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Expressed sequence tag-PCR markers for identification of alien barley chromosome 2H in wheat

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ABSTRACT. We developed EST-PCR markers specific to barley chromosome 2H, for the purpose of effectively tracing alien chromosomes or chromosome parts in the wheat genetic background. The target alien chromosome 2H confers high resistance to pre-harvest sprouting, which is a worldwide natural disaster in wheat. A total of 120 primer pairs were selected by combining the wheat group 2 chromosomes of the EST database and the genome sequences of the new model plant *Brachypodium distachyon*. Seventy-seven of 120 primer pairs were polymorphic and 31 of 120 primer pairs were monomorphic between a set of wheat-barley chromosome 2H disomic addition/substitution lines and their parents by agarose gel electrophoresis and polyacrylamide gel electrophoresis. Thirty of 77 polymorphic primer pairs including primer pair P120 derived from the *basi* gene were chromosome 2H-specific. These markers are expected to be valuable in screening of wheat-barley

chromosome 2H recombination lines and pre-harvest sprouting resistant varieties.

Key words: Wheat; Barley; Chromosome 2H; EST-PCR marker

INTRODUCTION

Barley (*Hordeum vulgare*, 2n = 14, HH) was one of the earliest domesticated cereal crops, and it continues to be widely cultivated today. It possesses many important agronomic traits, such as early flowering time, cold hardiness, disease resistance, and pre-harvest sprouting resistance. These traits are a potentially valuable resource of useful genes for the genetic improvement of wheat (*Triticum aestivum*, 2n = 42, AABBDD), a relative of barley. It has been reported that the *basi* gene on the long arm of barley chromosome 2H confers reduced precocious germination by coding an endogenous, bifunctional α-amylase/subtilisin inhibitor to modulate α-amylase activity (Hejgaard et al., 1984). It is especially effective at inhibiting wheat α-amylase activity (Henry et al., 1992). Hence, the introduction of the alien chromosome 2H to wheat is a reasonable strategy to improve wheat pre-harvest sprouting. A set of wheat-barley disomic substitution lines, 2H(2A), 2H(2B) and 2H(2D), were previously reported by Yuan et al. (2003), providing a valuable starting point for the development of wheat-barley chromosome 2H translocation or recombination lines.

An effective, precise and easy-to-use technique for the detection of alien chromosomes or chromosome parts in a large population would significantly facilitate the production of recombination lines or translocation lines. STS/EST-PCR (sequence tag site/expressed sequence tag-PCR) markers are a novel kind of functional marker that is less time-consuming than traditional cytogenetic techniques, such as genomic *in situ* hybridization. They are also different from random DNA markers (Andersen and Lübberstedt, 2003; Gupta and Rustgi, 2004), such as RAPDs (and simple sequence repeats, which are usually derived from genomic DNA rather than transcribed sequences. STS/EST-PCR markers are often designed from coding sequences, including anonymous cDNA clones, ESTs and published gene sequences. STS-PCR markers are normally functional markers derived from RFLP probes or other sequences, with the exception of ESTs. These markers were formerly developed for the construction of genetic maps of barley or for screening new wheat germplasms to which a barley chromosome was introduced (Blake et al., 1996). These markers distinguished barley products from their wheat counterparts.

More recently, the large accumulation of ESTs in both public and private databases has become a resource for EST-PCR marker development. A high-density barley genetic map was constructed (Sato et al., 2004, 2009) using more than 1000 EST markers. These EST markers were first generated from nine unique cDNA libraries of three barley strains; they were then mapped with a population derived from a cross between two of the three EST donors. Next, these markers were assigned to the seven barley chromosomes, and 90 markers were physically mapped onto segments of chromosome 7H, using 19 deletion stocks. Extensive testing of these markers was performed, including analysis of alien chromosomes in the wheat genetic background and identification of several 5H deletion lines (Nasuda et al., 2005). More than 16,000 EST loci have been physically mapped to specific regions of wheat chromosomes using deletion lines (Qi et al., 2004) and 2600 confirmed loci were mapped onto group 2 homologous chromosomes (Conley et al., 2004). This mapping resulted in 769 loci (29.6%)

of 651 EST probes mapped to chromosome 2A, 959 loci (36.9%) from 728 EST probes mapped to chromosome 2B, and 872 loci (33.5%) from 725 EST probes mapped to chromosome 2D. Chromosome 2B contained the most deletion bins in the set. These mapped EST probes are likely to be a better source of EST-PCR markers for tracing barley chromosome 2H in the wheat genetic background than barley ESTs, due to their information-rich character.

Brachypodium distachyon has been considered a new model plant because of its small genome size. DNA sequences from *B. distachyon* are conserved in the genomes of many grass species, including wheat (Draper et al., 2001; Foote et al., 2004; Opanowicz et al., 2008). The 4X draft genome assembly of *B. distachyon* has been completed, and the BLAST server is available at http://blast.brachybase.org/. It may be possible to develop useful EST-PCR markers for tracing chromosome 2H by combining the wheat EST database and the genome sequences of the new model plant *B. distachyon*.

We developed a series of EST-PCR markers for the detection of barley chromosome 2H, or chromosome 2H parts, in the wheat genetic background.

MATERIAL AND METHODS

Plant materials and DNA extraction

Four wheat-barley derivatives and their parents, including common wheat cultivar 'Chinese Spring' (CS) and barley cultivar 'Betzes', were used for EST-PCR marker screening. These wheat-barley derivatives included the CS-Betzes 2H disomic addition line (2n = 44, Islam et al., 1981) and disomic substitution lines 2H(2A), 2H(2B) and 2H(2D) (2n = 42, Yuan et al., 2003). All of these plants are preserved at the College of Plant Science, Jilin University.

DNA extraction was carried out as described by Doyle and Doyle (1990), with minor modifications. DNA was extracted, purified and stored at -20°C for later use.

The origin of EST-PCR markers

One hundred and twenty ESTs, allocated to eight bins of wheat chromosome 2B, were selected and downloaded. To avoid problems arising from sequences that map to multiple loci, ESTs that could be unambiguously mapped to chromosomes of homologous groups other than group 2 were ignored.

The sequence of the *basi* gene (X16276) has been accessible in public nucleotide databases since 1995 (Leah and Mundy, 1989). We directly designed a pair of primers to this sequence, designated as P120.

Primer design

We designed 120 primer pairs from 120 downloaded ESTs (including the *basi* gene sequence) with Primer Premier 5.0 (PREMIER Biosoft International, USA). To improve efficiency, each EST was analyzed to predict intron position by comparing it with genomic sequences of *B. distachyon*. Primer pairs were obtained by flanking the introns, whereas ESTs that failed intron prediction were designed randomly using the entire sequence. Early in our analysis, 75 of 120 primer pairs were commonly designed, due to the unavailability of the

B. distachyon database prior to the public release date. Differing strategies led to significant variation in efficiency, as discussed below.

Marker development

PCRs were generally carried out in a volume of 25 µL, containing 100 ng DNA as template, 0.2 mM of each primer, 1 U Taq polymerase (Takara, Japan), 0.2 mM dATP, dCTP, dGTP, and dTTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$. PCR program conditions were as follows: an initial step of 94°C for 5 min to denature DNA, followed by 35 cycles of 94°C for 45 s, Tm°C 45 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The annealing temperature was altered a few degrees as necessary. PCRs for each primer pair were carried out at least twice to obtain satisfactory reproducibility.

Separation and visualization of amplicons for each primer pair was performed with the "triple level separation system" established in our laboratory. Our protocol to visualize the polymorphism includes 2-4% agarose gel electrophoresis (AGE) with ethidium bromide staining, 6% polyacrylamide gel electrophoresis (PAGE) and 6% single-strand conformation polymorphism (SSCP) with silver staining. The product of each primer pair was run through this system. Usually, AGE was carried out first. Depending on the separation result, the PAGE only or both PAGE and SSCP procedures were utilized until a satisfactory result was obtained. Primer pairs that remained monomorphic after the separation in this system were discarded.

Because primer pair P120 was derived from the *basi* gene, the PCR products were reclaimed, cloned into a sequencing vector and sequenced following routine PCR. An alignment of the sequences generated was carried out to insure that these fragments originated from the *basi* gene and to discover whether fragments from the wheat background were homologous with the *basi* gene.

RESULTS

Comparative analysis of gene identity between wheat and barley

The majority of the 120 EST primer pairs were successfully used for amplification in wheat (90%) and barley (65%), indicating a close relationship between barley and its wheat counterpart in the *Triticum* genus. This result agrees with the conclusion that there is extensive microcolinearity among species in the grass family (Feuillet and Keller, 2002). However, some primers failed with both the wheat and barley as template (Table 1), which might be due to the lack of accuracy in some base pairs of the EST or the annealing of a primer at an unpredictable intron-exon junction site.

Table 1. Comparing gene identity between wheat and barley using EST primers.

	PCR product types[a]		Percentage	
Monomorphism			25.8% (31 pairs)	
Polymorphism	Different	Non-2H-specific	14.2% (17 pairs)	Total 64.2% (77 pairs)
		2H-specific	25.0% (30 pairs)	
	Totally different[b]		25.0% (30 pairs)	
	Failed in PCR		10.0% (12 pairs)	
Total			100.0% (120 pairs)	

[a]Amplicons from wheat and barley were separated and classified by comparing the molecular weight; [b]primers that amplified successfully in wheat but not in barley.

Compared with the relatively high success rate of 65% in *Thinopyrum intermedium* (Wang et al., 2010), another close relative of wheat, few EST primers (25.8%) amplified the same or nearly the same size EST products between wheat and barley, indicating a difference between the wheat ESTs and their orthologs in barley. This result is in agreement with the fact that both the *Thinopyrum* and *Triticum* genera belong to the subtribe Triticinae in tribe Triticeae, whereas the *Hordeum* genus belongs to the subtribe Hordeinae.

Chromosome 2H-specific EST-PCR markers

Amongst those EST primers that yielded different PCR products from wheat in barley, 30 pairs (25%) produced clear bands and could be further mapped onto chromosome 2H by a set of wheat-barley disomic addition/substitution lines. These EST primers were designated as 2H-specific EST-PCR markers. Thirty markers distributed amongst eight bins on chromosome 2B were selected, with several bins containing good marker density (Figure 1). Detailed EST marker information is reported in Table S1.

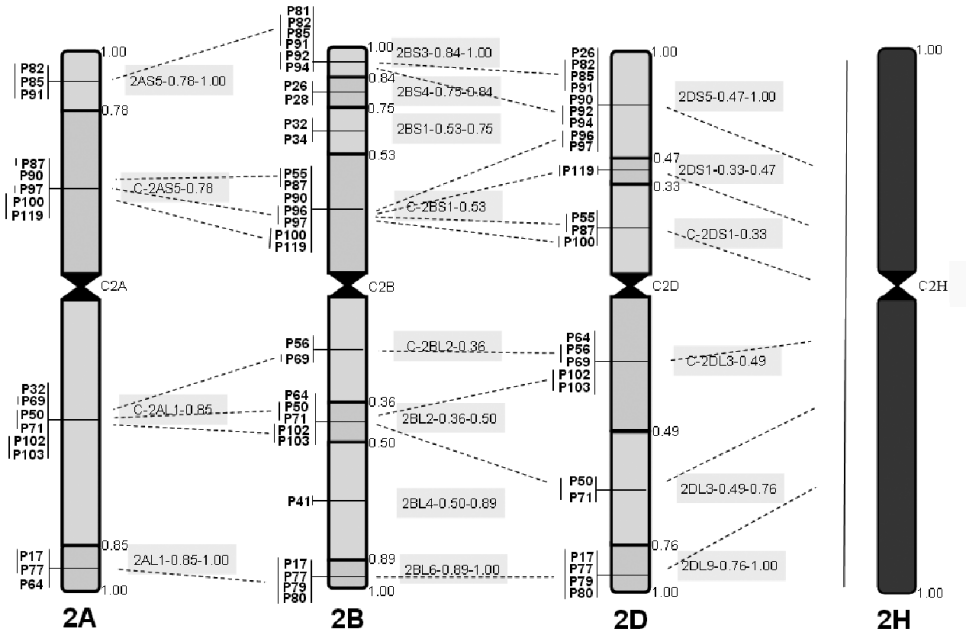

Figure 1. Physical linkage map of group 2 chromosomes. The map was drawn with 2H-specific EST-PCR markers. Wheat chromosomes were divided into several regions (bins) measured by fraction length (FL; defined by Endo and Gill, 1996) at right. Note that the order of markers within an identical bin (wheat chromosomes) or arm (2H) is unknown; however, the colinearity of markers among different sub genomes is presented by dashed lines. Markers were marked in bold to indicate that chromosome assignment information revealed in our study was consistent with that in the mapped EST database.

Each 2H-specific EST marker has unique characteristics and can be classified into different groups. First, according to the separation method, markers were divided into three

groups: AGE-level markers, PAGE-level markers and SSCP-level markers (Figure 2). Most of the markers show polymorphisms on the PAGE level, indicating that PAGE is a suitable method for EST-PCR marker development. Second, considering the information provided by the markers, they were then further divided into two groups: simple polymorphism markers and complex polymorphism markers. The former often produce few bands and only exhibited polymorphisms between the alien chromosome and the wheat genetic background (e.g., P28 and P69; Figure 2), whereas the latter usually produced several bands and could be mapped onto chromosome 2H and wheat chromosomes at the same time (e.g., P71 and P82; Figure 2). Obviously, AGE-level complex EST-PCR markers like P79 are more convenient for practical use.

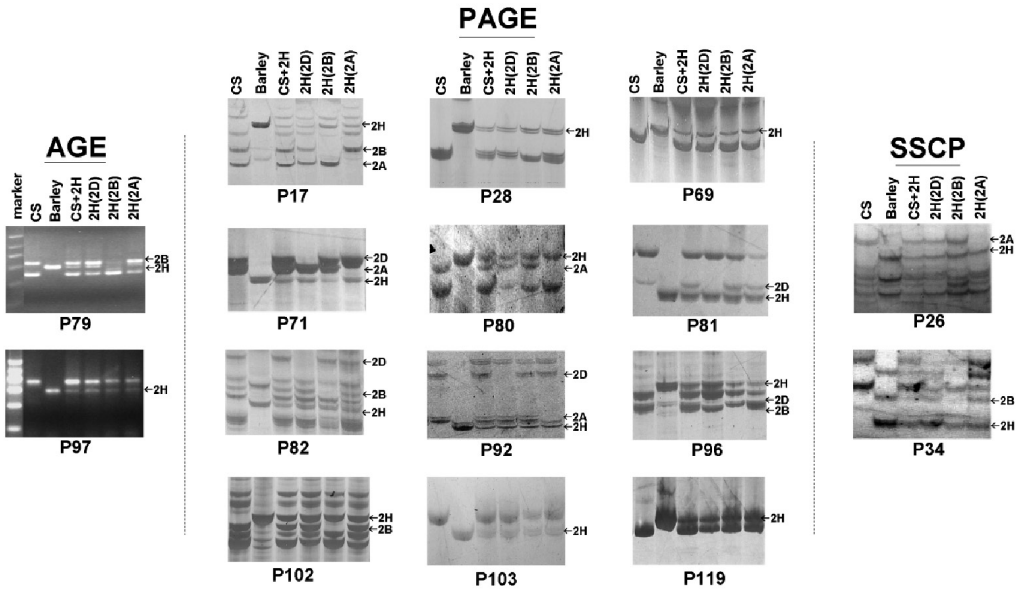

Figure 2. Electrophoresis patterns of 2H-specific EST-PCR markers in different polymorphism levels and polymorphism types. The fragments produced by certain primer pairs were easily assigned to chromosomes based on the presence or absence of PCR products from 2H addition/substitution lines. The arrows indicate the fragment that can be assigned to a certain chromosome. AGE = agarose gel electrophoresis; PAGE = polyacrylamide gel electrophoresis; SSCP = single-strand conformation polymorphism.

P120: developing the *basi* gene into a functional marker

The primer pair P120, designed from the *basi* gene sequence, was amplified into two clear and reproducible bands in six basic samples. One band was amplified from the wheat background (P120-Ta; Figure 3), and the other 433-bp band was successfully mapped onto chromosome 2H (P120-Hor; Figure 3). A sequence comparison (Figure 4) showed that P120-Hor was homologous to the template sequence from which the primer was derived, with 100% identity, indicating that the *basi* gene was successfully amplified. The fragment amplified from the wheat background appears to be the product of false priming, which suggests that the *basi* gene is a unique gene in the barley genome.

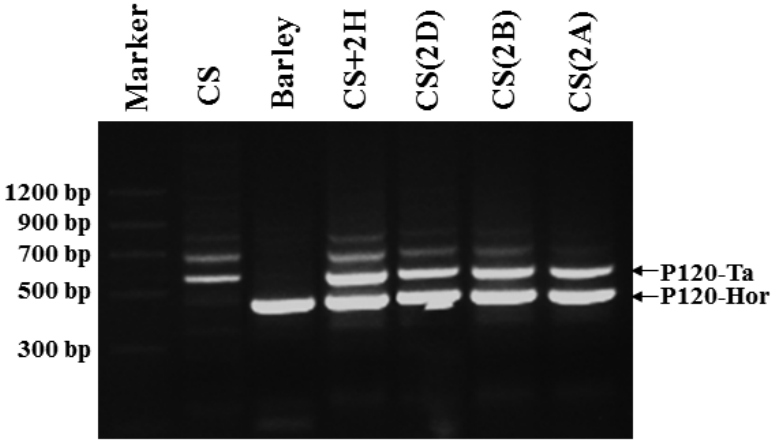

Figure 3. Electrophoresis patterns of marker P120. P120-Hor indicates barley sequence. P120-Ta indicates wheat sequence.

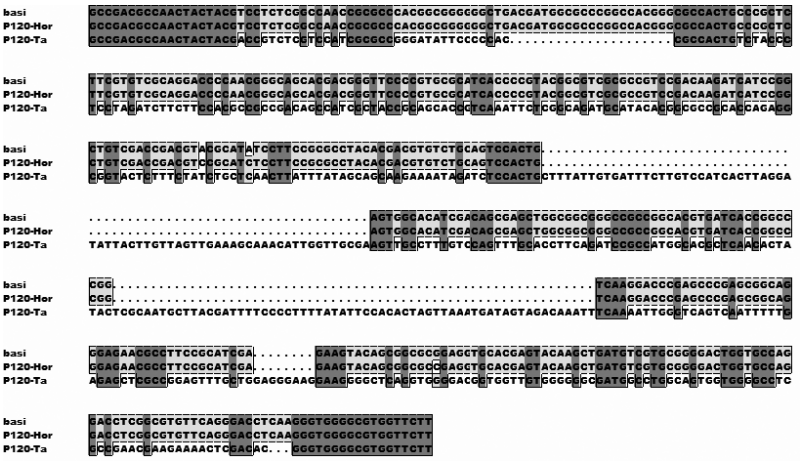

Figure 4. Alignments of sequences from recovered fragments of marker P120. Basi indicates the original sequence of the *basi* gene from which the primer pairs were designed. P120-Hor indicates barley sequence. P120-Ta indicates wheat sequence.

DISCUSSION

Comparative genomics

The grass family, an economically important crop, is a large and diverse family. There is comprehensive colinearity and microcolinearity within this family, allowing for convenient development of transportable markers sharing sequence information (e.g., ESTs) amongst grass species, such as rice, wheat and barley. Our study provides a starting point for the development of EST-PCR markers using mapped wheat ESTs (wESTs). We predict that the majority

of primers derived from wheat ESTs would successfully amplify in both wheat and barley. Moreover, since an EST is a part of a specific gene, we also predict that orthologous regions amplified by EST primers are homologous to ESTs (in both sequence and position), in most cases (refer to sequence analysis result in Wang et al., 2003). In fact, successful amplification was carried out with 65% of 120 wEST primer pairs and thus proves that this is a reasonable methodology.

Factors that influence the efficiency of development

As described in Material and Methods, the first 75 primer pairs were randomly designed within the EST instead of using the intron-flanking method, as with the latter 45 pairs. This difference of development strategy results in a significant difference in the percentage of polymorphic markers. In the first 75, 16% (12/75) were polymorphic, whereas in the later 45 primer pairs, 40% (18/45) were polymorphic. One reasonable explanation is that intronic regions of a gene are often less conserved than exons. Aside from the development strategy, the amplicon separation also influences marker development. Methods with better resolution usually yield a higher polymorphism rate.

The necessity of developing functional markers from genes of interest

The ultimate goal of this project is to produce new wheat germplasms with the precocious germination resistance trait or other useful traits. Therefore, functional markers that are directly linked to the genes affecting these traits would be helpful (Hagras et al., 2005). However, the development of markers linked to other regions of chromosome 2H, besides the *basi* locus, is still necessary. The reasons are 2-fold; first, unlike gene transformation, it is likely that a relatively large DNA segment will be introduced into the acceptor plant in translocation lines rather than only a gene region. Secondly, other parts of the alien chromosome contain genes that control traits of interest, which could be used as a potential resource.

Mapped wheat ESTs are a valuable resource for investigators working on crop breeding. This database is not only useful for the study of functional marker development but also for cloning of new genes, QTL mapping, and transcription analysis, and so on. We concentrated on the development of an EST-PCR marker set that is specific to chromosome 2H of barley, with the purpose of effectively tracing this alien chromosome in a wheat background. Of 120 markers, we reported 30 primer pairs, giving a total marker polymorphism rate of 25%. These markers are informative and able to unambiguously distinguish 2H-specific amplicons from their wheat homologues, and they will be put into practical usage for screening wheat-barley translocation lines.

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SUPPLEMENTARY MATERIAL

Table S1. Details of expressed sequence tag (EST)-PCR makers specific for barley chromosome 2H.

Marker name	EST accession[a]	Primer pair		Polymorphism type	Bin location[b]				Chromosome assignment[c]	Function prediction	
		Sequences	Tm (°C)		2A	2B	2D	Other		Blastn/x	E value
P79	BQ169948	F: 5'-AAAATGAAACATCTCCTCGC-3' R: 5'-AGTCAAATAACACAACCAATAAG-3'	54	AGE	-	2BL6-0.89-1.00	2DL9-0.76-1.00	-	2H,2B	Unknown protein	-
P97	BE586093	F: 5'-ATTGCTGATGACGCTGTTAT-3' R: 5'-CTTCTCGTTGTCTTGGGTT-3'	56	AGE	C-2AS5-0.78	C-2BS1-0.53	2DS5-0.47-1.00	-	2H	Unknown protein	-
P120	X16276[d]	F: 5'-CGCCGACGCCAACTACTA-3' R: 5'-ACCCTTGAGGTCCCTGAAC-3'	56	AGE	-	-	-	-	2H	Alpha-amylasesubtilisin inhibitor (BASI) [*Hordeum vulgare*]	0
P17	BF484009	F: 5'-CTTAGAAGTAGCCCAGCAACG-3' R: 5'-GACTCGCAGCAGGCAAAA-3'	57	PAGE	2AL1-0.85-1.00	2BL6-0.89-1.00	2DL9-0.76-1.00	-	2H,2A,2B	Delta-24-sterol methyl-transferase [*Triticum aestivum*]	9e-05
P28	BQ161381	F: 5'-CTTCCGAACAAATCCTGG-3' R: 5'-CAAGGTGGTTGTGAAAGTAGAA-3'	53	PAGE	-	2BS4-0.75-0.84	-	-	2H	Unknown protein	-
P32	BE403863	F: 5'-CTCGGTGCTCTTCATCAG-3' R: 5'-CCGTGTACTTGGACTTTGT-3'	55	PAGE	C-2AL1-0.85	2BS1-0.53-0.75	-	-	2H,2B	Unknown protein	-
P41	BE445284	F: 5'-AGATAACGGTGGTGAAATG-3' R: 5'-TGGAAGTAAAGGTAGGCTC-3'	54	PAGE	-	2BL4-0.50-0.89	-	-	2H	Calcium/proton exchanger CAX1-like protein [*Zea mays*]	7e-20
P50	BQ169894	F: 5'-GCCCTACTCCTCTTCCCGTA-3' R: 5'-TGGTCTGTCCAAAGTGAGCC-3'	56	PAGE	C-2AL1-0.85	2BL2-0.36-0.50	2DL3-0.49-0.76	-	2H	Unknown protein	-
P55	BQ159522	F: 5'-AATCAACAAACGAGCAAGAG-3' R: 5'-TCACCAAGCCTAAGAATCAC-3'	54	PAGE	-	C-2BS1-0.53	C-2DS1-0.33	-	2H	Unknown protein	-
P69	BQ166410	F: 5'-GCTAACCATACTAAGGGTGTTC-3' R: 5'-CGAGTTCTTCGTTGAGGG-3'	53	PAGE	C-2AL1-0.85	C-2BL2-0.36	C-2DL3-0.49	-	2H	Putative protein kinase [*Sorghum bicolor*]	2e-55
P71	BQ160526	F: 5'-AGTGTATGTTCCCACCTCCC-3' R: 5'-GGTATCGGTGAAGGCACTATC-3'	56	PAGE	2AL1-0.85-1.00	2BL6-0.89-1.00	2DL3-0.49-0.76	-	2H,2A,2D	Senescence-ssociated putative a protein [*Narcissus pseudonarcissus*]	3e-46

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Table S1. Continued.

Marker name	EST accession[a]	Primer pair		Polymorphism type	Bin location[b]				Chromosome assignment[c]	Function prediction	
		Sequences	Tm (°C)		2A	2B	2D	Other		Blastn/x	E value
P77	BE499251	F: 5'-AGCCACGAGCAGAAGAGCAC-3' R: 5'-GAGGGCGTCGCTGTCCA-3'	60	PAGE	2AL1-0.85-1.00	2BL6-0.89-1.00	2DL9-0.76-1.00	-	2H,2B	Unknown protein	-
P80	BE637228	F: 5'-GCTTCTCCCCCTTCTGTAAT-3' R: 5'-GCAGCCAAACGAATAGTCAG-3'	55	PAGE	-	2BL6-0.89-1.00	2DL9-0.76-1.00	-	2H,2A	Unknown protein	-
P81	BE443737	F: 5'-GGGGTCAACGCTTCAGGT-3' R: 5'-GAGCCGCTGCTAACTGTGA-3'	57	PAGE	-	2BS3-0.84-1.00	-	-	2H,2D	Jasmonate-induced protein mRNA [*H. vulgare*]	1e-109
P82	BE444541	F: 5'-GCGGACCTGTTGACATTCT-3' R: 5'-ATCTCTTCCCTTGCTCACG-3'	55	PAGE	2AS5-0.78-1.00	2BS3-0.84-1.00	2DS5-0.47-1.00	-	2H,2B,2D	Unknown protein	-
P85	BF201235	F: 5'-GCAAACCCTGTATCACTAAAG-3' R: 5'-CAATCATGGCTCCAATAAGT-3'	53	PAGE	2AS5-0.78-1.00	2BS3-0.84-1.00	2DS5-0.47-1.00	-	2A,2B	Rubisco subunit binding - protein alpha subunit	3e-50
P87	BE425962	F: 5'-GAAGTTTTTGTCAGTGGGCT-3' R: 5'-AATGGATGGAACCCTGCT-3'	54	PAGE	C-2AS5-0.78	C-2BS1-0.53	C-2DS1-0.33	-	2H	Unknown protein	-
P90	BE586093	F: 5'-AGTTCGCTCTGTTCCTTCG-3' R: 5'-CTGCCTTCTGCTCTTCTCGT-3'	54	PAGE	C-2AS5-0.78	C-2BS1-0.53	2DS5-0.47-1.00	-	2H	Unknown protein	-
P91	BQ170491	F: 5'-TGTCATCCAACCATAGCAGAG-3' R: 5'-TCGACCAGCACCATCGA-3'	55	PAGE	2AS5-0.78-1.00	2BS3-0.84-1.00	2DS5-0.47-1.00	-	2H,2A, 2B,2D	Triticin [*T. aestivum*]	0.39
P92	BF474028	F: 5'-TGTTTGCCATCGTTATCTT-3' R: 5'-GAATCAAAGAATGTATGAAACTG-3'	51	PAGE	-	2BS3-0.84-1.00	2DS5-0.47-1.00	-	2H,2A,2D	Unknown protein	-
P94	BG606132	F: 5'-AAGAGTGGGCAAGTCGGC-3' R: 5'-CTCACGGGGCGACTTGG-3'	58	PAGE	-	2BS3-0.84-1.00	2DS5-0.47-1.00	-	2H	Leucine-rich repeat trans-membrane protein kinase [*Arabidopsis thaliana*]	3e-20
P96	BE518419	F: 5'-GGCGAACAACTACTACCGTG-3' R: 5'-CAAGTAGCCCAGGGAGGAG-3'	55	PAGE	-	C-2BS1-0.53	2DS5-0.47-1.00	-	2H,2B	Unknown protein	-

Continued on next page

Table S1. Continued.

Marker name	EST accession[a]	Primer pair		Polymorphism type	Bin location[b]				Chromosome assignment[c]	Function prediction	
		Sequences	Tm (°C)		2A	2B	2D	Other		Blastn/x	E value
P100	CD373439	F: 5'-TCCTCCGAGTTCCTCAAGTT-3' R: 5'-GAGTAGGTGCTGGGGTCAAA-3'	53	PAGE	C-2AS5-0.78	C-2BS1-0.53	C-2DS1-0.33	-	2H	Unknown protein	-
P102	CD492149	F: 5'-AGACCAGCCAGTCCTAAAAG-3' R: 5'-TACTGTTTGCGGGTAGGG-3'	54	PAGE	C-2AL1-0.85	2BL2-0.36-0.50	C-2DL3-0.49	-	2H,2B	Unknown protein	-
P103	BG607196	F: 5'-TATTGATGAATGCGTAGCCC-3' R: 5'-TAGTCTGCGGCGAGGATT-3'	57	PAGE	C-2AL1-0.85	2BL2-0.36-0.50	C-2DL3-0.49	-	2H	Mei2-like protein [*H. vulgare*]	2e-84
P119	BG263521	F: 5'-CATCCTCCGCAAGTCCCA-3' R: 5'-ACACAGAGCCATGCCATCAA-3'	59	PAGE	C-2AS5-0.78	C-2BS1-0.53	2DS1-0.33-0.47	-	2H	Probable mitogen activated protein kinase [*Oryza sativa*]	5e-86
P26	CD453258	F: 5'-ATCGGCGGGTCAATCCTC-3' R: 5'-CCCAACACTGCCTAGCAATCT-3'	59	SSCP	-	2BS4-0.75-0.84	2DS5-0.47-1.00	-	2H,2A,2B	Unknown protein	-
P34	BE426646	F: 5'-ACCCGATGTATCTGCTGTAA-3' R: 5'-GCCACCATTCTGCCTAAA-3'	52	SSCP	-	2BS1-0.53-0.75	-	4AL13-0.59-0.66	2H,2B	Putative manganese transport protein [*Oryza sativa*]	1e-45
P56	BE403458	F: 5'-CCTTACTATTGCTGGTGTCCGC-3' R: 5'-GGAAAATGTGGAACCTGCGA-3'	60	SSCP	-	C-2BL2-0.36	C-2DL3-0.85-1.00	-	2H,2A	Putative bHLH transcription factor [*A. thaliana*]	0.57
P64	BE591372	F: 5'-CAAATCTGTACTCGGCAATAA-3' R: 5'-TGTAGGTATGCTGCGGTAAT-3'	53	SSCP	2AL1-0.85-1.00	2BL2-0.36-0.50	C-2DL3-0.49	-	2H	Unknown protein	-

[a]The EST listed here is one end of the cDNA clone used as a probe, for example 5'-end EST; [b]Bins onto which the EST is mapped. Data were collected from an EST deletion mapping database (http://wheat.pw.usda.gov/cgi-bin/westsql/map_ locus.cgi;); [c]one or more bands of the PCR products of a certain EST primer are assigned to chromosomes in this study, and the chromosome assignment may not be fully exhibited as bin location data of the EST (or different from that, e.g., P80) for some methodological reasons. [d]P120 was derived from BASI mRNA sequence X16276 rather than EST. Tm = melting temperature; AGE = agarose gel electrophoresis; PAGE = polyacrylamide gel electrophoresis; SSCP = single-strand conformation polymorphism.