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Single nucleotide polymorphic marker enabling rapid and early screening for the homoeolocus of β-amylase-R1: a gene linked to copper efficiency on 5RL

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Abstract This study describes the development of a PCR marker to detect the β-amylase-R1 gene of rye. It provides an easy and rapid means for the identification of plants containing the β-amylase-R1. Because rye chromosome segments do not normally recombine with wheat chromosomes, this marker provides a means for tracking all linked genes on that alien 5RL chromosome segment. Reaction conditions were optimised for an annealing temperature of 60°C for a high stringency. The reaction was also optimised for low reaction volumes reducing the cost of the reagents required for the reaction. This PCR test can be used in breeding or mapping programs for the rapid screening of progeny containing translocations of 5RL and hence select for the copper efficiency trait of rye.

Introduction

Copper is an important micronutrient for the growth and development of plants. Its role in plants appears to be most notably for the production of viable pollen and therefore influences grain yield in cereal crops (Brown and Clark 1977; Graham et al. 1987; Grundon 1991). Harry and Graham (1981) used glasshouse pot experiments to study the effects of copper deficient soil on rye, wheat and triticale. They observed that rye gave maximum yield, irrespective of the copper status of the soil, triticale gave an intermediate yield and wheat no yield when severely deficient conditions are applied compared with the control (i.e. maximal yield). Wheat is severely affected by copper deficiency and is completely male sterile under conditions when copper levels are still sufficient for near maximal yield of rye (Graham 1975, 1976, 1981, 1984; Owuoche et al. 1994). Genetic variation in copper efficiency has been reported among wheat cultivars (Nambiar 1976; Owuoche et al. 1994; Soon et al. 1997), but not to the extent to which rye is efficient. A characteristic of copper deficiency is a significant loss in grain yield of up to 20%, without prior symptoms (Graham and Nambiar 1981). As well as having an influence on plant fertility, it has also been claimed that crops growing on nutrient deficient soils are more susceptible to root diseases (Graham 1980; Graham et al. 1987) and may also be more susceptible to airborne fungal pathogens than plants growing with adequate copper (see Graham 1983).

Merker (1975) reported that the genes controlling copper efficiency of rye and the presence of the hairy peduncle were located on chromosome 5 of rye. Graham (1978) also suggested that genes controlling copper efficiency were located on chromosome 5 of rye (5R). Several lines expressing the copper efficiency trait of rye and carrying different wheat rye translocations involving 5RL of rye have been isolated. Two different 4BS.4BL-5RL translocations arose spontaneously as set out below:

1. Cornell Wheat Selection 82a1-2-4-7 carrying a segment of 5RL from Rosen rye joined onto chromosome 4B (Driscoll and Sears 1965).
2. Viking wheat with a hairy peduncle and carrying a segment of 5R from an unknown rye source (Riley et al. 1970).

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Another type of translocation with a segment of 5RL from Imperial rye translocated onto wheat chromosome arm 5BS (5BL.5BS-5RL) was isolated after irradiation treatment (Sears 1967).

Polymerase chain reaction (PCR) markers are useful in the detection of specific DNA sequences in plants (Wang et al. 1992). They are a very easy and rapid way of screening in population analysis (Steiner et al. 1995). In breeding populations the early screening of progeny is essential in reducing numbers, especially with those traits that are only detectable at maturity. Early generation selection using PCR markers enables more practical allocation of resources in a breeding program. Single nucleotide markers are a reliable and rapid means of selection of individuals in breeding populations, especially if the primers are amenable with a quick nucleic acid preparation, such as sodium hydroxide.

At the commencement of this study only a single sequence from a cDNA of β-amylase from the *β-amy-1* series of *T. aestivum* was in the nucleotide database (National Centre for Biological Information (NCBI) nucleotide database http://www.ncbi.nlm.nih.gov/). Two sequences for β-amylase from *S. cereale* were also listed in the database a longer sequence and a shorter sequence both sequenced from a cDNAs. It was considered too difficult and time consuming, if not impossible, to develop a marker for each of the β-amylase genes of *T. aestivum* due to the presence of all these homoeoloci an alternate approach was sort. Therefore, β-amylase-R1 of *S. cereale* was a much better candidate for the development a PCR marker.

Methods

Plant materials

Seeds of wheat lines *Triticum aestivum* (var. Chinese Spring), *Secale cereale* (var. Imperial Rye), Chinese Spring hairy neck (CSHN; Cornell Wheat Selection 82a1-2-4-7 (4BS.4BL-5RL)) and two addition lines ditelosomic 5RL and Rye + A (disomic 5R) all in Chinese Spring backgrounds were provided by Associate Professor K. W. Shepherd from the University of Adelaide.

Genomic DNA extraction

Plant genomic DNA was prepared from 0.4 g leaf tissue harvested from seedlings that were three to five weeks old using the method of Guidet et al. (1991).

Genomic DNA extraction by sodium hydroxide extraction

Three pieces of leaf material of about 5 mm in length were homogenised in 50 µl of 0.5M NaOH in a 1.5 ml plastic centrifuge tube (Eppendorf) and then 5 µl of the homogenate was transferred to a new tube containing 495 µl of 100 mM Tris–HCl (pH 8.0) and shaken vigorously for 30 s. A 1 µl aliquot was then used in a 25 µl PCR reaction (adapted from Wang et al. 1992).

Identification of a single nucleotide polymorphism to β-amylase-R1

Sequences of wheat and rye β-amylases were obtained from a search of the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/). The sequences were aligned using alignment software (http://www2.ebi.ac.uk/clustalw/) with the default settings. A target site was identified that would discriminate between wheat β-amylase and rye β-amylase on the basis of a single nucleotide polymorphism, and primers were designed with the aid of primer design software (http://www-genome.wi.mit.edu/cgi-bin/primer3-www.cgi) using the default settings. The primers were selected to generate a sufficiently large fragment that was easy to score and as these homoeoloci show a high degree of similarity so that it would increase specificity to the *β-amy-1* series of rye. In addition these sites were ideal for a high annealing temperature of 60°C also increasing the stringency of the reaction.

PCR amplification of a unique band

Each PCR reaction contained 1 µl of isolated genomic or alkali extracted DNA and was carried out in a 25 µl reaction volume for alkali extracted DNA or 12 µl for isolated genomic DNA. The reaction mixture contained 4 mM of each of the following oligonucleotide primers BAMRF (5′-ATA GCA CGC ATG CTC ACA AG-3′) and BAMRR (5′-GCG CCA CTG GAT CAA CAG-3′) were the β-amylase-R1 specific primers, AAMRF (5′-AGG GGT TTA ATT GGG AAT CG -3′) and AAMRR (5′-AGT GTC GTG GTT GTC GAT GA-3′) were the α-amylase specific primers (reaction control primers), 1 × reaction buffer (Amersham), 0.8 mM $MgCl_2$, 0.2 mM dNTP (Sigma), 0.5 or 1.0 U *Taq* DNA polymerase (Amersham). More consistent results were obtained with 1 U of *Taq* DNA polymerase when nucleic acid preparations were prepared by the alkali method. All amplifications were completed in an

Eppendorf Mastercycler® gradient (Eppendorf). The amplification conditions were 4 min (7 min for alkali extraction) at 94°C, followed by 30 cycles of a denaturing step of 1 min at 94°C, an annealing step of 1 min at 60°C, an extension step of 1 min at 72°C. After the program was completed the final step was a constant 4°C. The products were visualised using gel electrophoresis.

Verification of amplification product

Template from an amplification product of β-amylase-R1 from the CSHN was purified after gel electrophoresis. The DNA was purified using a Bresaspin gel extraction kit (Geneworks, Australia) and cloned into the p-GEM® plus Vector System I kit (Promega) according to the manufacturer's specifications. Samples were then prepared for sequencing at the Institute of Medical and Veterinary Science (IMVS; http://www.imvs.sa.gov.au) as directed by their procedures, with the addition of either the BAMRF (5′-ATA GCA CGC ATG CTC ACA AG-3′) or BAMRR (5′-GCG CCA CTG GAT CAA CAG-3′) primers. All amplifications were completed in an Eppendorf Mastercycler® gradient (Eppendorf).

Results

Development of a polymerase chain reaction marker detecting the homoeoloci of β-amylase-R1 gene of *Secale cereale*

Alignment of β-amylase from wheat and rye

Two sequences were available for β-amylase-R1 from the NCBI nucleotide database for *S. cereale* (AC# Z11772 and AC# X56785) and one for β-amylase from *T. aestivum* (AC# X98504). Rorat et al. (1991) suggested that a protein encoded by the AC# X56785 sequence was the most likely candidate for the β-amylase-R1 gene on 5RL. Since there was only a single sequence available for the ubiquitous β-amylase for wheat, the ubiquitous sequence for rye was also included in the alignment. These sequences were aligned with the aid of alignment software (http://www2.ebi.ac.uk/clustalw/) using the default parameters and the alignments of these are shown in Fig. 1. The alignment revealed a block of about 550 nucleotides where the rye sequences (AC# X56785 and AC# Z11772) shared 86 and 97% identity with wheat sequence (AC# X98504), respectively.

Generation of a unique band for β-amylase-R1 and optimisation of PCR conditions

Reaction conditions were optimised for an annealing temperature of 60°C for a high stringency. The reaction was also optimised for low reaction volumes reducing the cost of the reagents required for the reaction. The BAMRF and BAMRR primers were based on a single nucleotide polymorphism present between the wheat and rye β-amylase *β-amy-1* series loci (Fig. 1).

Verification of PCR

DNA isolated from wheat lines of *T. aestivum* (var. Chinese Spring), the ditelosomic 5RL, disomic 5R addition lines, CSHN and *S. cereale* (var. Imperial Rye), was used in amplification reactions and visualised (Fig. 2). After amplification using CSHN DNA and the BAMRF and BAMRR primers the band was excised from a gel, cloned and sequenced. Sequences from both the forward and reverse directions were aligned and compared for consistency (data not shown). Also the predicted protein sequence of the *β-amy-1* series of *S. cereale* (Accession number X56785) was aligned to the forward direction sequence and assessed using intron identification software (DNAWSIE; http://www.ebi.ac.uk) to identify possible introns (Fig. 3). Two introns were identified in this sequence. Intron 1 is 84 nucleotides in length and intron 2 is 108 nucleotides in length. Both predicted introns are large enough to be plant introns and they begin with the consensus intron start bases GT and end with AG. Finally the sequence was aligned with both the forward and reverse sequences generated and the introns were included in the alignment (Fig. 3).

Generation of a control band for PCR reaction

Scoring of the β-amylase-R1 PCR reaction relies on presence or absence of a specific amplification product. Thus if there no amplification product that can be visualised using an agarose gel then either the β-amylase-R1 gene is not present or the amplification reaction failed. To distinguish between these two possibilities, a second set of primers was introduced into the reaction mixture. These primers were designed to target the α-amylase genes of *T. aestivum* which are located in triplicate on chromosome groups 6 and 7 (Ainsworth et al. 1987) and therefore would always be present in a wheat genome (Fig. 2).

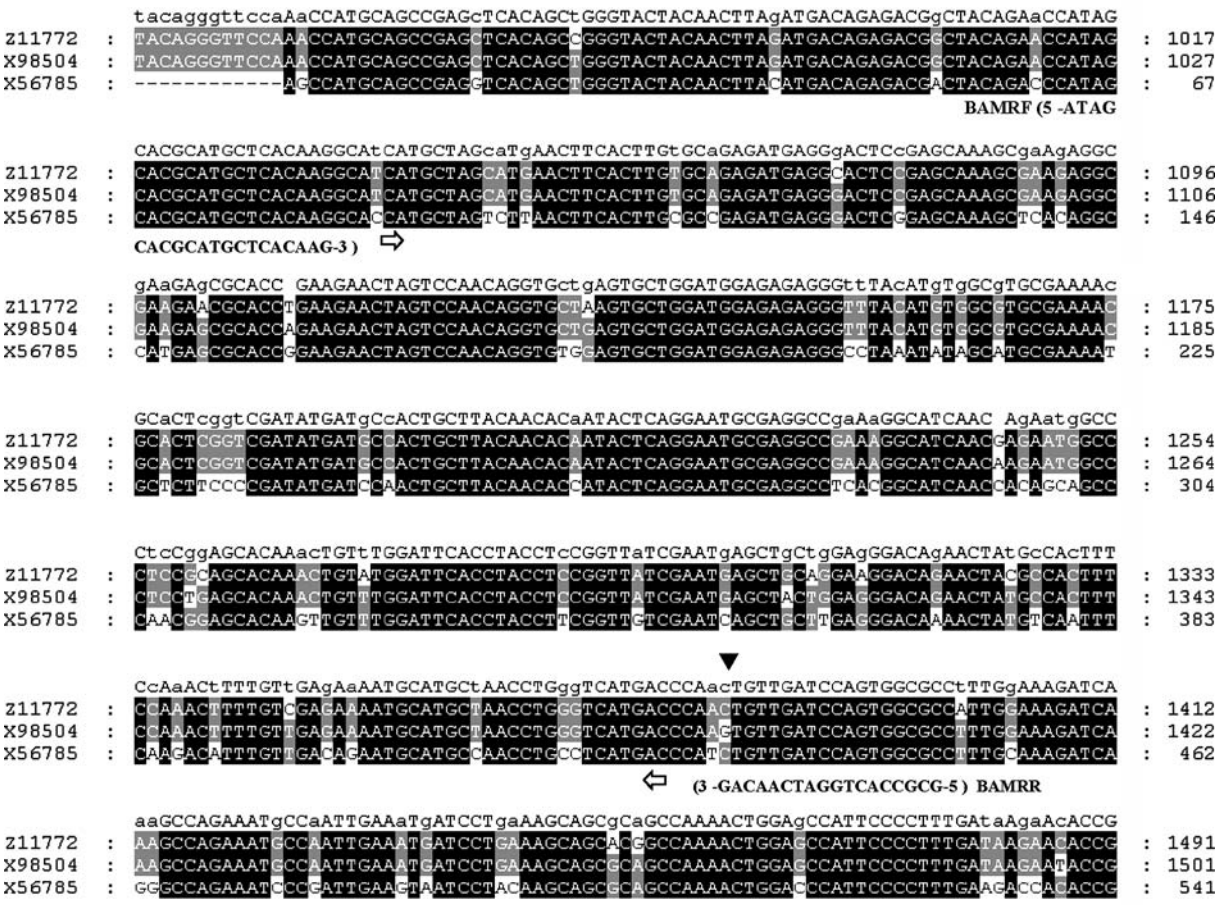

Fig. 1 Alignment and primer location of sequences obtained for β-amylase-R1 of *S. cereale* (Z11772.1 GI:393449 and X56785.1 GI:21191) and β-amylase of *T. aestivum* (X98504.1 GI:1771781) (http://www.ncbi.nlm.nih.gov). ▼ Indicates the position of the single nucleotide polymorphism

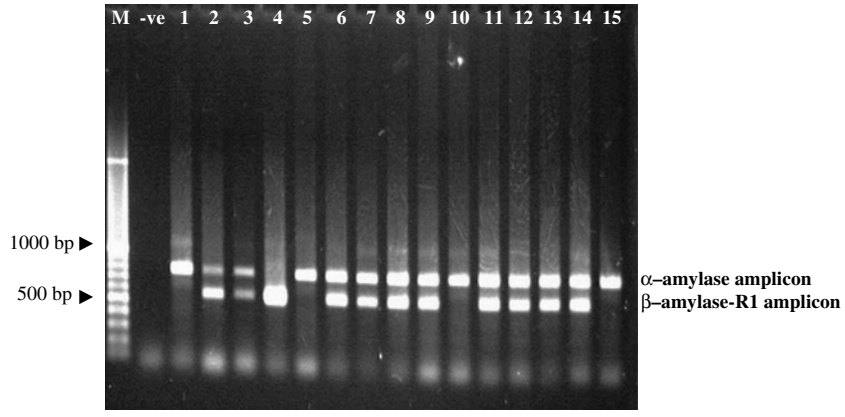

Fig. 2 PCR using the BAMRF, BAMRR, AAMF and AAMR primers on sodium hydroxide isolate DNAs of various segregating progeny for the Cornell Wheat Selection 82a1-2-4-7 (4BS.4BL-5RL) translocation, visualised using gel electrophoresis and ethidium bromide staining. (M = Marker 100 bp ladder, -ve = no DNA control, 1 = Chinese Spring, 2 = CSHP, 3 = Ditelosomic 5RL, 4 = Rye, 5,10,15 = homozygous normal wheat 4B and 6,7,8,9,11,12,13,14 = Heterozygous for the normal wheat 4B and the 4BS.4BL-5RL translocation)

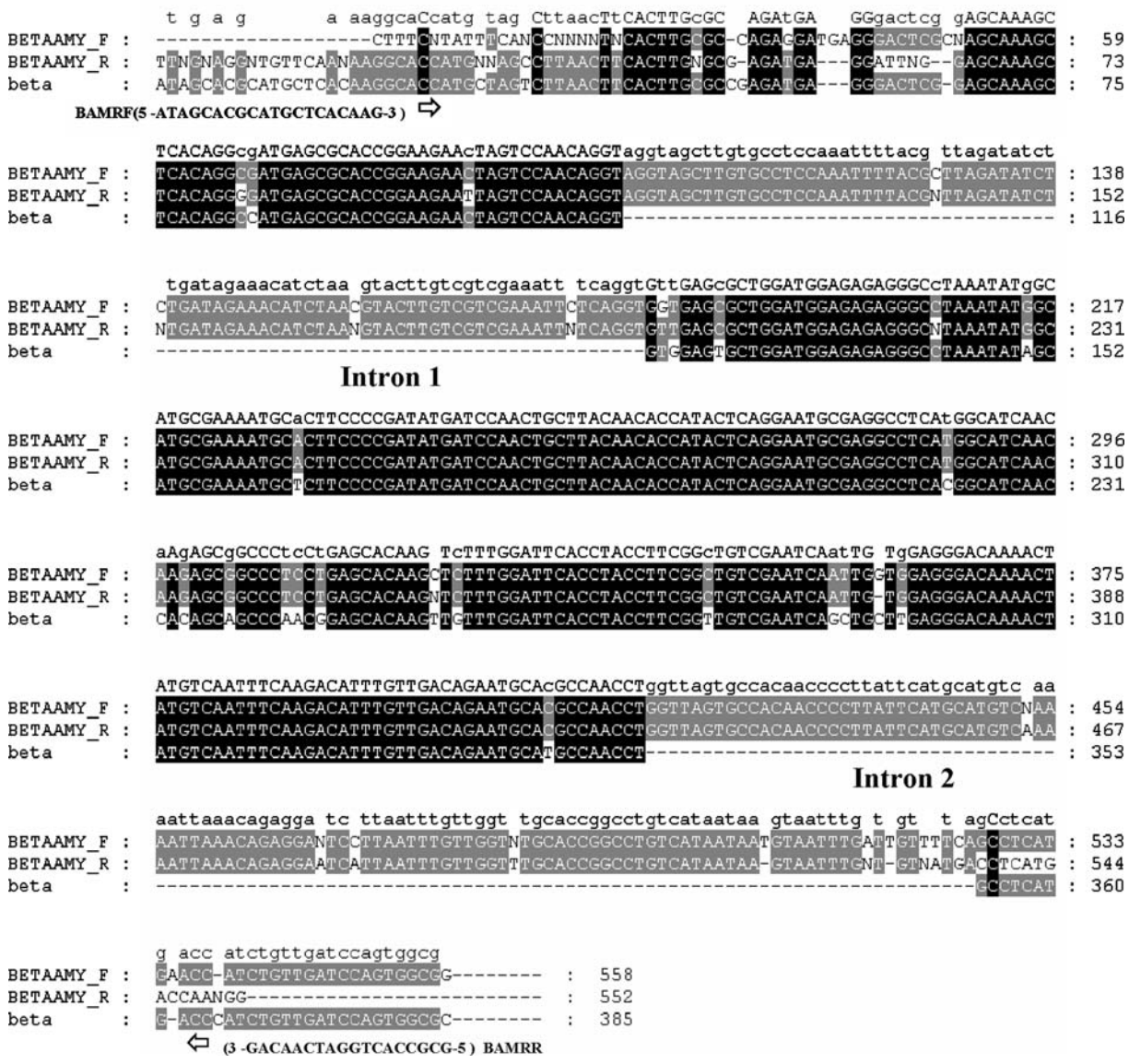

Fig. 3 Alignment of forward and reverse sequences from a cloned amplicon from a PCR of a genomic DNA amplification with BAMRF (β-amylase-R1 forward) and BAMRR (β-amylase-R1 reverse) showing the identified introns when compared with the consensus cDNA of β-amylase (*beta*, derived from Z11772.1 GI:393449 and X56785.1 GI:21191, http://www.ncbi.nlm.nih.gov)

Optimisation of sodium hydroxide extracted DNA

One of the limiting steps in screening large numbers of progeny using PCR is the isolation of nucleic acid for testing. Alkali extraction is a rapid means of isolation of sufficient nucleic acid for a PCR. The β-amylase-R1 PCR marker was tested on alkaline extracted DNA to assess the application of these primers in a rapid screening procedure (Fig. 2). The modifications required to optimise the PCR using alkali extracted DNA, were the need to use 1U of *Taq* DNA polymerase per reaction and to increase in the initial denaturing step from 5 to 7 min. Furthermore, it was established that this simple extraction procedure along with these PCR reaction condition could be used to rapidly verify the presence of β-amylase-R1 in any stock and therefore confirm the presence of chromosome 5RL and or the segment containing β-amylase-R1 gene.

Discussion

The development of a specific PCR marker for the β-amylase-R1 gene of *S. cereale* enables the simple and rapid identification of plants containing the β-amylase-R1 gene of rye and consequently indicating the presence

of the distal portion of 5RL. The predicted size of the amplicon bases on the primers derived from β-amylase cDNA was 385 nucleotides. Figure 2 shows that the actual size of the fragment generated which appears to be between 500 and 600 bases in length. The amplicon generated from CSHN genomic DNA was sequenced and the size of the fragment was determined to be 560 bases. Over 80% of plant protein-coding genes contain introns (Goodall et al. 1991) and so the difference between the predicted size of the PCR fragment and the actual size is due to the presence of one or more introns in the genomic DNA sequence. The sequence of the fragment described in this chapter was aligned with the predicted protein sequences for both β-amylase *β-amy-1* series from *S. cereale* (Accession numbers Z11772 and X56785; Figs. 1 and 2) using intron identification software. A single intron was predicted for the translated protein sequence Z11722 and two for the X56785 sequence. When the translated protein sequence of Z11722 was aligned many of the predicted amino acids were different from those that were predicted for the translation of the CSHN sequence and with only a single intron predicted that would produce a fragment shorter than the one sequenced in the experiment described in this chapter. These facts suggest that Z11722 is not the β-amylase gene present on 5RL.

The translation of the X56785 sequence revealed the presence of two introns. Plant introns are commonly between 70 and 1000 nucleotides in length with many being shorter than 150 nucleotides (Goodall and Filipowicz 1990; Goodall et al. 1991). Most characterised introns begin with GU (GT for DNA) and end with AG, and though there are some exceptions to this rule found in both plants and animals (Goodall et al. 1991). With the inclusion of two predicted introns in the X56785 sequence and the alignment of the product described in this chapter a better alignment was achieved. The Z11722 scored 172.34 bits over the entire sequence and X56785 scored 205.43 bits (higher bits- the better the identity) over the entire sequence. It is not surprising that the X56785 sequence scored had a higher bits score as the two sequences showed a very high identity over this region. As a higher bits score indicates a better match it is most likely that the X56785 sequence is that of the cDNA from the β-amylase located on 5R. Rorat et al. (1991) suggested that the location of this isoform of β-amylase was on chromosome 5 of rye. The results present in this chapter support this observation and since this is the only rye derived β-amylase gene presented in the CSHN the only conclusion is that this is the same gene.

Monocot exons contain an average of 44% A + T content in contrast to introns which have a 59% A + T content (Goodall et al. 1991). For the introns detected here, intron 1 has 59% A + T and intron 2 has 60%. The inclusion of these introns in the alignment of X56785 and the forward and reverse sequence (Fig. 3) corresponds to the fragment size generated by the PCR. On the 3′ end of the forward sequence the BAMRR primer can be identified and on the 5′ end of the reverse sequence the BAMRF primer is also evident.

Primers described in this chapter are able to distinguish between the β-amylase *β-amy-1* series sequences from *T. aestivum* located on 4BL and 4DL and the β-amylase-R1 of the *β-amy-1* series of *S. cereale* located on 5RL. This PCR discriminates between the two *β-amy-1* series sequences of *S. cereale* and *T. aestivum* on the basis single nucleotide. The choice of location for primer selection was based on their ability to generate a sufficiently large fragment that was easy to score. In addition these homoeoloci show a high degree of similarity so the annealing temperature of the selective primer needed to be high enough to ensure specificity for the *β-amy-1* series of rye.

A second set of primers was introduced to act as a control for the PCR reaction. Since the PCR is qualitative it is impossible to distinguish between a failed reaction and a negative result. With the introduction of the second set of primers it was possible to determine which results were truly negative.

The application of a rapid DNA extraction procedure would be an enormous benefit in screening larger numbers of progeny in molecular marker screening. In addition to speeding up the screening process, the current procedure for PCR reduces the amount required amount of DNA and an added advantage is the use of an agarose gel system compared with PAGE systems used with SSR marker systems. This marker system would be useful the identification of genetic stocks in a wheat breeding or mapping program, such as ditelosomic 5RL, and disomic 5R rye addition lines without the necessity to do cytological analyses or grow to maturity every plant to determine whether they were segregating for the rye chromosome.

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