Candidate-gene cloning and targeted marker enrichment of wheat chromosomal regions using RNA fingerprinting – differential display

Kulvinder S. Gill and Devinder Sandhu

Abstract: The usefulness of the RNA fingerprinting – differential display technique in gene cloning and targeted marker enrichment in wheat is demonstrated. A small region of chromosome 5BL was targeted that contains *Ph1*, a chromosome-pairing regulator gene. The cultivar Chinese Spring (CS) and mutant *ph1b* are almost identical except for chromosome 5BL, which, in the mutant line, carries an interstitial deletion encompassing the *Ph1* gene. Poly(A)$^+$ RNA of the two lines from anthers at developmental stages ranging from pre-meiotic mitosis to anaphase II was PCR-amplified using 38 pairwise combinations of 19 primers. The ^{35}S-labeled amplified products were size-separated on denaturing polyacrylamide–urea gels. A total of 3154 fragment bands were observed, of which 43 were present in CS but absent in the *ph1b* mutant. These 43 fragment bands were eluted, re-amplified, and used as probes in gel-blot DNA analyses of wheat group 5 nullisomic–tetrasomic lines and the *ph1b* mutant. Twenty-four of these 43 probes were single- or few-copy sequences. Eight of the 24 probes mapped to wheat group 5 and five mapped to the deletion of the *ph1b* mutant. Three of these five probes were further localized to the submicroscopic region containing the *Ph1* gene, by using two deletion lines flanking the region. Northern-blot analysis revealed that the gene corresponding to one of these three probes expresses mainly during meiosis and is from the B genome.

Key words: RNA fingerprinting – differential display, wheat, gene cloning, marker enrichment, *Ph1* gene.

Résumé : L'utilité de l'approche combinant les empreintes d'ARN et l'affichage différentiel (« RNA fingerprinting – differential display ») pour des fins de clonage et d'identification sélective de marqueurs est illustrée ici. Une petite région du chromosome 5BL contenant le gène *Ph1*, un régulateur de l'appariement chromosomique, a été ciblée. Le cultivar Chinese Spring (CS) et un mutant *ph1b* sont pratiquement identiques à l'exception du chromosome 5BL, le mutant ayant une délétion qui comprend le gène *Ph1*. L'ARN poly(A)$^+$ des deux lignées a été extrait des anthères à divers stades allant de la mitose pré-méiotique à l'anaphase II et amplifié par PCR à l'aide de 38 combinaisons de 19 amorces. Les produits d'amplification marqués au ^{35}S ont été séparés sur gels dénaturants de polyacrylamide–urée. Au total, 3154 bandes ont été observées dont 43 qui étaient présentes chez CS mais absentes chez *ph1b*. Ces 43 fragments ont été élués, réamplifiés et employés comme sonde dans des analyses Southern sur des lignées nullisomiques–tétrasomiques pour les chromosomes du groupe 5 et sur le mutant *ph1b*. Vingt-quatre de ces 43 sondes détectaient des séquences présentes en une ou quelques copies seulement. Huit des 24 sondes ont été cartographiées sur les chromosomes du groupe 5 et cinq des sondes provenaient de régions délétées chez le mutant *ph1b*. Trois de ces cinq sondes ont de plus été assignées à une région submicroscopique contenant le gène *Ph1* en utilisant deux lignées ayant des délétions bordant cette région. Des analyses Northern ont révélé que le gène correspondant à l'une de ces trois sondes s'exprime principalement lors de la méiose et en provenance du génome B.

Mots clés : empreintes d'ARN – affichage différentiel, blé, clonage de gènes, enrichissement pour des marques, gène *Ph1*.

[Traduit par la Rédaction]

Introduction

RNA fingerprinting – differential display (RNAF–DD) is a powerful technique for identifying and isolating genes that are differentially expressed in different tissues or under altered growing conditions (Liang and Pardee 1992). The key element of this technique is to use a set of oligonucleotide primers, comprising one primer that anchors to the polyadenylated tail of a subset of mRNAs and another that is short and arbitrary in sequence so that it anneals at a differ-

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ent position relative to the first primer. RNA fingerprinting by arbitrarily primed PCR and its variants has been successfully used to isolate differentially expressed genes in a large number of experimental systems (Liang et al. 1993; Diachenko et al. 1996). A major complication with this procedure is the appearance of false positives that are normally differentiated from the real positives either by Northern-blot hybridization or by quantitative reverse-transcriptase (RT) PCR (Liang and Pardee 1992). This problem is bigger for genes with rare transcripts. Furthermore, Northern-blot hybridization or RT-PCR based methods will only confirm differential expression of genes and will not reveal their location in the genome. The precise inter- and (or) intrachromosomal map locations of many agronomically important traits are known. Therefore, mapping-based identification of the real positives will be more accurate than identification based on expression analysis alone.

Because of its unique structural and functional organization and the availability of various aneuploid stocks, wheat is an ideal system for gene cloning and marker enrichment via the RNAF–DD technique. Most wheat genes are present in clusters that encompass physically small chromosomal regions (Gill et al. 1996*a*, 1996*b*; Sandhu 2000). On average, there are four to six major "gene-rich" regions per wheat chromosome. Most of the major gene-rich regions of wheat have been identified and bracketed by the breakpoints of single-break deletion lines (Gill et al. 1996*a*, 1996*b*; Boyko et al. 1999; Faris et al. 2000; Sandhu 2000). Most of the useful segments of the genome can, therefore, be targeted for gene cloning and marker enrichment by the RNAF–DD technique.

The precise physical location of any single- or few-copy DNA–RNA fragment can be revealed in wheat using a variety of aneuploid stocks. The chromosomal location of most fragments can be determined by mapping on nullisomic–tetrasomic lines (where a pair of chromosomes is missing, the loss of which is compensated for by a double dose of one of its two homoeologous chromosomes) (Sears 1954). Sub-arm localization is then accomplished using single-break deletion lines that are available for all wheat chromosomes. Essentially all fragments can be mapped, because the procedure is less dependent upon the level of polymorphism.

The principal gene regulating chromosome pairing in polyploid wheat, *Ph1*, maps in a gene-rich region present on the long arm of chromosome 5B (Sears 1977; Jampates and Dvořák 1986; Gill et al. 1993). Fine mapping of the *Ph1* gene relative to the breakpoints of various deletion and mutant lines is shown in Fig. 1. Two mutant lines have been isolated for the *Ph1* gene. The *ph1b* mutant was generated in the hexaploid wheat cultivar Chinese Spring (CS) (Sears 1977), and the mutant *ph1c* was generated in the tetraploid wheat cultivar Cappelli (Jampates and Dvořák 1986). Both mutants resulted from interstitial deletions encompassing the gene-containing chromosomal region (Gill and Gill 1991; Gill et al. 1993) (Fig. 1). The size of the deletion in *ph1c* is about 0.89 μm and is smaller than that in *ph1b*, which is 1.05 μm in length (Gill et al. 1993). Two single-break terminal deletion lines, 5BL-1 and 5BL-11, have also been isolated, the breakpoints of which bracket about 0.32 μm of the chromosomal region around the *Ph1* gene (Gill et al. 1993; Endo and Gill 1996). The *Ph1* gene is absent in the deletion line 5BL-1 but is present in 5BL-11. The fraction length (FL) of the retained 5BL chromosome arm of 5BL-1 is 0.55, compared with 0.59 for 5BL-11 (FL is the fraction given by the chromosome length in the mitotic root tip compared with that in normal CS). The breakpoint of deletion 5BL-1 maps just proximal to C-band 5BL2.1. The deletions in the mutants *ph1b* and *ph1c* are also proximal to C-band 5BL2.1. Therefore, the *Ph1* gene is present in the chromosomal region distal to the breakpoint of deletion line 5BL-1 but proximal to C-band 5BL2.1 (FL 0.51). This chromosome region is very small and cannot be resolved by light microscopy. The distal breakpoints of the two mutants (*ph1b* and *ph1c*) are present in this region, as the breakpoint of *ph1b* is distal to that of *ph1c* (Fig. 1). The precise location of the *Ph1* gene is, therefore, between the breakpoints of 5BL-1 and the distal breakpoint of the interstitial deletion in *ph1c*. This region will be referred to as the "*Ph1*-gene region". Based on long-range mapping, the region was estimated to be smaller than 550 kb (Gill et al. 1996*a*).

The objectives of this study were to clone and characterize cDNAs from the *Ph1*-gene region.

Materials and methods

Plant material

The *Ph1* gene mutant line *ph1b* and its wild-type counterpart CS were used for RNAF–DD. Wheat homoeologous group 5 nullisomic–tetrasomic lines along with CS, *ph1b*, *ph1c*, and the two deletion lines 5BL-1 and 5BL-11 were used for gel-blot DNA hybridization analysis of the putative positive cDNAs. The plant material was obtained from the Wheat Genetic Resource Center, Manhattan, Kans.

Poly(A)$^+$ RNA isolation

Because of its involvement in chromosome pairing, the *Ph1* gene is expected to be expressing during or just before prophase I of meiosis. With that assumption, inflorescence spikes at developmental stages ranging from pre-meiotic mitosis to metaphase–anaphase II were selected. Developmental stage was determined by microscopic observations of anthers squashed in 1% acetocarmine solution. Total RNA of *ph1b* and CS was isolated from whole spikes, using the guanidinium thiocyanate – cesium chloride method (Sambrook et al. 1989), with the following modifications. The tissue was ground to a fine powder in liquid nitrogen and suspended in guanidinium thiocyanate buffer containing 1% mercaptoethanol and 0.5% sodium lauryl sarcosinate. Cell debris was removed by centrifuging the slurry at 10 000 rpm for 15 min. The supernatant was layered on 5.7 M cesium chloride – 0.01 M EDTA solution in an ultracentrifuge tube and centrifuged in a swinging bucket rotor at 40 000 rpm for 16–24 h. The RNA pellet was washed with 70% ethanol and resuspended in TE (1× TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.1% SDS. Poly(A)$^+$ RNA was isolated following the standard protocol (Sambrook et al. 1989).

RNAF–DD

The RNAF–DD reactions were carried out using the Delta RNA fingerprinting kit (CLONTECH Laboratories, Inc.). The manufacturer's recommended protocols were used, except that the isotope ^{35}S was used instead of ^{33}P. First-strand cDNA was synthesized from poly(A)$^+$ RNAs of the *ph1b* line and CS, using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (CLONTECH Laboratories, Inc). A total of 19 primers were used for the experiment, of which 10 were "P" primers and nine were "T" primers (Table 1). Thirty-eight different primer combinations,

Fig. 1. C-banding pattern (left) and ideogram (center) of chromosome 5B of wheat (Gill et al. 1991). The diagram on the right represents the indicated portion of the long arm magnified to show the segments deleted in the mutants (*ph1b* and *ph1c*) and the deletion breakpoints of the 5BL-1 and 5BL-11 lines. Part of the information presented is from Gill et al. (1993). The *Ph1*-gene region is marked.

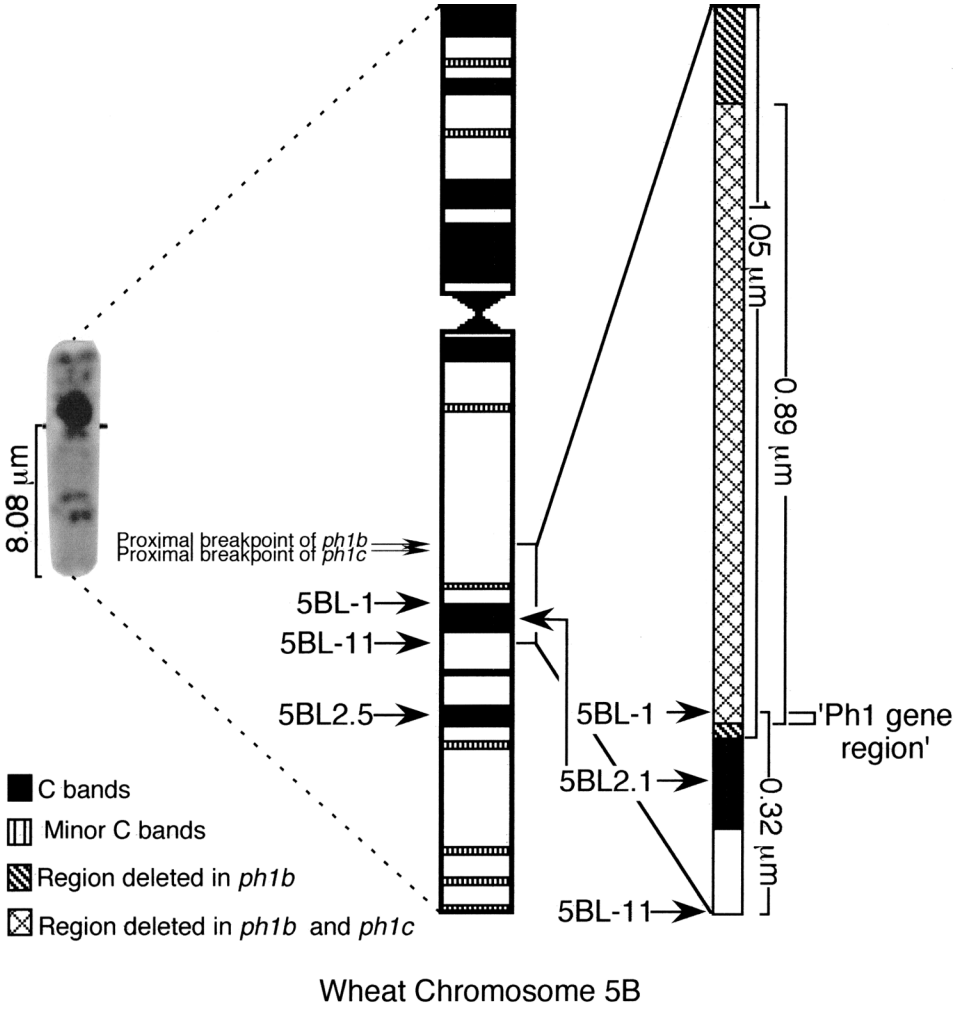


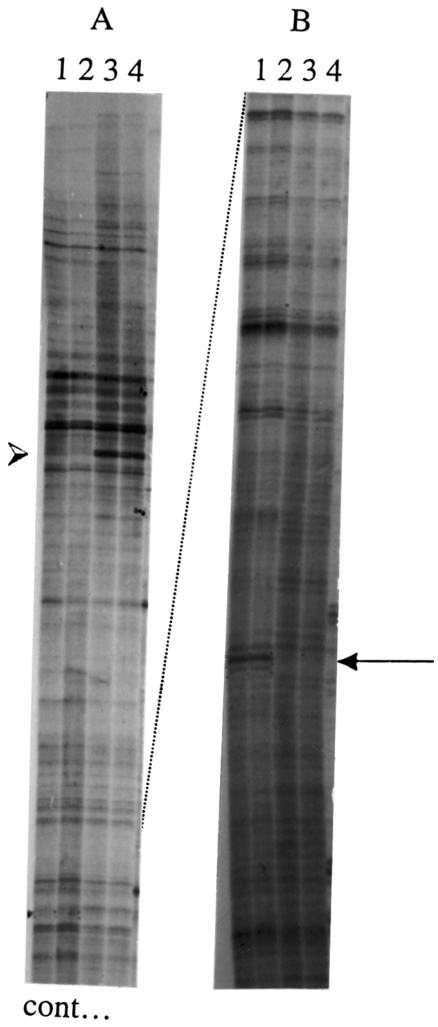
Table 1. DNA sequences of the P and T primers used in RNA fingerprinting – differential display reactions.

P-primer sequences	T-primer sequences
5′-ATTAACCCTCACTAAATGCTGGGGA-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTAA-3′
5′-ATTAACCCTCACTAAATCGGTCATAG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTAC-3′
5′-ATTAACCCTCACTAAATGCTGGTGG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTAG-3′
5′-ATTAACCCTCACTAAATGCTGGTAG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTCA-3′
5′-ATTAACCCTCACTAAAGATCTGACTG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTCC-3′
5′-ATTAACCCTCACTAAATGCTGGGTG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTCG-3′
5′-ATTAACCCTCACTAAATGCTGTATG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTGA-3′
5′-ATTAACCCTCACTAAATGGAGCTGG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTGC-3′
5′-ATTAACCCTCACTAAATGTGGCAGG-3′	5′-CATTATGCTGAGTGATATCTTTTTTTTTGG-3′
5′-ATTAACCCTCACTAAAGCACCGTCC-3′	

each having a P and a T primer, were used. The T primers have a common 19-bp sequence at the 5′ end followed by nine thymidine bases. The two bases at the 3′ end were variable and were present in all possible pairwise combinations of A, C, and G. The P primers shared a common sequence of 16 bases at the 5′ end, but contained a variable sequence of 9–10 bases at the 3′ end. The variable sequences of the P primers were selected based on commonly occurring sequence motifs in the coding regions of eukaryotic mRNAs.

The PCR conditions used were 1 cycle of 94°C for 5 min, 40°C for 5 min, and 68°C for 5 min; 2 cycles of 94°C for 2 min, 40°C for 5 min, and 68°C for 5 min; followed by 25 cycles of 94°C for

Fig. 2. Autoradiogram of an RNAF–DD gel. Lanes 1 and 2 present an RNAF pattern of CS and lanes 3 and 4 present an RNAF pattern of the *ph1b* mutant: lane 1, 4.5 ng of CS template; lane 2, 20 ng of CS template; lane 3, 4.5 ng of *ph1b* template; lane 4, 20 ng of *ph1b* template. The arrow indicates a fragment band present in CS but missing in *ph1b.* The arrowhead indicates a fragment band present in *ph1b* but missing in CS. (A) The upper half of the gel. (B) The lower half of the gel.



2 min, 60°C for 1 min, and 68°C for 2 min. The reaction mixture was then incubated at 68°C for an additional 7 min before cooling to 4°C.

The amplified products were size-separated in a 0.4-mm thick denaturing 5% polyacrylamide – 8 M urea gel, following standard sequencing gel protocol (Sambrook et al. 1989). Only about one-fifth of the amplification product was loaded on the gel, which was pre-run for 30 min at a constant current of 33 mA. The gel was run at 70 W for 3–4 h, rinsed in TE buffer for about 5 min, and blotted onto Whatman 3MM paper. The gel side of the paper was covered with plastic wrap and dried using a model 583 gel dryer (Bio-Rad). The plastic wrap was removed and an X-ray film was then placed on the dried gel and exposed for 3–7 days. The fragment bands present in the CS lane but absent in the lane with the *ph1b* mutant were identified and cut out of the gel. DNA from these gel pieces was eluted by boiling in 50 mL of TE for 10 min. The eluted DNA was re-amplified, using the same set of P and T primers that were used for the RNAF–DD reaction.

Identification of the positive clones

To identify the cDNA clones mapping to the interstitial deletion of the *ph1b* mutant, the positive fragment bands were tested by gel-blot DNA hybridization. The re-amplified DNA was used as probe on blots containing 10 μg of restriction enzyme digested genomic DNA of CS, the *ph1b* and *ph1c* mutants, the wheat group 5 nullisomic–tetrasomic lines, and the two single-break deletion lines (5BL-1 and 5BL-11). The *ph1c* mutant and the deletion lines 5BL-1 and 5BL-11 were included to identify cDNA clones mapping in the *Ph1*-gene region, which is much smaller than the deleted region in the *ph1b* mutant. For the genomic DNA digestion, two restriction enzymes, *Dra*I and *Hin*dIII, were used for the analysis of each positive fragment band. The gel-blot DNA hybridization procedure was as previously described (Gill et al. 1993).

DNA cloning

The pGEM®-T Easy Vector System I (Promega) was used to clone fragments mapping in the *Ph1* gene region. Inserts from three to six white colonies for each positive fragment were again tested by the gel-blot DNA hybridization technique on the blots described earlier, to identify individual fragments mapping in the target region.

Northern-blot analysis

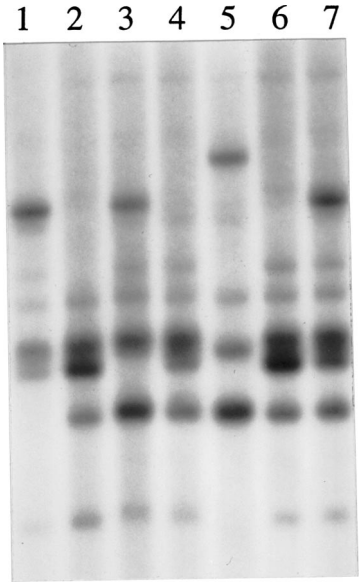
Approximately 2 μg of poly(A)$^+$ RNA of CS and the *Ph1* mutant, from the same developmental stage as was used for the RNAF–DD, was size-separated in a 1% agarose gel containing formaldehyde, following Sambrook et al. (1989). RNA was transferred to MSI membrane (Micron) following the manufacturer's recommendations. The blots were hybridized with ^{32}P-labeled DNA of the positive clones (as described earlier).

Results

The RNAF–DD technique worked well and produced consistently reproducible fragment bands (Fig. 2). Three independent reactions were performed for each primer combination and the replicated lanes were identical (data not shown). To check the template-concentration dependence of the reaction, duplicate RNAF–DD reactions with different concentrations were performed for five primer combinations. Lanes 1 and 2 in Fig. 2 show RNA fingerprinting patterns of CS; the reaction in lane 1 was performed with 4.5 ng of template cDNA and that in lane 2 with 20 ng. The lanes with different template concentrations usually showed identical band patterns, although band intensities were often higher in lanes with a higher template concentration. Since template concentration rarely changed the fragment-band patterns, only reactions with 20 ng of template cDNA were run for the remaining primer combinations.

A total of 38 primer combinations were used for the RNAF–DD reactions. For each primer combination, amplification products of CS and the *ph1b* mutant were run side by side and the band patterns were compared. On average, 83 fragment bands were amplified from CS with each primer combination. The fragment-band patterns in CS were almost identical to those in *ph1b*, except for a few bands. Of a total of 3154 fragment bands amplified by 38 primer combinations, 43 were not amplified from the *ph1b* mutant (see arrow in Fig. 2). An additional 35 fragment bands were also different between CS and the *ph1b* mutant; they were present in the *ph1b* mutant but absent in CS (see arrowhead in Fig. 2). These fragment bands were not characterized further.

Fig. 3. Gel-blot DNA hybridization of probe UNL2 on genomic DNA of chromosome group 5 aneuploid stocks and *Ph1* mutant lines: lane 1, Nulli 5A – Tetra 5B; lane 2, Nulli 5B – Tetra 5D; lane 3, Nulli 5D – Tetra 5A; lane 4, the *ph1b* mutant; lane 5, the *ph1c* mutant; lane 6, the 5BL-1 deletion line; and lane 7, the 5BL-11 deletion line.

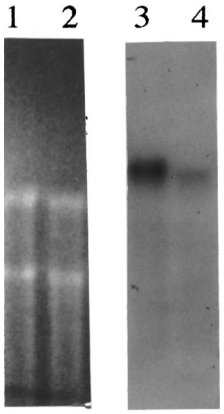


The 43 fragment bands that were differentially expressed in CS were eluted from the polyacrylamide gel and re-amplified. The amplified DNA fragments were used as probes for gel-blot DNA hybridization analysis of group 5 nullisomic–tetrasomic lines and the *ph1b* mutant, to identify fragments mapping in the *ph1b* deletion (Fig. 3). Nineteen of the 43 fragments detected numerous bands each, indicating that many copies are present in the wheat genome for the corresponding genes. The remaining 24 fragments represented single- or few-copy genes, as the number of detected fragments ranged from 3 to 15. Eight of the 24 fragment bands mapped to wheat homoeologous group 5, as missing fragment band(s) were detected in one or more of the group 5 nullisomic–tetrasomic lines (Fig. 3). Five of these eight probes mapped in the interstitial deletion of the *ph1b* mutant, because the corresponding 5B-specific fragment bands were absent in the mutant line. The remaining three probes mapped outside the interstitial-deletion region, as the 5B-specific fragment bands were detected in the *ph1b* mutant line also.

The *Ph1* gene is present in the region bracketed by deletion 5BL-1 and the distal breakpoint of the interstitial deletion of the *ph1c* mutant (*Ph1*-gene region). Therefore, the five positive probes were further mapped on the *ph1c* mutant and the two single-break deletion lines 5BL-1 and 5BL-11. Three probes mapped in the *Ph1*-gene region, because the 5BL-specific fragments were missing in the 5BL-1 deletion and the *ph1c* mutant. The other two probes mapped proximal to the *Ph1*-gene region, as the 5BL-specific fragments were present in the 5BL-1 deletion.

As each probe represents a band cut out of a sequencing gel, it may be a mixture of different fragments. Therefore, the three positive fragment bands were cloned using the pGEM®-T Easy Vector System I (Promega). From three to six of the cloned inserts corresponding to each fragment were again tested by gel-blot DNA analysis, to identify the true positives. Clones from two of the gel-purified fragments detected two types of patterns, indicating a mixture of at least two different types of fragments. All three clones from the third fragment showed only one hybridization pattern. The cloned fragments usually showed a cleaner hybridization pattern than the gel-purified fragments. The cDNA fragments mapping in the *Ph1*-gene region were identified for all three clones and were named UNL1, UNL2, and UNL3; these fragments were ca. 420, 500, and 550 bp, respectively, of the 3′ ends of the corresponding genes.

Fig. 4. Northern-blot analysis of CS and the *ph1b* mutant line with UNL2 as probe. The left-hand panel is an ethidium bromide stained gel containing poly(A)$^+$ RNA (the developmental stage is described in Materials and methods) of CS (lane 1) and the *ph1b* mutant (lane 2). The right-hand panel is a Northern-blot hybridization, using UNL2 as a probe on the blot made from the gel shown in the left-hand panel: lane 3, CS (lane 1 in the left-hand panel); lane 4, the *ph1b* mutant (lane 2 in the left-hand panel).



Northern-blot analysis was performed on poly(A)$^+$ RNA of CS and *ph1b*, using UNL1, UNL2, and UNL3 as probes. Poly(A)$^+$ RNA for the Northern-blot analysis was from 4-week-old leaves, and immature anthers as used for the RNA-DD reaction. All three probes detected differences in gene expression between CS and *ph1b*. The results of the Northern-blot analysis using UNL2 as probe are shown in Fig. 4. The gene corresponding to the probe UNL2 expresses during meiosis but not in 4-week-old leaves (data not shown). The genes corresponding to the other two probes, UNL1 and UNL3, were equally expressed during the two developmental stages tested.

Discussion

Here we demonstrate a successful application of the RNAF–DD technique for targeted marker enrichment of a wheat chromosomal region and, potentially, for gene cloning. The only difference between CS and the *ph1b* mutant lines is in chromosome 5B, which, in *ph1b*, contains an interstitial deletion encompassing the *Ph1* gene. The size of the deleted region was cytogenetically estimated to be 1.05 μm (Gill et al. 1993). The total length of haploid wheat metaphase chromosomes is 235 μm (Gill et al. 1991). Therefore, one out of every 224 random probes should map in the

deleted region. The observed number of random probes mapping in the deleted region of *ph1b* was 3 out of the total of 550 clones that were mapped on an *Aegilops tauschii* F_2 population (Gill et al. 1993; Boyko et al. 1999). In the present study, however, 5 out of 24 usable probes mapped in the interstitial deletion of *ph1b*. The present method, therefore, resulted in about a 38-fold enrichment of probes.

The breakpoint of 5BL-1 and the distal breakpoint of the interstitial deletion of the *ph1c* mutant bracket the *Ph1*-gene region (Gill et al. 1993). The region is submicroscopic and was estimated to be smaller than 550 kb in size (Gill et al. 1993, 1996*a*). This 550-kb region of chromosome 5B is about 1/29 000 of the wheat genome. If the genes were uniformly distributed throughout the wheat genome, one out of every 29 000 random DNA clones should map in this region. Since most of the wheat genes are present in clusters, which span only about 5–10% of the total wheat genome (Gill et al. 1996*a*, 1996*b*), one out of every 1450–2900 random clones should map in the *Ph1*-gene region. Previously, only 1 probe out of 550 random probes was identified for the *Ph1*-gene region (Gill et al. 1993). The present method identified 3 out of 24 usable probes for this region, thereby achieving about an 86- to 360-fold enrichment.

Most wheat genes are present in clusters (with from four to six gene-rich regions per chromosome), which encompass less than 10% of the total wheat genome (Gill et al. 1996*a*, 1996*b*). Many agronomically important genes also map in these gene-rich regions. Furthermore, only a fraction of each currently defined gene-rich region is expected to contain genes, resulting in only about 1% of the total wheat genome. Therefore, only the gene-containing regions should be targeted both for marker discovery and gene cloning. In addition to nullisomic–tetrasomic and ditelosomic lines, there are more than 400 deletion lines available for all wheat chromosomes (Sears 1954; Endo and Gill 1996). Almost all wheat gene-rich regions have been bracketed by the breakpoints of deletion lines (Gill et al. 1993, 1996*a*, 1996*b*; Faris et al. 2000; Sandhu 2000). Following the strategy described in this study, the gene-rich regions can be targeted using the flanking deletion lines, and the gene-containing regions within the gene-rich regions can be targeted further by isolating only the expressed sequences. Essentially any gene-containing region of wheat can be targeted, as the flanking deletion lines have been identified and are freely available.

The presence of false positives is a major problem with the RNAF–DD technique. Usually real positives are identified by either Northern-blot hybridization or RT-PCR based methods. These methods are technically demanding, especially for genes with rare transcripts. The aneuploid-based method of identifying real positives used in this study is relatively easy, more conclusive, and independent of expression-difference detection. Furthermore, Northern-blot analysis and RT-PCR based methods can only confirm differential expression of positive clones, not reveal their location on the chromosomes. For cloning a gene of unknown molecular function, mapping positive clones relative to the gene or its flanking markers is essential. The aneuploid-based method will not only identify the real positive clones, it will also reveal their physical location on the chromosomes. The map positions of almost all fragment bands can be revealed, as the strategy is less dependent on the extent of polymorphism. This method of positive-clone identification is particularly powerful for genes that have dispersed duplicated copies, because most copies can be physically mapped.

Most wheat genes have at least three structural copies corresponding to the A, B, and D genomes. Wheat genes will follow disomic inheritance if only one of these paralogues is functional. A complex polyploid inheritance will be observed if two or all three paralogues are functional. For the *Ph1* gene, only the 5BL copy seems to be functional, because chromosome pairing in plants nullisomic for chromosome 5A or 5D appears to be normal (Sears 1977). The *ph1b* mutant was backcrossed several times to its donor parent, CS, and, therefore, should be a true isogenic line for CS. High-density RFLP (restriction fragment length polymorphism) mapping has shown that chromosomes 5A and 5D are normal in the *ph1b* mutant. The differential expression between CS and *ph1b* for the three gene fragments isolated in the present study is most probably due to the absence of the 5BL copy of the corresponding genes. Therefore, the genes corresponding to the three fragments identified in this study are good candidates for the *Ph1* gene, because the corresponding genes are physically present in the same small submicroscopic region to which the *Ph1* gene maps. Furthermore, the corresponding genes are predominantly expressing from chromosome 5BL and not from its other two paralogues on chromosomes 5AL or 5DL. One of the three gene fragments is also meiosis specific, making it a better candidate for the *Ph1* gene than the other two.

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