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Development of STS markers closely linked to the *Ppd-H1* photoperiod response gene of barley (*Hordeum vulgare* L.)

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Abstract A BC_2 population of 353 plants segregating for the *Ppd-H1* photoperiod response gene was developed from a cross between the winter barley 'Igri' and the spring barley 'Triumph.' Bulk segregant analysis identified six AFLP markers closely linked to the *Ppd–H1* gene and three strongly amplified AFLP bands that mapped 0.8-cM distal, 0.6-cM proximal and 2.3-cm proximal to *Ppd-H1* were cloned and sequenced. Southern-blot analysis showed that the cloned fragments were single-copy sequences in 'Igri', the variety from which they were derived. Two of the sequences were absent from 'Triumph' while the third detected a single-copy sequence. The cloned fragments were used to design specific sequence tagged site (STS) primer pairs to assist in the construction of a high-resolution map of the *Ppd-H1* region.

Keywords Amplified fragment length polymorphism (AFLP) · Barley (*Hordeum vulgare*) · Photoperiod · Mapping · *Ppd-H1* · Sequence tagged site (STS)

Introduction

Genes affecting flowering time (heading date) are important components of plant adaptation and yield potential. Previous work using doubled-haploid (DH) lines from an 'Igri' (winter barley)×'Triumph' (spring barley) cross identified five major loci and eight quantitative trait loci (QTLs) affecting flowering time (Laurie et al. 1995). The largest effect on flowering time in field experiments was caused by the *Ppd-H1* photoperiod response gene on the short arm of chromosome 2H (Laurie et al. 1994, 1995). Two RFLP loci (*Xmwg858* and *Xbcd221b*) defined a 2.2-cM interval containing the *Ppd-H1* gene in the DH population. Subsequently, two additional RFLP markers were mapped in the *Ppd-H1* region in the same cross. The rice cDNA clone C213 gave a polymorphism (*Xrgc213*) that co-segregated with *Xmwg858*, distal to *Ppd-H1* (Laurie 1997), while an AFLP fragment cloned from wheat (PSR2606, K.M. Devos, unpublished data) mapped 1-cM proximal to *Xbcd221* and 2.2-cM proximal to *Ppd-H1*.

In order to develop additional markers for the *Ppd-H1* region in barley, two backcross populations were developed by introgressing the 'Igri' allele of *Ppd-H1* into a 'Triumph' genetic background. Bulk segregant analysis (Michelmore et al. 1991) using AFLP (Vos et al. 1995) was used to compare pools of early or late-flowering individuals selected from the BC_2 population. This identified six AFLP markers in the *Xrgc213–Xpsr2606* interval of which three were cloned and sequenced. The application of these markers in further work is discussed.

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Materials and methods

Production of the BC_2 population

Doubled-haploid line 92 (DH92) from the 'Igri'×'Triumph' mapping population was used as the donor parent for backcrossing to 'Triumph.' DH92 carried the 'Igri' allele of *Ppd-H1* and the *denso* (*sdw1*) dwarfing gene but had the 'Triumph' allele at three other major loci affecting flowering time [*Ppd-H2*, *sh* (*sgh1*) and Sh_2 (*sgh2*), Laurie et al. (1995); symbols in brackets are the equivalent symbols from Franckowiak (1996)]. *Ppd-H1* is probably the *Eam1* gene listed by Franckowiak (1996), but because this has not been proven by an allelism test we have retained the *Ppd-H1* nomenclature in this paper. A DH92 plant was pollinated with 'Triumph' to generate an F_1 generation which was grown in a glasshouse under long days together with the parental lines. Flowering time was recorded for each plant as the date on which awns had emerged 2-cm from the leading tiller. Awn emergence in the F_1 plants occurred about 2 days later than in DH92 and about 12 days earlier than 'Triumph', showing that the early ('Igri') allele of *Ppd-H1*

was essentially dominant. F_1 plants were back-crossed to 'Triumph' to produce a BC_1 population of 120 plants.

BC_1 plants were grown as a single randomized block in a glasshouse under long days (18-h light). Flowering time fell into two classes showing the segregation of *Ppd-H1*. DNA was extracted from leaves of individual early flowering plants using a CTAB method (Murray and Thompson 1980) and the samples were screened to determine their genotype at *Xpsr109*, an RFLP locus that was 7.6-cM distal to *Ppd-H1* in the original DH population (Laurie et al. 1995). Southern-blot analysis was carried out as described in Laurie et al. (1993). Three early flowering plants homozygous for the 'Triumph' allele of *Xpsr109* were identified and these were re-planted in fresh soil and allowed to produce new tillers. Newly emerged ears on the selected plants were emasculated and subsequently pollinated with 'Triumph' to produce 353 BC_2 seeds.

The BC_2 population was grown as a single randomized block in a glasshouse under long-day conditions (18 h light). Flowering time in the BC_2 population again showed segregation of plants into two phenotypic classes, allowing classification of the *Ppd-H1* genotype. Self-pollinated seed was collected and 12 to 16 seedlings from each BC_2 plant were pooled for DNA extraction and analysis using the methods described above.

Cloning and sequencing of AFLP fragments

AFLP markers closely linked to the *Ppd-H1* locus were identified by bulk segregant analysis (Michelmore et al. 1991) using the procedure of Vos et al. (1995) with *Pst*I or *Sse*83871 as the rare cutting restriction enzyme and *Mse*I as the frequent cutter. AFLP markers were designated by primer combination and band molecular weight. For three AFLP fragments linked to *Ppd-H1* the relevant bands were excised from acrylamide gels, re-amplified using appropriate primer combinations and cloned into pGEM-T vector (Promega). For each marker, 20 white colonies were tested to ensure that they amplified a fragment of the expected size using the appropriate primer combination and did not amplify using the *Mse*I primer alone. Plasmids from positive colonies were prepared and their inserts sequenced using ABI PRISM BigDye reactions following the manufacturer's protocol. PCR-amplified inserts were also labelled with ^{32}P and used to probe Southern blots as described by Laurie et al. (1993).

STS amplification conditions

PCR-A. PCR amplification using STS primer pairs was carried out in 50-µl reactions containing 100 ng of genomic DNA, 50 pmol of each primer, 0.2 mM dNTPs, 2.5 u of *Taq* polymerase (Boehringer) or 2.5 u of *Pfu* polymerase (Stratagene) and 1× cresol loading buffer (5% glycerol, 40 µg/ml of cresol red). Reaction conditions were 96°C for 5 min followed by 30 cycles of 96°C for 1 min, 55°C for 30 s and 72°C for 1 min. PCR-B. As above but with the 1× cresol buffer excluded from the PCR reaction.

Results

Identification of recombinants in the *Xmwg858*/*Xrgc213–Xpsr2606* interval

Analysis of the BC_2 population using previously identified RFLP markers showed that there was a clear distinction between plants heterozygous for markers in the *Xmwg585*/*Xrgc213–Xpsr2606* interval, which were early flowering (Fig. 1a), and plants homozygous for 'Triumph' alleles, which were late flowering (Fig. 1b). *Xbcd221b* was mapped between *Ppd-H1* and *Xpsr2606*

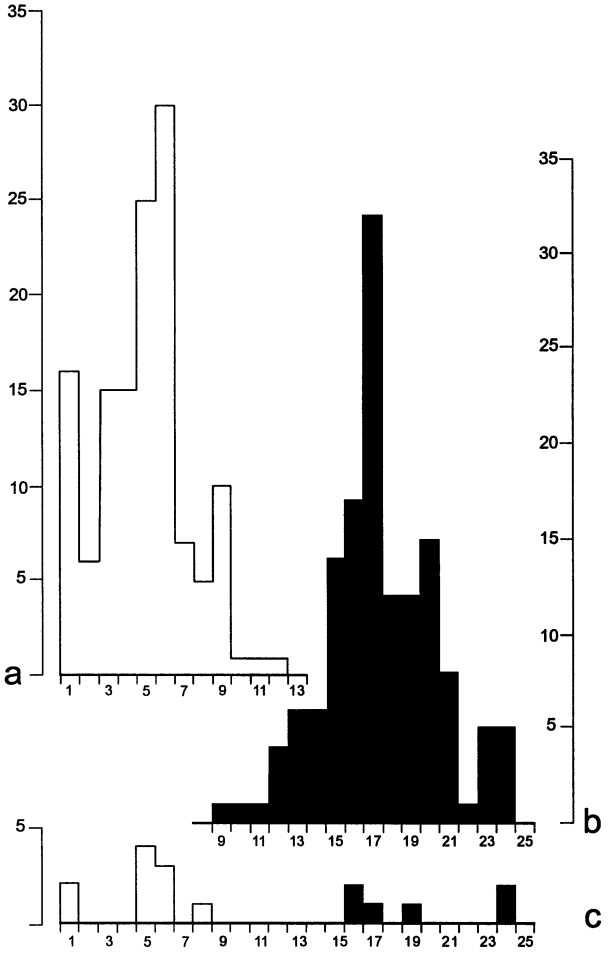

Fig. 1a–c Histogram of flowering time in the BC_2 population. **a** Plants heterozygous for markers in the *Xrgc213–Xpsr2606* interval and for *Ppd-H1* (see Fig. 2); **b** plants homozygous for 'Triumph' alleles in the *Xrgc213–Xpsr2606* interval and for the 'Triumph' allele of *Ppd-H1*; **c** plants with recombination in the *Xrgc213–Xpsr2606* interval. The horizontal scale is in days with the earliest flowering plants at day 1

in previous work (Fig. 2a) but this probe detected a presence/absence polymorphism with the band present in the 'Triumph' parent. Thus, *Xbcd221b* was not used for the BC_2 population.

As in previous experiments there was a separation of about 12 days between the means of the early and late-flowering groups. Variation within each *Ppd-H1* class may have been caused by segregation for the *denso* (*sdw1*) gene, which is known to affect flowering time in this cross (Laurie et al. 1995), and by additional QTLs. Eight QTLs affecting flowering time were identified in the original 'Igri'×'Triumph' DH population and at least one was expected to be segregating in the BC populations. In addition, a small number of BC_2 seeds produced weak plants with intermediate flowering times (days 10 to 13 in Fig. 1a, and days 9 to 11 in Fig. 1b). Progeny testing of these plants showed a clearer separation of flowering times and confirmed that they were non-recombinant for the *Xmwg858*/*Xrgc213–Xpsr2606* interval.

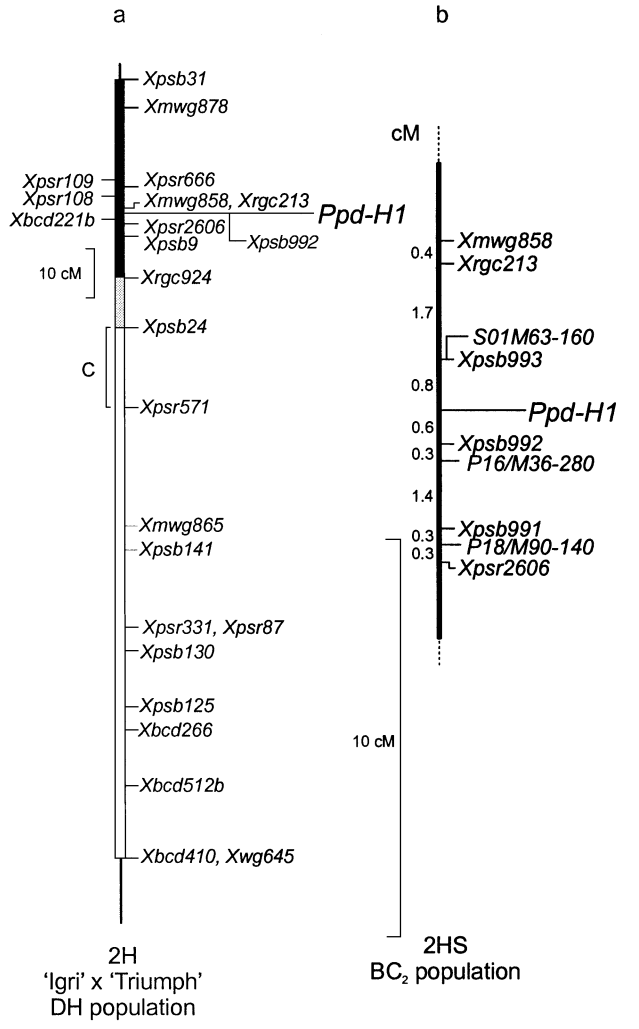

Fig. 2 a Genetic map of chromosome 2H from the original DH population [Laurie et al. (1995) plus three additional markers] showing the location of the *Ppd-H1* photoperiod response locus. The DH92 line used as the donor parent for the BC populations had 'Igri' alleles of markers *Xpsb31–Xpsb24* (black box) and 'Triumph' alleles of markers *Xpsr571–Xwg645* (white box). The parental origin of the intervening region (grey box) is unknown. **b** Genetic map of the *Ppd-H1* region in the BC_2 population (drawn to larger scale). *Xmwg858*, *Xrgc213* and *Xpsr2602* were mapped by RFLP. *Xpsb991* and *Xpsb993* were mapped by AFLP, RFLP and by PCR using STS primers. *Xpsb992* was mapped by AFLP and RFLP. *S01M63–160*, *P16M36–280* and *P18M90–140* were mapped solely by AFLP

Sixteen recombinants were found between *Xrgc213* and *Xpsr2606* and one further recombinant placed *Xmwg858* distal to *Xrgc213*. The flowering times of the 16 *Xrgc213–Xpsr2606* recombinants allowed their *Ppd-H1* genotypes to be assigned with confidence (Fig. 1c) and this was verified by analysing families of 12 plants grown from self-pollinated seed of each putative recombinant. Families from the ten early flowering recombinants showed segregation for flowering time, while families from the six late-flowering recombinants were uniformly late-flowering. Thus, all data from RFLP analysis were consistent with a location of *Ppd–H1* between *Xrgc213* and *Xpsr2606*.

Identification of AFLP markers linked to *Ppd-H1*

Equal amounts of DNA from 15 early and 15 late-flowering BC_2 plants that did not have recombination in the *Xrgc213–Xpsr2606* interval were used to make early and late pooled samples for bulk segregant analysis. The pools were screened using *Mse*I primers with three selective bases and *Pst*I primers with two or three selective bases (841 and 53 primer combinations, respectively). *Sse*83871 digests used the same primer as *Pst*I with one selective base (nine primer combinations). In total, the 903 primer combinations were estimated to have generated approximately 60000 bands of which 13 were polymorphic, being present in the early pool but absent in the late pool. Six polymorphisms were placed between *Xrgc213* and *Xpsr2606* when scored in the 16 recombinant plants and only these six were analysed in detail and mapped in the BC_2 population (Fig. 2b).

Cloning and mapping of selected AFLP fragments

Three strongly amplified polymorphic bands (P22M73–279, P63M72–266 and S01M77–220) were selected for cloning. The cloned fragments were designated PSB991, PSB992 and PSB993, respectively. PSB991 and PSB993 detected single-copy sequences in 'Igri' but gave no hybridization signal in 'Triumph' when used as probes on Southern blots. PSB992 detected a single-copy sequence in both varieties that could be mapped by RFLP. The sequences of the three cloned fragments (Fig. 3) were analysed for similarity to known genes or ESTs using the BLAST program (Altschul et al. 1990) but no significant homologies were found.

Southern-blot analyses of the BC_2 population using the cloned fragments corresponded exactly to the respective AFLP scores. This confirmed that the correct fragments had been cloned. PSB992 was also mapped in the original DH population where it co-segregated with the *Ppd-H1* locus (Fig. 2a). Thus, *Xpsb992* was more tightly linked to *Ppd-H1* than *Xbcd221*, the closest proximal marker identified by Laurie et al. (1995).

This analysis, in combination with results obtained using previously known RFLP markers, showed that one of the three selected BC_1 plants had a cross-over between *Xpsb993* and *Ppd-H1* so that the 115 BC_2 plants from this individual were homozygous for the 'Triumph' allele at all markers distal to *Ppd-H1*. The remaining two BC_1 plants had undergone recombination between *Xpsr109* and *Xmwg858*. In Fig. 2b the genetic distances between markers in the *Xrgc213–Ppd-H1* interval were therefore calculated from 238 plants, while distances in the *Ppd-H1–Xpsr2606* interval were from the full population of 353 plants. The genetic distance between *Xrgc213* and *Xpsr2606* increased from 3.3 cM in the

PSB991 (AF242588); STS size 185bp

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  1 GTACATGCAG GTTGGTATAT GAACGATATC ATTCTATGCC TTTTCACTAG
 51 ATATCTGAGG GTTGTTGCTG AGTACTTTCA TTACATAGTT CTTGTTACCC
101 CACCCCAAAT CTACAAGGGC ACTCGCCTCT TACCAATGGT GTTTTTCTGT
151 ATATACGCGG CAGGGGTTTG TTCTGTATAT CCAACGGTGC TCTGAATATG
201 TGCAAGACAT AATCAGGTGT GCATTGGAGA TATGATCCAC CC
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PSB992 (AF242589); STS size 202bp

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                                                  *
  1 GGCCAGCTTC AATCATTCAA AAAGTGCGGC TGCTTCGGCA ACAACACCAC
 51 CAGCCACATG TTCAAGCCTA GCTGTAGAAG CCGTGATGAA GTGCCCTTTA
101 TGATCTCTGA TAATTGCTCC ACAAGCACCG GAGCTTGTTT CAACAGAAAA
151 GGAAGCATCC ACGTTTAGCA CTAGATGCCC AGCTAGGACA GAAGGCCATC
201 TATTTGACTT TGCATCTACC AAGACCTT
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PSB993 (AF242590); STS size 112bp

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  1 ATCTCCTCGT GGCCGGCCGG CTGTATACAT TCGTATCTGA TCCTTCCTCT
 51 TTGTCCATGT GTTGAAACCA AACACGGTTC GATCTGAGCA GATGATGCAC
101 TCCTTCCTAT GCCAGGTAAA GTCTTGCACT CTTGATCAAG TTTCAACCTT
151 ACCAAGTTGC AAATTTTCTG CTCCTTTCCT CAC
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Fig. 3 Nucleotide sequences of the three cloned AFLP fragments. The PSB991 (P22M73–279) and PSB993 (S01M77–220) sequences were present only in 'Igri.' For PSB992 (P63M72–266) the sequence from 'Igri' is shown and * shows the position of a C to T transversion distinguishing the 'Triumph' and 'Igri' amplicons. *Underlined* nucleotides show the positions of the STS primers. GenBank accession numbers are in brackets

original DH population to 5.8 cM in the BC_2 population. The reason for this is unclear but it was not due to male/female differences in recombination because in both cases recombinants were derived from female gametes.

The sequences of the three cloned fragments were used to design sequence tagged site (STS) primer pairs for PCR amplification (Fig. 3). These were tested on genomic DNA of the parents (Fig. 4) and on the BC_2 population. Primer pairs for PSB991 and PSB993 gave the same presence/absence segregation patterns as the respective Southern hybridizations and sequences of PCR amplicons were identical to those of the respective clones, confirming that the correct sequences were amplified.

Using the PCR-B conditions (see Materials and methods) the PSB992 STS primer pair gave a presence/absence polymorphism in which the 'Igri' allele was amplified (Fig. 4, lanes 1 and 2). Again, the amplicon sequence was identical to that of the PSB992 clone. Using the PCR-A conditions it was possible to amplify a band from 'Triumph' (Fig. 4, lanes 5 and 6) which differed from the 'Igri' sequence by a single C to T transversion at position 36 (Fig. 3). We were unable to confirm this difference using a high-fidelity *Pfu* polymerase as this amplified only the 'Igri' allele using PCR-A or -B conditions (Fig. 4, lanes 3 and 4). The sensitivity of the 'Triumph' allele to PCR conditions and type of DNA polymerase provided a diagnostic assay for the 'Igri' allele and suggested that the 'Triumph' allele may have a mismatch to one or both primer sequences.

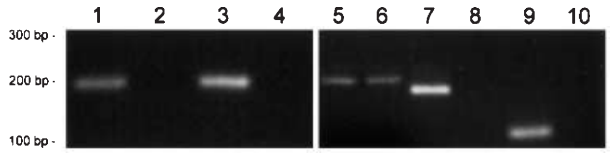

Fig. 4 PCR products from STS primer pairs. *Lanes 1, 3, 5, 7 and 9* are DH92 ('Igri' allele), *lanes 2, 4, 6, 8 and 10* are 'Triumph.' PSB992; *lanes 1 and 2, Taq* polymerase, PCR-B; *lanes 3 and 4, Pfu* polymerase, PCR-A (PCR-B gave the same result); *lanes 5 and 6, Taq* polymerase, PCR-A. PSB991; *lanes 7 and 8, Taq* polymerase, PCR-A. PSB993; *lanes 9 and 10, Taq* polymerase, PCR-A

Discussion

The BC populations allowed novel markers to be identified that were more closely linked to *Ppd-H1* than previously known RFLPs. Sequences of cloned AFLP fragments were used to develop diagnostic STS primers which provide useful markers for *Ppd-H1* and which can be used to select recombinants in the *Ppd-H1* region from further BC populations. By combining the latter with the development of additional markers a much-higher resolution map of the *Ppd-H1* region can be obtained. Our aim is to use closely linked markers to identify YAC or BAC clones spanning the region of interest which can then be sequenced to identify candidate genes for *Ppd-H1*. This type of map-based approach has recently been proved to work successfully in barley (Büsches et al. 1997). The novel markers identified in this paper could be used to identify YAC and BAC clones but, as in 'Triumph', the PSB991 and PSB993 sequences were absent from 'Ingrid' or 'Morex', the two spring barleys for which large insert libraries exist (Büsches et al. 1997; and http://www.genome.clemson.edu/lib-frame.html, respectively). However, all three cloned sequences were present in the winter barley 'Franka' which was used to construct the YAC library described by Michalek et al. (1997).

The phenotypes of F_1 and BC plants, combined with previous data from controlled environment experiments, showed that 'Igri' carries a largely dominant photoperiod responsive allele of *Ppd-H1* and 'Triumph' possesses a weaker or possibly null allele. However, although the PSB993 sequence was absent from 'Triumph' this cannot be due to a deletion that includes the *Ppd-H1* locus because recombinants were found between *Xpsb993* and *Ppd–H1*. Therefore, further analysis of the *Ppd-H1* region in both winter and spring barleys should identify additional sequences that can be used to isolate clones from large-insert libraries.

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