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ORIGINAL PAPER

A *Pseudo-Response Regulator* is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.)

James Beales · Adrian Turner · Simon Griffiths · John W. Snape · David A. Laurie

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Abstract *Ppd-D1* on chromosome 2D is the major photoperiod response locus in hexaploid wheat (*Triticum aestivum*). A semi-dominant mutation widely used in the "green revolution" converts wheat from a long day (LD) to a photoperiod insensitive (day neutral) plant, providing adaptation to a broad range of environments. Comparative mapping shows *Ppd-D1* to be colinear with the *Ppd-H1* gene of barley (*Hordeum vulgare*) which is a member of the pseudo-response regulator (*PRR*) gene family. To investigate the relationship between wheat and barley photoperiod genes we isolated homologues of *Ppd-H1* from a 'Chinese Spring' wheat BAC library and compared them to sequences from other wheat varieties with known *Ppd* alleles. Varieties with the photoperiod insensitive *Ppd-D1a* allele which causes early flowering in short (SD) or LDs had a 2 kb deletion upstream of the coding region. This was associated with misexpression of the 2D *PRR* gene and expression of the key floral regulator *FT* in SDs, showing that photoperiod insensitivity is due to activation of a known photoperiod pathway irrespective of day length. Five *Ppd-D1* alleles were found but only the 2 kb deletion was associated with photoperiod insensitivity. Photoperiod insensitivity can also be conferred by mutation at a homoeologous locus on chromosome 2B (*Ppd-B1*). No candidate mutation was found in the 2B *PRR* gene but polymorphism within the 2B *PRR* gene cosegregated with the *Ppd-B1* locus in a doubled haploid population, suggesting that insensitivity on 2B is due to a mutation outside the sequenced region or to a closely linked gene.

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J. Beales and A. Turner contributed equally to the work.

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J. Beales · A. Turner · S. Griffiths · J. W. Snape · D. A. Laurie (✉)
Crop Genetics Department, John Innes Centre,
Norwich Research Park, Colney, Norwich NR4 7UH, UK
e-mail: david.laurie@bbsrc.ac.uk

Introduction

The high yielding hexaploid wheat (*Triticum aestivum* L.) varieties of the "green revolution" were characterized by rust resistance, semi-dwarf stature and adaptation to a broad range of agricultural environments conferred by insensitivity to photoperiod (day length neutrality) (Borlaug 1983). In wheat terminology, "insensitivity" means that plants have a short delay in flowering in short day (SD) conditions (10 h or less light) compared to long day (LD) conditions (14 h or more light), provided that any requirement for vernalization has been met. For example, in 127 varieties studied by Worland et al. (1994) the difference in flowering time between SDs and LDs was 21–35 days for photoperiod insensitive varieties and 64–200 or more days for photoperiod sensitive varieties. Photoperiod insensitivity is widespread in the world's wheat varieties and predominates in regions where spring wheat is grown as a crop over the winter (SD) period and where autumn sown winter wheat needs to mature in the following year before the onset of high summer temperatures (Law 1987; Law and Worland 1997; Worland and Snape 2001). A study of 158 wheat landraces by Kato and Yokoyama (1992) also concluded that early flowering conferred by an alteration of photoperiod response was important for regional adaptation by avoiding stresses associated with high temperature.

The major source of photoperiod insensitivity in wheat is the semi-dominant *Photoperiod-D1a* (*Ppd-D1a*) allele on

chromosome 2D [Welsh et al. 1973; Worland et al. 1998; Worland 1999; following the nomenclature recommended by McIntosh et al. (2003); *Ppd-A1*, *-B1* and *-D1* are the former *Ppd3*, *2* and *1*, respectively, and the *a* suffix indicates the photoperiod insensitive allele]. Photoperiod insensitivity is likely to be the result of mutation because the wild ancestors of wheat, and many cultivated wheat varieties, are sensitive to photoperiod and are stimulated to flower by exposure to LDs (Thomas and Vince-Prue 1997).

The importance of the *Ppd-D1a* allele is illustrated by the detailed studies of Worland and Law (1986) and Worland et al. (1998). Near-isogenic lines were created by backcrossing the *Ppd-D1a* allele from 'Mara' into the photoperiod sensitive variety 'Cappelle-Desprez'. The *Ppd-D1a* allele caused earlier flowering which was associated with an average yield disadvantage in the UK (−1.8%; range −16 to +9%), a yield advantage in Germany (+7.7%; range −13.5 to +28.4%) and a considerable yield advantage in the former Yugoslavia (+33%; range +10.1 to +59.6%). The ranges show that photoperiod insensitivity was always beneficial to yield in the Southern European setting but was variable from year to year in the more northerly locations depending on conditions and conferred a yield advantage even in the UK in years with hotter drier summers. The ability of photoperiod insensitive varieties to flower earlier in the year is advantageous in warmer environments because plants can complete development and grain filling before the onset of high summer temperatures and associated water deficit. High summer temperatures in wheat growing regions are likely to become more prevalent with climate change, making it important to understand the genetic basis of photoperiod insensitivity and the range of flowering time variation that is available to wheat breeders.

The region of wheat chromosome 2D containing the *Ppd-D1* locus is colinear with the region of barley (*Hordeum vulgare* L.) 2HS that contains the photoperiod gene *Ppd-H1* (Laurie 1997; Börner et al. 1998). The barley *Ppd-H1* gene has recently been cloned and identified as a member of the pseudo-response regulator (*PRR*) family (Turner et al. 2005). *PRR* proteins are characterized by a pseudo-receiver domain near the amino-terminus and a 43 amino-acid CCT domain near the carboxy-terminus of the protein (Mizuno and Nakamichi 2005). *PRR* genes are distantly related to other CCT domain genes important in flowering control, namely the *CONSTANS*-like family of transcription factors (Robson et al. 2001; Griffiths et al. 2003) and the wheat vernalization gene *VRN2* (Yan et al. 2004). Five *PRR* genes are present in *Arabidopsis* and rice (Matsushika et al. 2000; Murakami et al. 2003, respectively) and they are best characterized in the former where *PRR1* (*TOC1*) is part of the central oscillator of the circadian clock (Strayer et al. 2000). The remainder are likely to comprise parts of the interlocking feedback loops associated with the circadian clock, although their full roles have not yet been determined (Mizuno and Nakamichi 2005; Zeilinger et al. 2006). Cockram et al. (2007) provide a recent review of flowering pathways in temperate cereals and Arabidopsis.

A homologue of the barley *Ppd-H1* gene would be an excellent candidate for the wheat *Ppd-D1* locus, but wheat and barley show an important phenotypic difference in photoperiod response. The semi-dominant *Ppd-D1a* mutation in wheat allows plants to flower rapidly in SD or LD conditions while in barley the recessive *ppd-H1* mutation confers later flowering in LDs (e.g. 16 h light) but has no phenotypic effect in SDs (10 h light) (Laurie et al. 1995; Turner et al. 2005). Late flowering in barley can be explained by parallels to the well-understood photoperiod pathway in *Arabidopsis* where the circadian clock, which is set by light/dark cycles or temperature fluctuations, controls the timing of *CONSTANS* (*CO*) expression. *CO* levels peak late in the day and coincide with light only in LD conditions. CO protein is stable only in the light and therefore activates its downstream targets specifically in LDs (Suárez-López et al. 2001; Valverde et al. 2004). A key target of CO is *FLOWERING LOCUS T* (*FT*) (Samach et al. 2000). Barley plants homozygous for the recessive *ppd-H1* allele show a small change in the circadian expression pattern of *CO* homologues, with expression delayed in the day so that the expression peak occurs in the dark even under LDs. This is associated with a marked decrease in *FT* expression consistent with the late flowering phenotype (Turner et al. 2005). If the wheat and barley *Ppd* genes are homologous the two species must have contrasting types of mutation. One possibility is that the *Ppd-D1a* mutation in wheat causes misexpression of a functional protein, leading to activation of the photoperiod pathway and induction of *FT* under SD or LD conditions. To test this hypothesis, the wheat chromosome 2D homologue of the barley *Ppd-H1* gene was isolated and analyzed.

Photoperiod insensitivity can also be conferred by a locus on chromosome 2B (*Ppd-B1*) (Welsh et al. 1973; Scarth and Law 1983; Mohler et al. 2004) and a third locus (*Ppd-A1*) may be present on chromosome 2A but has not been genetically mapped (Law et al. 1978; Scarth and Law 1984). This suggests that *Ppd* genes form a homoeoallelic series on the group 2 chromosomes. This was investigated by cloning and sequencing the *PRR* genes from chromosomes 2B and 2A.

Materials and methods

Plant materials

Seeds of the various wheat varieties and genetic stocks were provided by the John Innes Centre Germplasm

Resources Unit or by Elizabeth Sayers and Simon Orford (John Innes Centre). Seeds of 'Norstar' were kindly provided by Prof. Brian Fowler, Univerity of Saskatchewan, Canada. DNA was extracted from seedlings using the phenol/chloroform method of Sharp et al. (1988).

Cloning and sequencing wheat *PRR* genes

Library screening, BAC DNA preparation and Southern hybridization followed the methods given in Allouis et al. (2003). Libraries with inserts of approximately 2–4 kb were made from selected BAC clones using the TOPO Shotgun Cloning Kit (Invitrogen) and following the manufacturer's protocols. Clones were sequenced using BigDye version 3.1 (Appled Biosystems, USA). PCR amplicons were sequenced directly or after cloning in pGEM-T Easy vector (Promega Corp., USA) following the manufacturer's protocols. Nucleotide and protein sequences were analyzed using the software packages GCG10 (Wisconsin Package V10.1, Genetics Computer Group, Madison, WI, USA) and by BLAST using programs at the NCBI web site (http://www.NCBI.org), TIGR (http://www.tigr.org) and the Triticeae Repeat database (TREP; http://wheat.pw.usda.gov/ITMI/Repeats). Protein domains were analyzed using Prosite (http://www.us.expasy.org). The sequences illustrated in Fig. 1 were aligned using the pileup command in GCG and adjusted manually to identify homologous regions.

GenBank sequence accessions are; *Ppd-A1* 'Chinese Spring' DQ885753, 'Mercia' DQ885756, 'Cappelle-Desprez' DQ885754, 'C591' DQ885755. *Ppd-B1* 'Chinese Spring' intact copy DQ885757, 'Chinese Spring' truncated copy DQ885758, 'Récital' DQ885763, 'Timstein' DQ885765, 'Cappelle-Desprez' DQ885759, 'Cheyenne' DQ885760, 'Mercia' DQ885761, 'Renan' DQ885764. *Ppd-D1* 'Chinese Spring' DQ885766, 'Mercia' DQ885768, 'Norstar' DQ885770, 'Ciano 67' DQ885767, *Aegilops tauschii* DQ885771.

Expression analysis

RNA of 'Mercia' and the 'Mercia (Ciano 67 2D)' single chromosome substitution line was extracted from seedlings grown for 24 days after vernalization for 45 days at 5°C with 8 h light/16 h dark. Seedlings were grown in a controlled environment room under SD conditions (9 h light; 136 µmol m^{-2} s^{-1} light intensity at bench level). Plants were compared from three replicates using a single RNA extraction for four plants per time course sample in each replicate. The time course was one 24 h period. RNA was extracted using the Qiagen RNeasy kit (Qiagen Ltd., UK; http://www.qiagen.com). DNA was removed by digestion with DNAse I prior to reverse transcription. cDNA was synthesized using Superscript II (Invitrogen Corp., USA; http://www.invitrogen.com) 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. 1/40 by volume of the final cDNA aliquot was then subjected to real-time PCR as described below. In the case of 18 s rRNA analysis, cDNA samples were diluted 1:100 and 1/40 of this dilution was analyzed.

Primers for each gene and the respective reaction conditions are listed in Table S1. Where possible, primers amplifying across intron positions were selected to detect any genomic contamination. Genome-specificity was tested by PCR using Chinese Spring nullisomic-tetrasomic lines (Fig. S2) and verified by direct sequencing of amplicons. RNA was quantified using an Opticon 2 real-time PCR instrument (Genetic Research Instrumentation Ltd, UK; http://www.gri.co.uk) with SybrGreen I (Molecular Probes Inc., USA) as a fluorogen. Reactions included 10 µl 2× Sigma Jumpstart SybrGreen Mastermix (Sigma-Aldrich Co. Ltd., UK; http://www.sigmaaldrich.com), 5 pmol of each primer, and an aliquot of each 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) × 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 were collected and analyzed using Opticon Monitor v2 software (http://www.gri.co.uk) with baseline subtraction and blank (no cDNA template) 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 Ct value (the cycle value at which each sample reached the fluorescence threshold) was extracted for each sample and imported into Microsoft Excel®. Delta Ct values were generated by subtracting the minimum Ct 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 Ct. *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 18 s rRNA levels within samples.

5′ Reverse Amplification of cDNA ends (RACE) was performed using the Smart™ RACE cDNA Amplification Kit (Takara-Clontech, USA) using the manufacturer's protocols. First strand cDNA synthesis was performed on 1 µg of DNAse-treated total RNA from seedlings of either 'Mercia' or the 'Mercia (Ciano 67 2D)' single chromosome substitution line, grown as described above. Synthesis was performed on 'Mercia' RNA taken 3 h after the start of the light

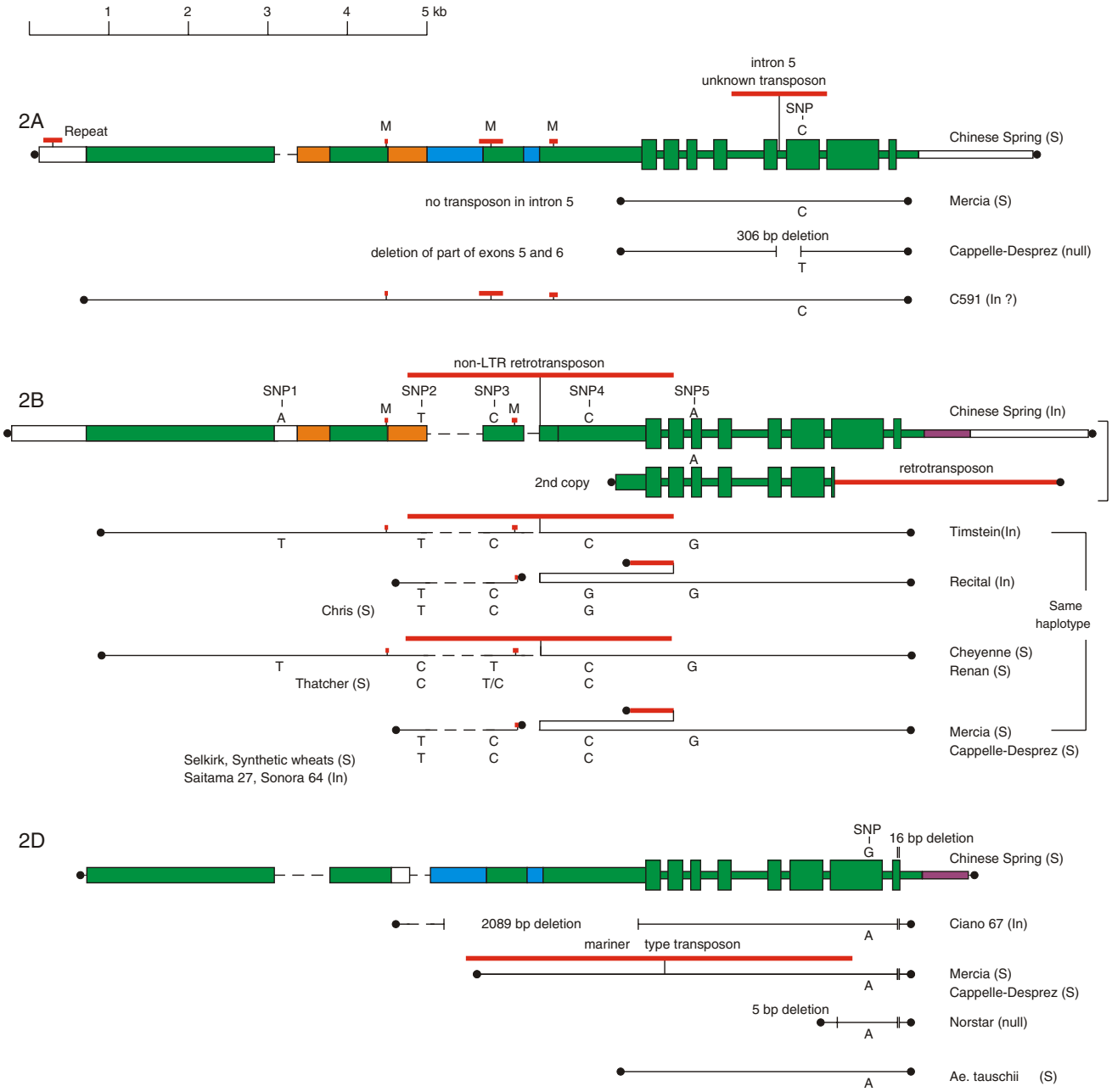

Fig. 1 Sequence features of *Pseudo-Response Regulator* (*PRR*) genes from chromosomes 2A, 2B and 2D of hexaploid wheat. The 'Chinese Spring' sequence is shown as *rectangles* (*tall rectangles* represent coding regions, *medium rectangles* the candidate promoter and 5′ UTR segments and *small rectangles* the introns and 3′ UTR regions. Segments well conserved between all three genome are shown in *green*, those conserved between A and B in *orange*, between A and D in *blue* and between B and D in *purple*. *White segments* are genome specific sequences. *Red lines* show the size and insertion positions of various transposons described in the text (*M* indicates MITE). *Dotted lines* are gaps in the sequence alignment that remain after extraction of the transposons. Regions sequenced from other varieties are shown as *black lines* below each 'Chinese Spring' sequence. *Black dots* show the limits of the sequence obtained from each variety. *Vertical bars* show the extent of deletions. The size of the 'Cappelle-Desprez' 2A deletion is relative to 'Mercia'. *SNP* shows the positions of single nucleotide polymorphisms. Varieties with photoperiod insensitive (*In*) or photoperiod sensitive (*S*) alleles on the respective chromosomes are marked. GenBank sequence accessions are given in the section "Materials and methods". Varieties named on the left in the 2B gene were sequenced only for the regions containing SNPs 2, 3, and 4

period, and on 'Mercia (Ciano 67 2D)' RNA taken 12 h after the start of the light period (see Fig. 4). Primary PCRs were conducted on the synthesized cDNAs using gene-specific primers cctgcggcgacggcggctgctggtggtga for 'Mercia' and gaagtcggcggcaccatttgacaggcag for 'Mercia (Ciano 67 2D)'. Secondary (nested) PCRs were performed on aliquots of the appropriate primary PCRs using nested gene-specific primers cgaacctcggtcgcatccgggagtcccag for 'Mercia' and cctgcg-

gcgacggcggctgctggtggtga for 'Mercia (Ciano 67 2D)'. RACE fragments were purified from agarose gels using the Qiagen gel-extraction kit and cloned into pGEM-T (Promega Corp., USA; http://www.promega.com). DNA from seven colonies of each RACE synthesis was sequenced using M13 forward and reverse primers.

PCR assay for the 2,089 bp deletion in the 2D *PRR* gene

Assays used a common forward primer (Ppd-D1_F acgcctcccactacactg) combined with two reverse primers (Ppd-D1_R1 gttggttcaaacagagagc and Ppd-D1_R2 cactggtggtagctgagatt) in a single 25 µl reaction containing 5 pmol of each primer with a PCR profile of 94°C for 2 min followed by 30 cycles of 94°C for 40 s, 54°C for 30 s, 72°C for 1 min. Products were separated on 1.2% agarose gels in TAE buffer. Ppd-D1_R1 is within the deletion and only amplified from the intact sequence, giving a 414 bp product. Ppd-D1_R2 is within intron 1 and amplified a 288 bp product from the allele with the deletion. Ppd-D1_R2 can potentially amplify a 2,377 bp product from the intact gene but in practice this reaction was out-competed by the amplification of the smaller products.

PCR assay for a 16 bp deletion in exon 8 distinguishing cultivated wheat varieties from *Ae. tauschii*

Amplification with Ppd-D1exon8_F1 (gatgaacatgaaacggg) and Ppd-D1exon8_R1 (gtctaaatagtaggtactagg) using a PCR profile of 94°C for 2 min followed by 30 cycles of 94°C for 40 s, 54°C for 30 s, 72°C for 1 min generated a 320 bp amplicon from genotypes with the deletion and 336 bp from genotypes with the intact gene. The 16 bp deletion removes a *Hpa*II restriction site. 'Chinese Spring' and other cultivated types give 326 bp plus 22 bp bands. *Ae. tauschii* types give 257, 69 and 22 bp bands. Products were separated on 1.2% agarose gels in TAE buffer.

PCR assays for the 2B *PRR* genes from 'Chinese Spring'

Amplification with Ppd-B1exon3SNP_F1 (agacgattcattccgctcc) and Ppd-B1exon3SNP_R1 (tctgaatgatgatacaccatg) using a PCR profile of 94°C for 2 min followed by 40 cycles of 94°C for 40 s, 55°C for 30 s, 72°C for 1 min generated a 955 bp amplicon. The SNP in exon 3 (A in 'Chinese Spring' and G in the other varieties sequenced) was distinguished by digestion with *Tsp*509I (65°C overnight) which gave 471, 328 and 155 bp bands from the 'Chinese Spring' allele and 483 and 471 bp bands from the other varieties. The partly deleted second gene in 'Chinese Spring' was detected by amplification with Ppd-B1_2ndcopy_F1 (taactgctcgtcacaagtgc) and Ppd-B1_2ndcopy_R1 (ccggaacctgaggatcatc) using a PCR profile of 94°C for 2 min followed by 40 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 40 s. This gave a 425 bp product. In the absence of the 'Chinese Spring' allele a faint band of 475 bp was sometimes produced. Products were separated on 1.2% agarose gels in TAE buffer.

Results

Isolation of wheat *PRR* genes

High density filters of wheat BAC clones from a library of the variety 'Chinese Spring' (Allouis et al. 2003) were screened using a CCT domain probe from the barley *Ppd-H1* gene. DNA was prepared from individual BAC clones and Southern blots of restriction digests were probed with the barley sequence and compared to Southern blots of genomic DNA from 'Chinese Spring' nullisomic/tetrasomic (NT) lines. This identified candidate clones from chromosomes 2A, 2B and 2D. Representatives of each were subcloned and sublibraries from individual BACs were reprobed with a full-length barley cDNA. Hybridizing subclones were sequenced and any remaining gaps filled by PCR amplification from the individual BAC clones. Three distinct sequences were found which were confirmed as originating from chromosomes 2A, 2B or 2D using sequence specific PCR assays on 'Chinese Spring' NT lines. Overlapping segments from other wheat genotypes were amplified by PCR, sequenced and assembled into full gene sequences (Fig. 1). PCR was also used to amplify sequences from an accession of the D genome donor *Ae. tauschii*.

Polymorphisms in the chromosome 2D *PRR* gene

Photoperiod insensitivity conferred by the *Ppd-D1a* allele has been widely used in wheat breeding and its effects have been studied in detail (Worland and Snape 2001). Among the materials developed by Worland et al. were photoperiod insensitive single chromosome substitution lines in which the endogenous 2D of 'Chinese Spring' or 'Mercia' was replaced with the 2D chromosome from 'Ciano 67'. To determine if the 2D *PRR* gene was a candidate for the *Ppd-D1* locus we compared sequences from these three varieties. The gene from 'Ciano 67' (photoperiod insensitive) differed from 'Chinese Spring' and 'Mercia' (photoperiod sensitive) by a deletion of 2,089 bp upstream of the coding region (Fig. 1). Butterworth (2000) analyzed 114 DH lines from a 'Mercia' × 'Mercia (Ciano 67 2D)' cross in two controlled SD experiments (10 h natural light) and under field conditions. In all experiments the lines could be unambiguously characterized as early or late flowering. A PCR assay for the deletion (Fig. 2a) showed that the 'Ciano 67' allele cosegregated completely with early flowering.

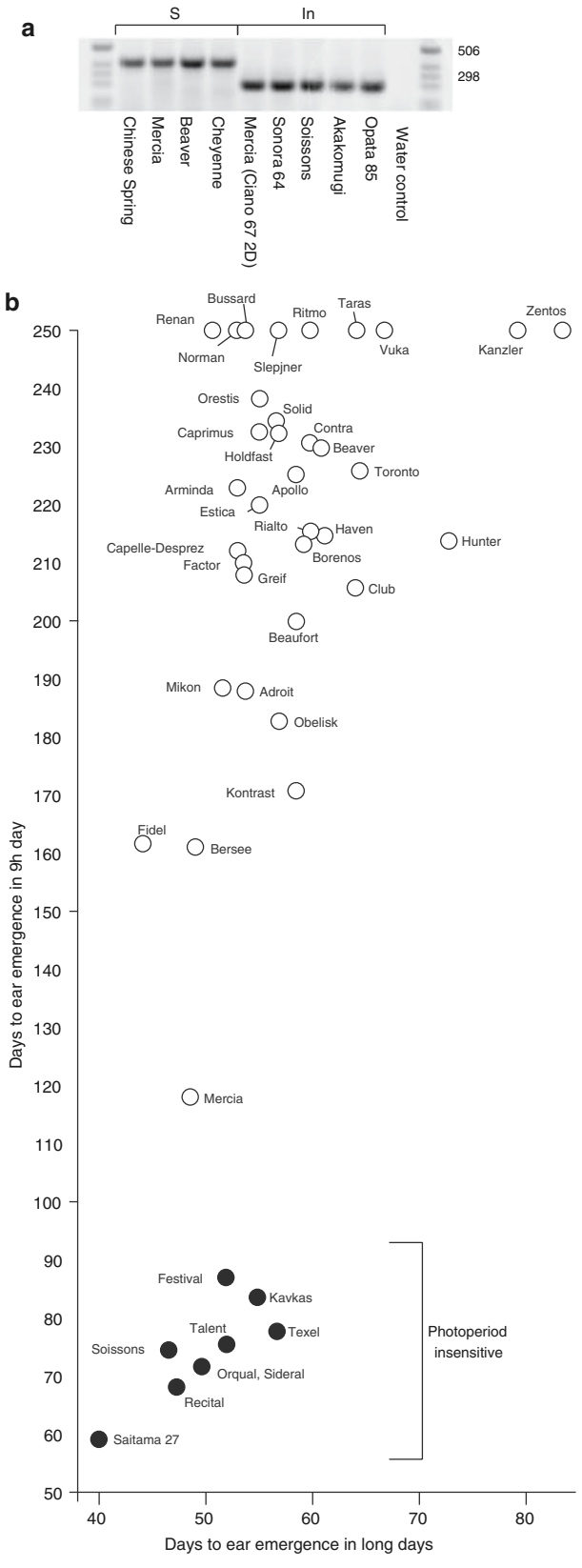

Fig. 2 Detection of the 2,089 bp upstream deletion in the chromosome 2D *PRR* gene. **a** PCR assay for the photoperiod sensitive (*S*) or insensitive (*In*; *Ppd-D1a*) 2D allele. Primer sequences and reaction conditions are given in the materials and methods. **b** Detection of the deletion in wheat varieties classified for photoperiod sensitivity by Worland et al. (1994). *Solid circles* show varieties with the deletion. *Open circles* show varieties with the intact sequence

The deletion was present in the CIMMYT varieties 'Sonora 64' and 'Opata 85' and the French varieties Récital and Soissons which are known to have the photoperiod insensitive *Ppd-D1a* allele (Welsh et al. 1973; Börner et al. 2002; Hanocq et al. 2004; Snape unpublished data, respectively). The *Ppd-D1a* allele is believed to have entered European material from Strampelli's breeding programme in Italy in the early part of the twentieth century, using the Japanese variety 'Akakomugi' as a source (Worland 1999). The Italian wheat 'Mara' used by Worland et al. (1998) and 'Akakomugi' had the deletion as did another early flowering Japanese wheat 'Saitama 27'. Varieties with a photoperiod sensitive allele on 2D ('Chris', 'Era', 'Lancer' and 'Selkirk'; Welsh et al. 1973) had the intact sequence. We then analyzed a further 48 European wheat varieties representing the range of flowering time described by Worland et al. (1994) who compared vernalized plants grown in SD or LD conditions. Lines classified as photoperiod insensitive in that paper (less than 35 days difference in days to ear emergence between SDs and LDs) all carried the deletion (Fig. 2b).

The 2,089 bp upstream deletion was the only polymorphism associated with photoperiod insensitivity but three additional polymorphisms were found in the 2D gene (Fig. 1). 'Chinese Spring' was distinct in having a SNP in exon 7 predicted to cause a conservative Ala to Thr change upstream of the CCT domain. Intron 1 was much larger in 'Mercia' that in the other varieties sequenced due to the presence of a *mariner*-like transposable element (MLE) identified by internal homology of predicted open reading frames, by the presence of 31 bp terminal inverted repeats (TIRs) whose ends were similar to the TIRs of *Stowaway* MITEs and a TA host duplication typical of *Stowaways* and MLEs (Feschotte and Wessler 2002; Feschotte et al. 2003). In SD conditions 'Mercia' can be clearly distinguished from varieties with the 'Ciano 67' type 2,089 bp deletion. However, 'Mercia' is earlier flowering in SDs than the remaining photoperiod sensitive varieties in Fig. 2b. This is not caused by the intron 1 insertion because this was also present in 'Cappelle-Desprez' which flowers late in SDs (212 days, Fig. 2b). The third polymorphism was a 5 bp deletion in exon 7 found in an EST from the photoperiod sensitive variety 'Norstar'. This was confirmed by sequencing genomic DNA of this variety and is likely to give a non-functional protein as the deletion produces a frame shift predicted to generate a stop codon upstream of the CCT domain. None of the early flowering

lines with the 2,089 bp upstream deletion also carried the exon 7 deletion.

When compared to barley and the hexaploid wheat 2A and 2B *PRR* genes, the 2D gene of all the cultivated wheat varieties that were sequenced had a 16 bp deletion in exon 8 (Fig. 3a). A PCR assay (illustrated in Fig. 3b) showed that this deletion was present in all the cultivated wheat varieties we examined. The deletion included the last two bases of the CCT domain and was predicted to change the last amino acid of the CCT domain (Gln to Leu) and to produce a new COOH terminus for the protein. The new protein is likely to be of similar length as the 3′ region has an alternative stop codon 17 bp downstream of the original (Fig. 3a). The deletion was not found in the *Ae. tauschii* accession that was sequenced and the intact sequence was also detected in two synthetic wheats (one from the JIC germplasm collection and one of the parents of the ITMI mapping population) that derive from crosses between tetraploid wheat and *Ae. tauschii*. It is unclear if the deletion has any effect on protein function but it can be excluded as the causal basis of the photoperiod insensitive phenotype.

Analysis of gene expression in wheat plants with contrasting *Ppd-D1* alleles

To understand how photoperiod insensitivity might be conferred we used quantitative RT-PCR to study the expression of wheat homologues of well-characterized *Arabidopsis* photoperiod pathway genes. We selected *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) and the 2D *PRR* gene itself to compare 'Mercia' with the 'Mercia (Ciano67 2D)' (*Ppd-D1a*) single chromosome substitution line. *GI* is closely associated with the circadian clock, *CO* is under circadian control and the protein is only active when the expression peak, which normally occurs late in the day, coincides with light. CO protein promotes transcription of *FT*, a key inducer of flowering (Valverde et al. 2004). *GI*, *CO* and *FT* have all been shown to have conserved roles in other species including rice (Hayama et al. 2003; Kojima et al. 2002; Yano et al. 2000).

Wheat *GI* and *FT* homologues were previously described by Zhao et al. (2005) and Yan et al. (2006) but wheat homologues of the barley *HvCO1* gene [the counterpart of the rice *CO* homologue *Hd1* (Griffiths et al. 2003)]

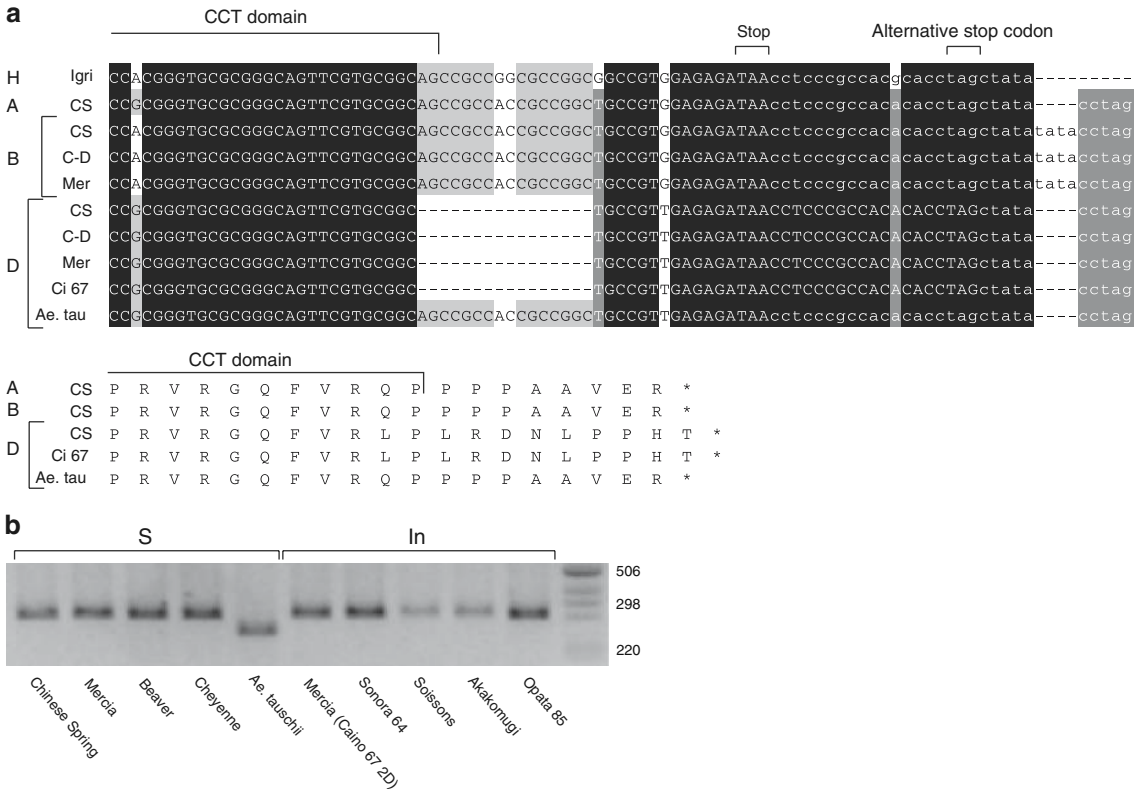

Fig. 3 Position of the 16 bp deletion in exon 8 of the chromosome 2D *PRR* gene. **a** The position of the deletion in relation to the 2A, 2B and barley 2H (AY970701) sequences. *Uppercase letters* show the predicted coding region of each gene including the stop codon. Predicted protein sequences are shown below. Wheat varieties included are *CS* 'Chinese Spring', *C-D* 'Cappelle-Desprez', *Mer* 'Mercia', *Ci 67* 'Ciano 67' and *Ae. tauschii*. **b** PCR assay for the 16 bp deletion. Primers and reaction conditions are given in the section "Materials and methods"

have not been described. Partial *TaCO1* and *TaFT* sequences were isolated by PCR using primers based on the equivalent barley *HvCO1* or *HvFT* genes (Griffiths et al. 2003; Turner et al. 2005, respectively). PCR primers and conditions used for the assays are described in Table S1.

To obtain an overall picture of gene expression for the group 2 *PRR* genes we first used a primer pair that was not genome specific and would amplify from transcript of the 2A, 2B and 2D genes. This showed reduced diurnal fluctuation in the photoperiod insensitive 'Ciano67' (*Ppd-D1a*) substitution line (Fig. 4a). When gene specific primers were used the 2D gene was found to be misexpressed with the peak shifted from the light to the dark period (to 21 h compared to 3 h in the 'Mercia' wild type, Fig. 4d). The 2A and 2B transcripts behaved similarly to 'Mercia' in terms of the timing of peak expression (Fig. 4b, c) and were similar to previous experiments in barley (Turner et al. 2005). The reduction in the amplitude of gene expression was not statistically significant. This suggested that the 2,089 bp deletion caused the 2D gene to be misexpressed but had little effect on the 2A or 2B genes.

For the analysis of other photoperiod pathway genes we used assays that were not genome specific in order to obtain an overall picture of expression. This showed that *TaGI* expression was unaltered (Fig. 4e). There was no effect on the timing of the *TaCO1* expression peak (Fig. 4f) but the amplitude of expression was significantly reduced. There was a clear effect on *TaFT1* expression (Fig. 4g) with high levels in the photoperiod insensitive (*Ppd-D1a*) genotype. Although this supports the original hypothesis that photoperiod insensitivity results from activation of *FT* expression in SD conditions it is not clear whether this is achieved by a change in *TaCO* activity. An alternative mechanism could involve TaCO protein. In *Arabidopsis*, CO protein is degraded in the dark (Valverde et al. 2004). *TaFT* expression in the 'Mercia (Ciano67 2D)' line increased in the light period and decreased in the dark (Fig. 4g), suggesting that TaCO protein may accumulate in the SD light period and degrade in the dark in photoperiod insensitive wheat.

The 2,089 bp deletion in the chromosome 2D *PRR* gene ended approximately 50 bp upstream of the predicted ATG start codon, suggesting that the transcription start site might be affected. To test this, we first used the GC strand bias formula of Fujimori et al. (2005) to make a computational prediction of the start site (Fig. 5). This suggested three possible positions in the wild type gene with the highest GC bias peak within the deleted region. 5′ RACE products from wild type plants were cloned and sequenced, confirming that this was the normal transcription start. 5′ RACE products from plants with the 'Ciano 67' *Ppd-D1a* allele gave a more variable result, but all the start sites were within a second peak immediately upstream of the deletion (Fig. 5). Photoperiod insensitivity is therefore associated with misexpression that involves the use of an alternative transcription start site as well as the potential loss of promoter elements.

Polymorphisms in the chromosome 2A *PRR* gene

Loci conferring insensitivity to photoperiod have been reported on wheat chromosomes 2A and 2B. These effects have been ascribed to homoeoloci (*Ppd-A1* and *Ppd-B1*) but they are not as well characterized as *Ppd-D1* and their phenotypic effects are generally regarded as weaker. That is, they do not advance flowering in SDs as much as the *Ppd-D1a* mutation (Worland et al. 1998). To investigate the 2A and 2B genes we sequenced alleles of the *PRR* gene from selected genetic stocks in which variation in photoperiod response has been ascribed to these chromosomes.

Variation on chromosome 2A is the least well documented but the Indian wheat 'C591' has been reported to carry an insensitive allele on 2A (*Ppd-A1a*) (Islam-Faridi 1988). Sequences were obtained from the candidate photoperiod sensitive alleles of 'Chinese Spring', 'Mercia' and 'Cappelle-Desprez' and from 'C591'. No mutation was found in 'C591', and Mohler et al. (2004) reported that a supposed 'Mercia' ('C591' 2A) substitution line in fact carries the photoperiod insensitive *Ppd-B1a* allele. This suggests that the original identification of a photoperiod insensitivity locus on 2A may be incorrect.

Two 2A polymorphisms were found in photoperiod sensitive varieties (Fig. 1). 'Chinese Spring' had a 1.2 kb insertion in intron 5 that had no homology to other database sequences but is likely to be a transposable element because of short inverted repeats at its termini and a 3 bp host sequence duplication at the insertion point. This provides a convenient marker for the 'Chinese Spring' type 2A gene but is unlikely to have a phenotypic effect. In contrast, 'Cappelle-Desprez' is likely to carry a null allele on 2A as it had a deletion of part of exon 5, intron 5 and part of exon 6. This produced an in frame stop codon shortly after the deletion and a predicted protein lacking the CCT domain.

Polymorphisms in the chromosome 2B *PRR* gene

Photoperiod insensitivity on 2B has been characterized in greater detail than for 2A and 'Chinese Spring', the variety used to construct the BAC library, carries a photoperiod insensitive allele on this chromosome (Scarth and Law 1983, 1984; Mohler et al. 2004). Experiments using 2B single chromosome substitution lines showed that the Australian variety 'Timstein' has greater photoperiod insensitivity (flowering earlier than 'Chinese Spring' in SDs) while 'Cheyenne' carries a photoperiod sensitive allele and is late flowering in SDs (Scarth and Law 1984). Sequence was also obtained from 'Récital' which has photoperiod insensitive alleles on 2B and 2D (Hanocq et al. 2004) and the

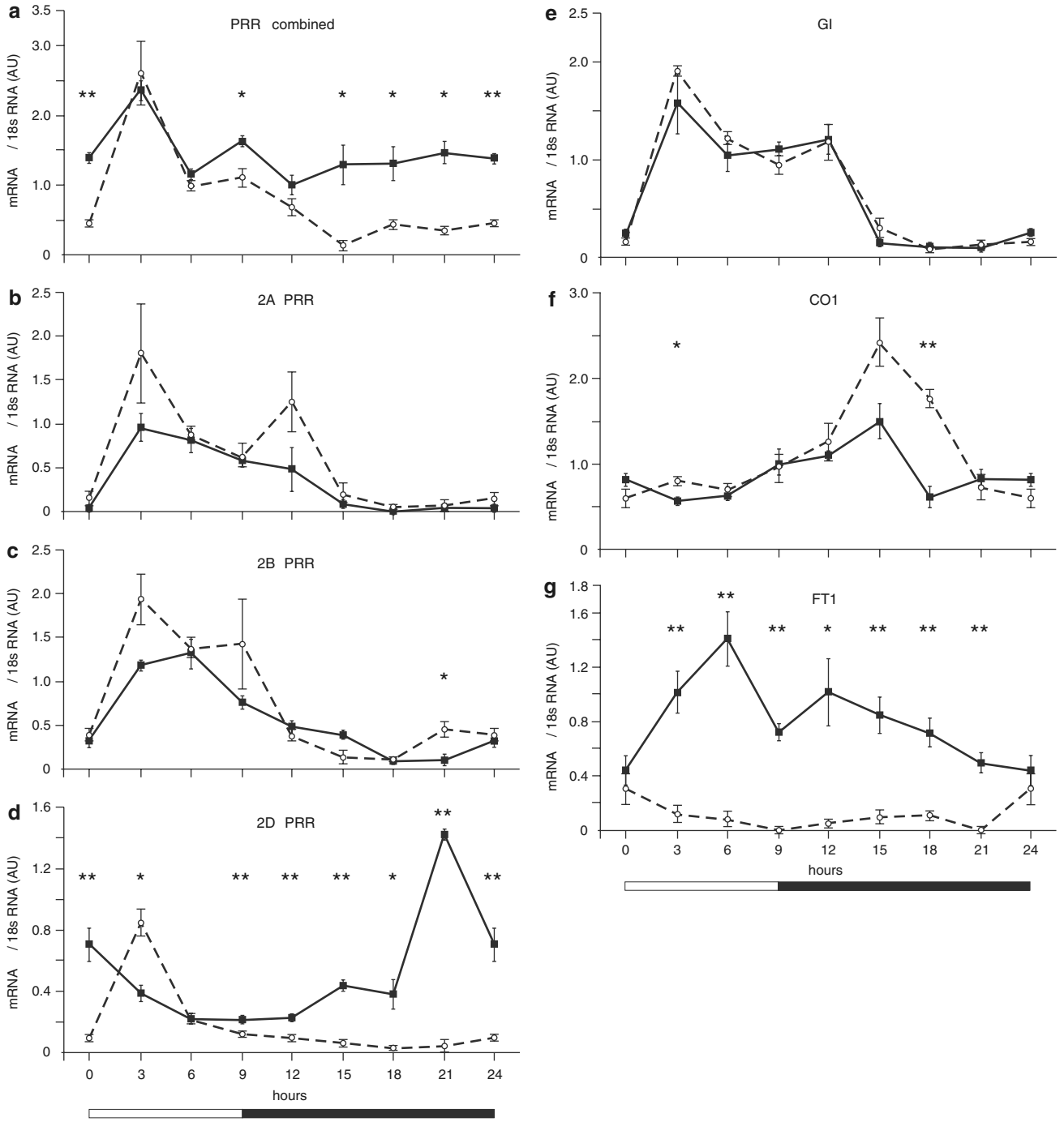

Fig. 4 Quantitative gene expression data from plants grown in short days (9 h light) at 22°C (day)/18°C (night) after vernalization. The graphs compare expression between wild type 'Mercia' (*open circles* and *dashed line*) and the photoperiod insensitive 'Mercia (Ciano 67 2D)' (*Ppd-D1a*) single chromosome substitution line (*solid circles* and *solid line*). Values are expressed as relative levels normalized against 18 S ribosomal RNA. SE bars are of 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 S1. **a** Composite expression levels of the 2A, 2B and 2D *PRR* genes measured using a primer pair that was not genome specific. **b** Expression of the 2A *PRR* gene. **c** Expression of the 2B *PRR* gene. **d** Expression of the 2D *PRR* gene. **e** Expression of *TaGI*. **f** Expression of wheat *TaCO1*. **g** Expression of wheat *TaFT*. Assays in **e** to **g** are not genome specific

photoperiod sensitive varieties 'Mercia', 'Cappelle-Desprez' and 'Renan' (Worland et al. 1994; Hanocq et al. 2004).

'Chinese Spring' differed from the other varieties in having a SNP in exon 3 giving a predicted conservative Asp-Asn change in the pseudo-receiver domain and in having a

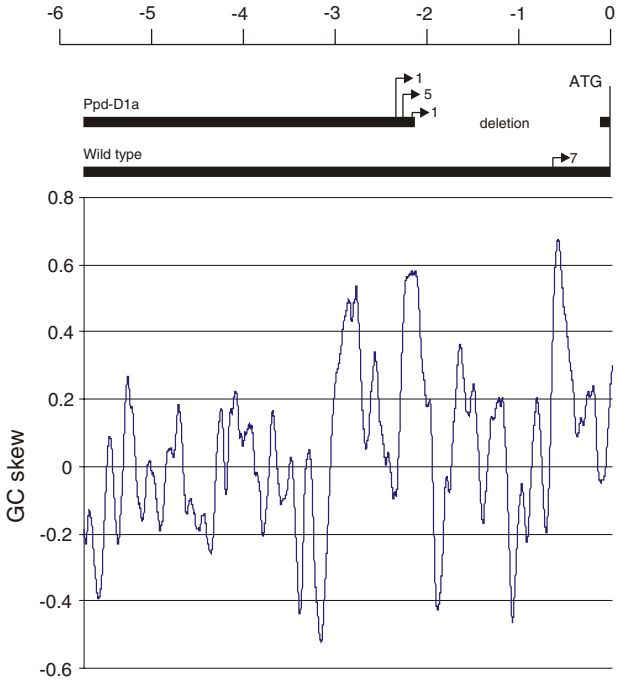

Fig. 5 Transcription start sites in the 2D *PRR* gene. The *wild type bar* represents 5,809 bp upstream of the predicted ATG start codon (−6 to 0 kb). The *Ppd-D1*a bar shows the position of the 2,089 bp deletion. The *graph* shows GC strand bias calculated as described in Fujimori et al. (2005) and averaged over a sliding 50 bp window. Positive peaks are candidate transcription start sites. *Arrows* show the starting positions of transcripts from 'Mercia' (wild type) and 'Ciano 67' (*Ppd-D1a*). The number of clones with each start site is also shown

second copy of the *PRR* gene that was truncated at the start of exon 7. The sequence adjacent to the truncation was highly homologous to wheat retrotransposon sequences (Fig. 1). 2B BAC clones could be classified using PCR primer pairs that amplified an exon 7/retrotransposon border fragment or an exon7/exon8 fragment. BACs were divided into those producing an exon 7/retrotransposon amplicon, those giving an exon7/exon8 amplicon and those giving both. This suggests the two copies are in close proximity and are possibly a partial tandem duplication. The sequence of the intact gene (Fig. 1) was obtained from a BAC lacking the exon 7/transposon amplicon. The truncated copy extends upstream at least as far as the first exon as a fragment of this size could be amplified from BAC clones using the retrotransposon sequence as a reverse primer. Sequencing of the PCR product showed that it was identical to the sequence obtained from the BAC subclones and therefore that both copies had an A at the SNP position in exon 3. This suggests that the partial duplication of the gene in 'Chinese Spring' is recent. None of the other varieties tested gave a PCR product with the exon 7/retrotransposon primers.

The intact 'Chinese Spring' gene contained a 3.5 kb insertion with homology to non-LTR retrotransposons upstream of the coding region. Although the insertion was in the same region as the 2 kb deletion in the 2D gene it cannot be the basis of the photoperiod insensitive phenotype on 2B because it was present in all the varieties analyzed. Four SNPs (1 to 4 in Fig. 1) were found upstream of the coding region. These provide useful markers for mapping and genotyping but none separated the photoperiod insensitive and sensitive types.

The exon 3 SNP and the presence of the exon 7/retrotransposon PCR amplicon were used to analyze a population of 102 DH lines from a 'Mercia' × 'Mercia (Chinese Spring 2B)' cross previously studied under controlled SD (10 h natural light) and field conditions by Butterworth (2000). Excluding five lines with ambiguous flowering times (those not classified consistently in different experiments), both 'CS' polymorphisms cosegregated with the early flowering phenotype. The *PRR* gene may therefore be closely linked to the *Ppd-B1* locus or photoperiod insensitivity may result from a mutation in the *PRR* gene outside the region that was sequenced.

Discussion

Temperate cereals such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are LD plants. That is, flowering is promoted by increasing photoperiod (Thomas and Vince-Prue 1997). Photoperiod response has been modified in both species during domestication and this has an important role in their adaptation to different environments and farming practices. In this paper we investigated members of the *PRR* gene family to assess the likelihood that they are the *Ppd* genes of wheat.

A candidate mutation was found in the 2D *PRR* gene where a 2,089 bp deletion upstream of the coding region was present in all the tested wheat varieties that are known to have the *Ppd-D1a* allele or that were known from previous phenotyping studies to be very early flowering in SDs. Quantitative RT-PCR assays showed that the 2D *PRR* gene was misexpressed and this was associated with altered *FT* expression. This was consistent with our original hypothesis for photoperiod variation in wheat and barley in that photoperiod insensitivity in wheat can be attributed to the induction of *FT* irrespective of daylength while late flowering in barley is due to a failure to induce *FT* correctly in LDs. Final proof that the deletion in 2D is the causal basis of photoperiod insensitivity requires transgenic studies. The mechanism by which the deletion causes misexpression could be by removal of regulatory elements, as previously shown for mutations conferring spring growth habit in the *Vrn1* gene (Yan et al. 2003; Fu et al. 2005), and/or the alteration of the transcription start site. In contrast to the results from barley, we found no convincing evidence for a

change in the timing of the wheat *CO*-like expression peak during the day, although differences in expression amplitude were found. Therefore, while the effect on *FT* expression was clear, the mechanism by which this effect is generated needs further study.

Previous work using single chromosome substitution lines suggested that more than one photoperiod insensitive mutation might exist for the 2D chromosome, although this was not supported by a detailed comparison of the insensitive phenotype derived from 'Mara' or 'Ciano 67' (Worland et al. 1998). Analysis of sequence variation confirms that these and other varieties have the same 2D mutation which suggests that a single photoperiod insensitive allele exists on 2D and that additional flowering time variation in the chromosome substitutions derives from additional minor QTL present on the introduced chromosome.

For chromosomes 2A and 2B we did not identify candidate mutations for photoperiod insensitivity although genetic mapping of a 2B cross showed that the *PRR* gene was closely linked to the *Ppd-B1* locus. This may mean that the *PRR* gene is *Ppd-B1* and the causal mutation is outside the region that was sequenced. Alternatively, the *Ppd-B1a* phenotype could be due to mutation of a closely linked gene. This can be resolved by higher-resolution mapping and further sequencing. Analysis of *Ppd-A1* would require the creation of populations segregating for a flowering time effect on 2A, which to date has not been identified.

Photoperiod sensitive and insensitive alleles of the 2D *PRR* gene were unusual in having a deletion in exon 8 when compared to the 2A, 2B and 2H genes. The exon 8 deletion was present in all the cultivated wheat varieties we tested while an intact form of the gene was found in *Ae. tauschii* and two synthetic wheats. Whether this has any phenotypic significance is unclear, but this deletion provides a valuable marker to investigate the evolution of cultivated wheat and it would be interesting to determine if the deletion is found in any *Ae. tauschii* accessions or in older wheat landraces.

Additional sequence variation was found in alleles from photoperiod sensitive varieties but it is unclear what effect these have on phenotype. Candidate null alleles exist for the 2A and 2D genes but the effect of these mutations in hexaploid wheat may be masked by the presence of intact genes on the other genomes. However, these mutations might produce a quantitative delay in flowering. Similarly, the effect of the large transposable element in the first intron of 2D gene in 'Mercia' is unclear. The 'Mercia' allele was expressed and had an expression profile similar to genes on the 2A and 2B chromosomes (Fig. 4), suggesting that the element was efficiently removed by splicing.

Selection for reduced photoperiod response similar to that seen in barley (Turner et al. 2005) would predict that loss of function mutations in wheat would be prevalent in ecogeographical regions with longer growing seasons. This can be tested by a detailed analysis of allele distribution in wheat germplasm and by development of near-isogenic lines for each allele to assess their pleiotropic effects in a common genetic background. This would provide a baseline data set for associating alleles with environments which would inform further investigations of gene function and searches for additional allelic variation.

The results from wheat and barley suggest that contrasting mutations in the group 2 *PRR* genes can be used to advance or retard flowering and that these genes therefore provide great adaptive flexibility. This further suggests that homologous genes in other species may have been targets of human selection for variation in flowering time. The equivalent gene in rice occurs in a region carrying the *Hd2* QTL in the 'Nipponbare' × 'Kasalath' cross (Lin et al. 2000) and the 'Kasalath' allele carries a mutation producing a premature stop codon in the CCT domain (Murakami et al. 2005). This provides a good candidate but it has not been proven that this is the basis for the *Hd2* effect. A QTL for flowering time is also present in the colinear region of *Lolium* chromosome 2 (Skøt et al. 2005). These observations suggest that mutations in *PRR* genes have been influential in a range of species and agricultural settings and that a detailed analysis of this gene family in grasses would be of great interest.

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