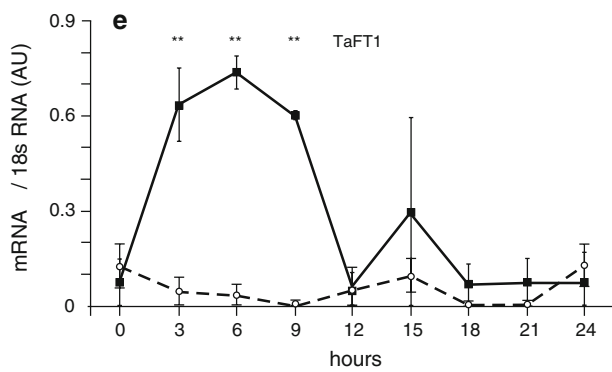
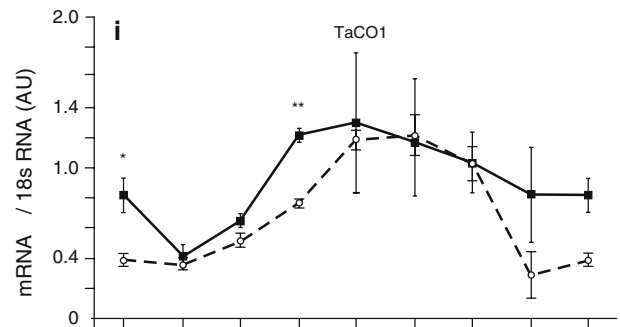
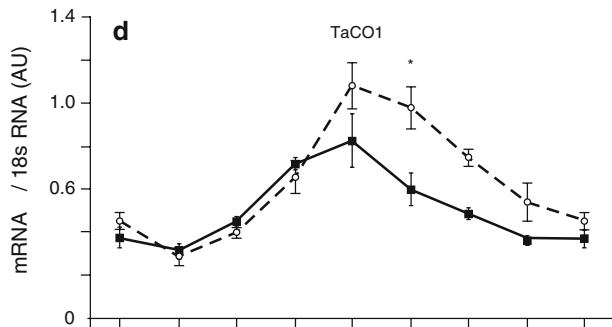
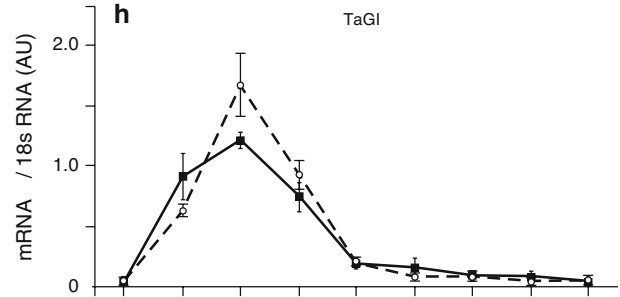
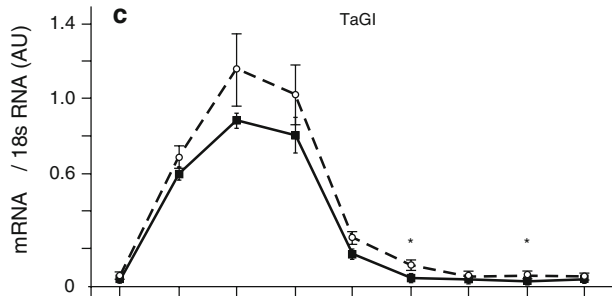
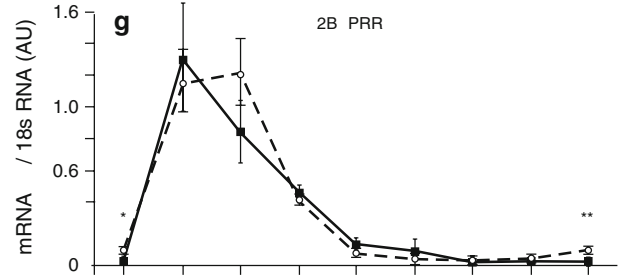
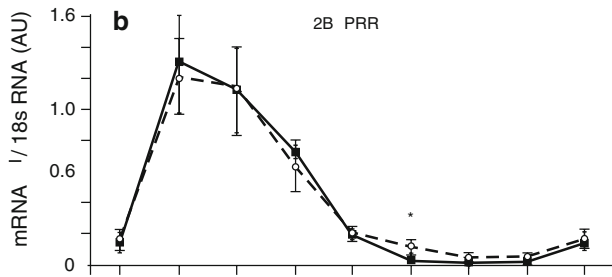
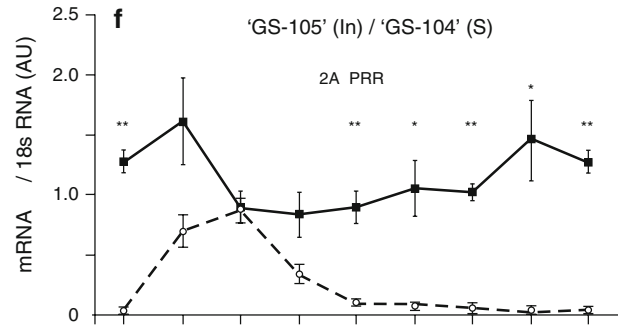
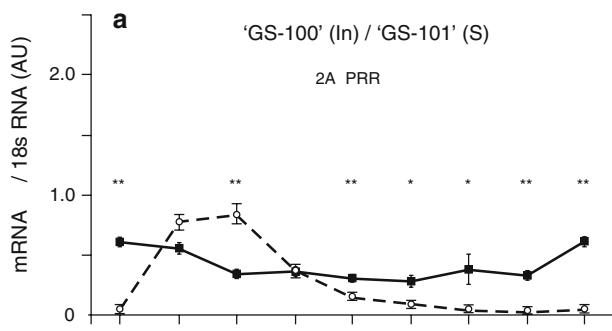
‘GS-104’/‘GS-105’ comparison (Fig. 4i). *TaFT1* expression was significantly increased during the light period at three time points (3, 6 and 9 h after dawn) in ‘GS-100’ and at one time point (3 h after dawn) in ‘GS-105’ (Fig. 4e, j). These results are similar to those previously found for the *Ppd-D1a* mutation (Beales et al. 2007), suggesting that the *Ppd-A1a* and *Ppd-D1a* deletions confer early flowering by a common mechanism resulting from the loss of normal circadian regulation of the *PRR* gene.

The 2B *PRR* gene showed very similar expression patterns between lines and had an expression peak coincident with that of the 2A *PRR* gene in the photoperiod sensitive lines (Fig. 4b, g). Significant variation was only observed at three points with very low expression and this is unlikely to be biologically meaningful. Therefore, the relative earliness of ‘GS-101’ and ‘GS-104’ (the photoperiod sensitive lines) in short days is not likely to be due to a weak photoperiod insensitive allele of either the 2A or 2B *PRR* genes. The graphs also show that the expression peak of the 2B *PRR* gene was not affected by the presence of a photoperiod insensitive allele on 2A.

Discussion

Sequencing and phenotypic analysis identified novel photoperiod insensitive mutations on the A genome of wheat (*Ppd-A1a* alleles). The finding of a common deleted region in the two A genome mutations described here and the D genome mutation (Beales et al. 2007) suggests that a single mechanism causes the insensitive phenotype and this conclusion is reinforced by the similarities in gene expression.

Recent work on circadian control of the Arabidopsis photoperiod pathway shows that *CYCLING DOF FACTOR 1* (*CDF1*) represses *CO* expression by binding to the *CO* promoter (Imaizumi et al. 2005). *CDF1* is expressed at its highest level at the start of the day and Nakamichi et al. (2007) have proposed that its transcription is repressed by the action of *PRR3*, *PRR5* and *PRR7* (the closest Arabidopsis homologue to *Ppd*). *CDF1* protein is removed from the *CO* promoter by a complex involving GI and FKF1 (Sawa et al. 2007). Consistent with this, overexpression of *PRR3*, 5 or 7 in Arabidopsis gave early flowering phenotypes (Matsushika et al. 2007). If this system is conserved in cereals the deregulated expression of *PRR* genes observed in photoperiod insensitive wheat mutations would be predicted to reduce *CDF1* expression, allowing *CO* transcription to increase in the early part of the day. Coincidence of *CO* expression with light would then induce *FT* (Valverde et al. 2004). A higher level of *TaCO1* transcript was observed for ‘GS-105’ (Fig. 4i) and this was associated with higher levels of *PRR* transcript (compare Fig. 4f and 4a). However, no increase in *TaCO1* expression was found



◀ **Fig. 4** Quantitative gene expression data from plants grown in short days (0–9 h light period) at 22°C (light)/18°C (dark). The graphs compare expression between wild type (*open circles, dashed line*) and photoperiod insensitive (*solid circles, solid line*) plants from the near-isogenic pairs of ‘GS-100’ (In) and ‘GS-101’ (S) (*left panel*) and ‘GS-105’ (In) and ‘GS-104’ (S) (*right panel*). Values are expressed as relative levels normalized against 18S ribosomal RNA. SE bars are of

for ‘GS-100’ (Fig. 4d) even though this was the earlier flowering of the genotypes. This suggests that additional factors such as CO protein production or turnover may also be important. Alternatively, there could be a direct effect on *TaFTI* expression. This requires further study.

The loss of a common region from the 2A and 2D deletions suggests that the removal of one or more regulatory regions underlies the photoperiod insensitive phenotype. The approximately 900 bp region defined by the three deletions is too large to allow causal regulatory motifs to be identified. However, one region of interest was identified by aligning the wheat sequences with barley (*Hordeum vulgare*) (AY943294), a *Brachypodium sylvaticum* BAC analysed by Turner et al. (2005) and the orthologous gene from rice (PRR37; AP005199). The alignment with *Brachypodium*, and especially with rice, was poor apart from a segment of approximately 100 bp (Fig. S2 in ESM) located about 120 bp upstream of the transcription start site previously identified by 5' RACE (Beales et al. 2007). The conserved region was analysed using the PLACE database (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>) but this did not identify a connection to the circadian clock or other genes known to be involved in photoperiod response. The significance of this or other deleted regions therefore needs to be analysed further by identifying new deletion alleles or by testing transgenic constructs from which specific promoter regions are removed.

Sequence comparisons show that the ‘GS-100’ allele could have arisen by deletion within a ‘GS-101’ type allele. The ‘GS-105’ allele had different deletion breakpoints and a distinct haplotype, showing that it derives from an independent source. Comparison with sequences from hexaploid wheat showed additional polymorphisms separating the hexaploid and tetraploid genotypes but four polymorphisms in the central part of the ‘GS-105’ sequence matched the hexaploid wheat sequence, suggesting a recombinant haplotype (polymorphisms 15, 16, 17, and 20; Table 1). However, while the ‘GS-105’ allele must have considerable evolutionary separation from the other sequences, the early flowering mutation may have occurred recently. To assess this, further haplotype analysis is necessary.

Plants with the ‘GS-105’ *Ppd-A1a* allele consistently flowered a few days later than plants with the ‘GS-100’ *Ppd-A1a* allele and this would be consistent with the increased *TaFT* expression observed in ‘GS-100’ (Fig. 4e, j). This could be due to differences between the deletions

three biological replicates and statistical variation between alleles is shown by * ($P < 0.05$) and ** ($P < 0.01$). PCR primers and reactions conditions are given in supplementary Table S2. **a, f** Expression of the 2A *PRR* gene. **b, g** Expression of the 2B *PRR* gene. **c, h** Expression of *TaGI*. **d, i** Expression of wheat *TaCO1*. **e, j** Expression of wheat *TaFT*. Assays in **a, b, f** and **g** are gene specific. Assays in **c, d, e, h, i** and **j** detect the composite A and B genome expression level

themselves, to background genotype or to variation within the respective proteins such as the Q to R change that was only seen in ‘GS-105’ (polymorphism 23, Table 1). This can be investigated by making new lines with the alleles in a common genetic background and by making different constructs for transformation. In the shorter term, diagnostic markers can be used to classify durum wheat germplasm and to assist the crossing of *Ppd-A1a* alleles into hexaploid wheat to assess their effects and interactions with photoperiod insensitive *Ppd-B1a* and *Ppd-D1a* alleles. This will be valuable in defining the range of flowering time variation that various *Ppd* alleles, alone and in combination, can provide.

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