

Application of real-time PCR-based SNP detection for mapping of *Net2*, a causal D-genome gene for hybrid necrosis in interspecific crosses between tetraploid wheat and *Aegilops tauschii*

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Available information on genetically assigned molecular markers is not sufficient for efficient construction of a high-density linkage map in wheat. Here, we report on application of high resolution melting (HRM) analysis using a real-time PCR apparatus to develop single nucleotide polymorphism (SNP) markers linked to a hybrid necrosis gene, *Net2*, located on wheat chromosome 2D. Based on genomic information on barley chromosome 2H and wheat expressed sequence tag libraries, we selected wheat cDNA sequences presumed to be located near the *Net2* chromosomal region, and then found SNPs between the parental *Ae. tauschii* accessions of the synthetic wheat mapping population. HRM analysis of the PCR products from F₂ individuals' DNA enabled us to assign 44.4% of the SNP-representing cDNAs to chromosome 2D despite the presence of the A and B genomes. In addition, the designed SNP markers were assigned to chromosome 2D of *Ae. tauschii*. The order of the assigned SNP markers in synthetic hexaploid wheat was confirmed by comparison with the markers in barley and *Ae. tauschii*. Thus, the SNP-genotyping method based on HRM analysis is a useful tool for development of molecular markers at target loci in wheat.

Key words: allopolyploidization, chromosomal synteny, high resolution melting analysis, single nucleotide polymorphism, synthetic hexaploid wheat

Common wheat (*Triticum aestivum* L.) is an allohexaploid species with AABBDD genome, derived through amphidiploidization between cultivated tetraploid wheat *Triticum turgidum* L. (AABB genome) and a wild diploid relative, *Aegilops tauschii* Coss. (DD genome). The common wheat speciation requires natural hybridization of the parental species, avoidance of hybrid breakdown, and formation of unreduced gametes (Matsuoka, 2011). It was recently found that cultivar Langdon (Ldn) of *T. turgidum* subspecies *durum* is available as an AB genome parent for efficient production of hexaploid wheat synthetics (Matsuoka and Nasuda, 2004), allowing us to produce a number of synthetic hexaploid wheat lines with D genomes derived from various accessions of *Ae. tauschii* (Takumi et al., 2009a; Kajimura et al., 2011). In the process of synthetic wheat production, several types of hybrid abnormalities including hybrid necrosis were reported (Nishikawa, 1960; Matsuoka et al., 2007). The abnormal growth pheno-

types observed in triploid hybrids between Ldn and *Ae. tauschii* accessions are divided into four types: two types of hybrid necrosis (type II and type III), hybrid chlorosis, and severe growth abortion (Mizuno et al., 2010a). Unlike type III necrosis, type II necrosis symptoms appear under low temperature conditions (Nishikawa, 1960; Mizuno et al., 2010a). Nishikawa (1962) assumed that complementary genes located on the AB and D genomes, respectively named *Net1* and *Net2*, control type II necrosis. *Net2* was recently confirmed to be located on the short arm of chromosome 2D (Mizuno et al., 2011).

Simple sequence repeat (SSR) markers have been greatly exploited in wheat and assigned to wheat chromosomes (Röder et al., 1998; Somers et al., 2004; Song et al., 2005; Torada et al., 2006). Despite such accumulated efforts, the number of SSR markers reported is not sufficient to identify closely linked markers useful in map-based cloning of *Net2* (Mizuno et al., 2011). To identify molecular markers linked to objective loci, genomic information on barley is widely available (Nasuda et al., 2005). In barley, about 3,000 expression sequence tags (ESTs) were mapped using a single doubled-haploid map-

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ping population (Sato et al., 2009). Recently, the number of wheat EST and full-length cDNA libraries became substantial (Carollo et al., 2005; Mochida et al., 2006, 2009), and a large amount of sequence information has become available to compare with barley EST data. Due to the conserved synteny between wheat and barley chromosomes, the high-density barley map is quite a valuable tool for searching wheat cDNA clones located near the objective SSR markers (Carollo et al., 2005; Mayer et al., 2011). Followed by screening of wheat cDNA sequences, polymorphic sites between the parental accessions of the mapping population are found, mostly as single nucleotide polymorphisms (SNPs) of the coding regions. SNPs are generally available after conversion to EST-PCR markers as cleaved amplified polymorphic sequences (CAPS), derived CAPS (dCAPS) and PCR-based landmark unique gene (PLUG) markers in wheat (Ishikawa et al., 2007), whereas the conversion does not necessarily work well for each EST.

High resolution melting (HRM) analysis is an efficient SNP detection system using real-time PCR apparatus (Wittwer et al., 2003). This technique has been successfully applied to EMS-induced mutant screening by targeting induced local lesions in genomes in common wheat (Dong et al., 2009; Botticella et al., 2011). Therefore, we considered that HRM analysis might help efficiently detect SNPs and construct high-density maps of the wheat genome. Here, we attempted to apply HRM analysis to develop SNP markers around the target chromosomal region using a *Net2*-mapping population of synthetic hexaploid wheat. A research objective of this

study is estimation of the usefulness of HRM analysis for wheat SNP mapping. To achieve our objective for the targeted *Net2* chromosomal region, the efficiency of the SNP mapping was compared between diploid and hexaploid wheat genetic backgrounds.

The F_2 mapping population for *Net2* ($n = 120$) was generated from a cross between wild-type (a synthetic wheat line from a cross between Ldn and *Ae. tauschii* KU-2075) and type II necrosis (a synthetic wheat line from a cross between Ldn and *Ae. tauschii* KU-2025) hexaploid lines (Mizuno et al., 2011). *Net2* was assigned between two SSR markers, *Xbarc102* and *Xgwm515*, on chromosome 2DS (Mizuno et al., 2011). First, 30 barley cDNAs presumably located near the *Net2*-chromosomal region were randomly selected using the high-density cDNA map of barley (Sato et al., 2009). Then, wheat EST libraries of the National BioResource Project (NBRP) KOMUGI web site (<http://www.shigen.nig.ac.jp/wheat/komugi>) were searched for wheat cDNA sequences homologous to the selected barley cDNAs. Gene-specific primers were designed based on the EST sequences, and the target cDNA sequences were partially amplified from total DNA of two *Ae. tauschii* accessions KU-2075 and KU-2025. The nucleotide sequences were determined using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA), and SNPs were found through sequence alignment using GENETYX-MAC version 12.00 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). In total, 15 cDNAs on chromosome 2DS were polymorphic between KU-2075 and KU-2025, and all of the polymorphic sites showed nucleotide substitutions

Table 1. List of barley EST accession numbers for sequencing and development of markers in *Ae. tauschii* orthologs

Barley EST-clone name	Accession number of barley EST	Sequenced length (bp)	Number of SNPs	Marker name in this study
baal13d17	BJ469115	800	2	HRM-1*†
bags20i15	AV917035	800	6	<u>HRM-2*†</u>
baal4a13	AV932776	500	2	HRM-3*, HRM-4*
baal7m13	AV933624	830	5	HRM-5*, <u>HRM-6*†</u>
bags23d08	AV913763	1100	2	HRM-7*†, <u>HRM-8*</u>
basd3h13	AV836281	700	1	HRM-9*†, <u>HRM-10*</u>
bah58e19	BJ481039	450	1	HRM-11
bags39a22	BJ465248	300	1	HRM-12
bags20g23	AV917006	500	15	HRM-13*†, HRM-14*
basd26l01	AV927083	450	13	HRM-15, HRM-16, <u>HRM-17*</u> , HRM-18*
bags21l01	AV913331	500	1	<u>HRM-19*†</u>
basd23f16	AV926678	1200	1	HRM-20*
bags5e16	AV914236	1100	2	HRM-21, HRM-22
bags23d01	AV913757	960	2	<u>HRM-23*†</u>
baal13d11	BJ469112	1200	28	<u>HRM-24*†</u>

* Polymorphic between KU-2075- and KU-2025-derived synthetic wheat.

† Polymorphic between two *Ae. tauschii* accessions, PI476874 and IG47182, and mapped markers.

Underlines indicate the mapped markers in the synthetic wheat genetic map.

Table 2. Primer sequences used for SNP mapping using HRM analysis

Marker name	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product size (bp)
HRM-1	ACTGGCAGGAGGCTTAACATATC	GTGGAGACCGACTTCAGGAC	103
HRM-2	AACTAGACGTGGATTGGGTAGATG	AGATACATCCATTTGAAGAACAAGC	110
HRM-6	TACATGAAGCCTTTGGAAGTTTTT	TCATGTGCAGTTATGTTCTAGTGTTT	120
HRM-7	TTTTGTAGATTAATGTTCTGTGCACTC	TGTTGTATCCTAGTAGCAACTTTTCTG	107
HRM-8	TCGTACATCACAATATCAATCCAAG	AGTCGGTTTAGAAGGGAACTAGC	102
HRM-9	CCTGAGAAGTCAATCCCATCA	GCAGACCATGATCCATCTGA	145
HRM-10	CTGAGAAAGTCAATCCCATCATCTAT	ACTACAAGACCAGTGCCCATTT	112
HRM-13	GTTATCAACATGGCATTAAACTCGT	GGTTTCCATTACAGCAGCATAGTA	96
HRM-17	GAGGTGGGTGTGTCTAAATATCAT	ATACATAGTTCTCACC GCCTAGGTA	111
HRM-19	TGAAAAGGTGAGCTTTCTACTGC	AGAAGGCATGCTAGAAAATATAGAGC	123
HRM-23	ACACTCTTATTGAGGAAGTGCTTTG	GCAGGTGGGAGAAAAAGATTCTA	106
HRM-24	ATGATCCTGCTGGTGAAGATGT	GAGGAAGATGCAGACGGAGTAG	93

(Table 1).

For SNP detection, 24 gene-specific primer sets were designed based on 15 read sequences without any consideration of the homoeologous sequences in the wheat A and B genomes (Tables 1 and 2). According to the manufacturer's manual (Roche, Basel, Switzerland), the amplified product length was about 100 bp, the annealing temperature was 60°C and the polymorphic site was placed in the middle of the amplicon. HRM analysis was performed using a LightCycler 480 Real-Time PCR System II and LightCycler 480 High Resolution Melting Master 2x reaction mix (Roche) according to the manufacturer's instructions. Melting analysis was performed from 65°C to 95°C with a data acquisition of 25 data points per degree Celsius, and melting curves and difference plots were obtained and normalized. Overall, 18 primer sets were polymorphic in the HRM analysis between the KU-2075- and KU-2025-derived synthetic wheat lines; that is, in the presence of A and B genomes from Ldn. The presence of other genomes decreased efficiency of the SNP detection in the D genome, which was probably due to additional recognition of polymorphisms between the A and B genomes and the D genome of allohexaploid wheat. Out of the 18 primer sets, 12 (66.7%) were ultimately available for genotyping of the F₂ individuals (Tables 1 and 2). The remained 6 primer sets failed to be used for the F₂ mapping, which also seemed to be due to insufficient sensitivity of the HRM analysis under the hexaploid wheat background.

In the HRM analysis, the presence of mismatched nucleotides in heteroduplex DNA of heterozygous individuals makes it possible to discriminate them from homozygous individuals (Wittwer et al., 2003). Two of the SNP markers examined in this study easily distinguished the three genotypes (heterozygous and both homozygous types) of the 120 synthetic wheat F₂ individuals using HRM analysis (Fig. 1, A and B). However, for six SNP markers, the melting curves and difference plots of both

homozygous genotypes showed indistinguishable patterns, although the curves and plots of heterozygous individuals were clearly distinct (Fig. 1, C and D). In these cases, addition of *Ae. tauschii* KU-2075 DNA at a final concentration of 5% (v/v) enabled us to clearly discriminate the homozygous alleles (Fig. 1, E and F). The presence of KU-2075 DNA led to heteroduplex formation in homozygous individuals with the KU-2025 genotype and helped shift their melting curves towards the heterozygous ones. In the four remaining markers, the melting curves and difference plots of one homozygous allele and heterozygous individuals showed indistinguishable patterns and were clearly different from those with another homozygous allele. Thus, they were genotyped as a dominant marker.

Genetic mapping was performed using MAPMAKER/EXP version 3.0b software (Lander et al., 1987). The threshold for log-likelihood scores was set at 3.0, and the genetic distances were calculated with the Kosambi function (Kosambi, 1944). Finally, eight of the 12 genotyped SNPs (66.7%) were assigned to the *Net2* chromosomal region in synthetic hexaploid wheat (Fig. 2). The four dominant markers (33.3%) were excluded from the linkage map due to segregation distortion in the F₂ population. Thus, the dominant markers failed to be assigned, whereas the eight successfully mapped markers segregated in a co-dominant manner. Five markers, HRM-10, HRM-17, HRM-19, HRM-23 and HRM-24, developed in this study were assigned within 12 cM of *Net2*, and in particular, HRM-24 was tightly linked to *Net2* (Fig. 2). Two SNP markers, HRM-2 and HRM-6, were assigned near the centromere and a third one on the long arm of chromosome 2D. The order of these markers corresponding to their order on barley chromosome 2H (Sato et al., 2009), indicating the conservation of chromosomal synteny in this region between wheat D genome and barley. Therefore, 44.4% of the SNP-representing cDNAs (8 out of 18) were able to be assigned to chromosome 2D in spite

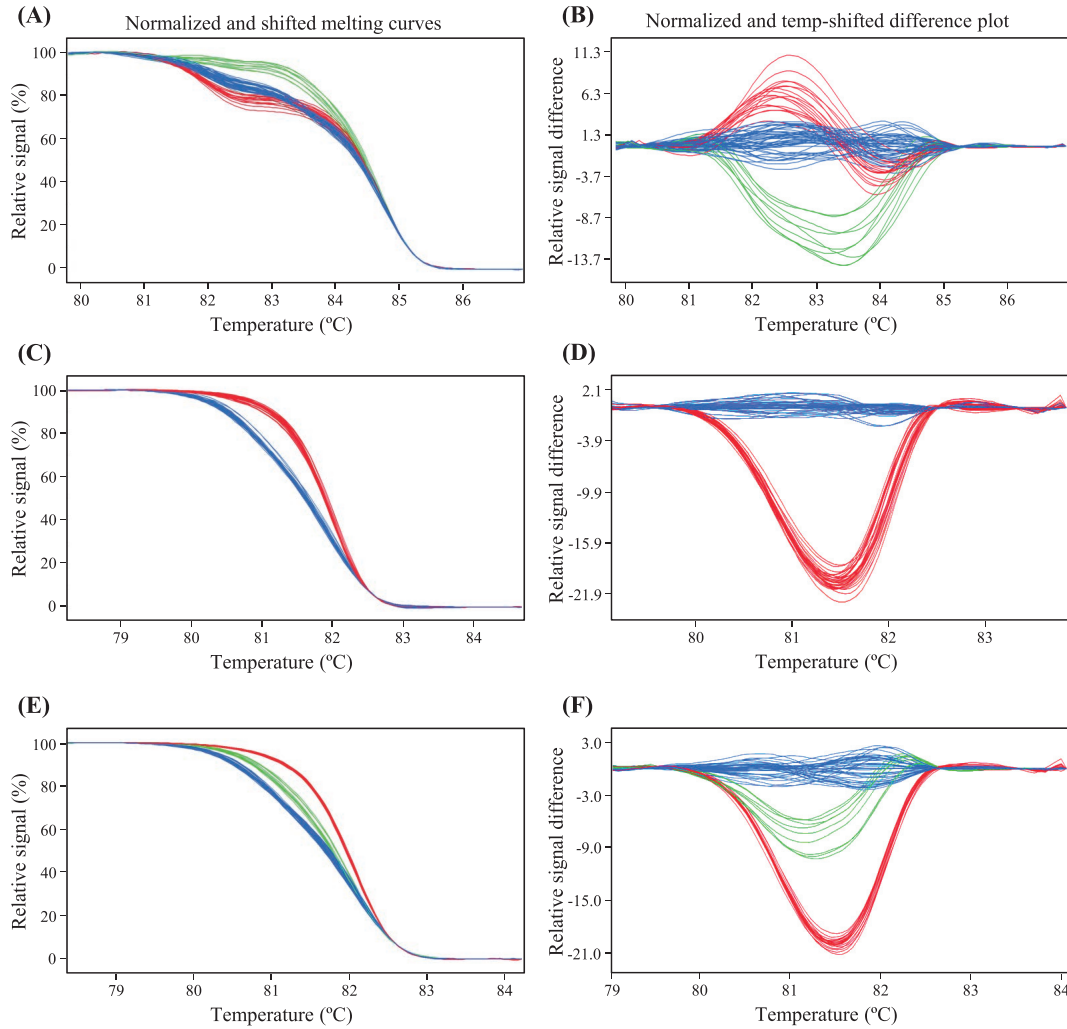


Fig. 1. High resolution melting analysis of PCR products of 120 F_2 individuals from a cross between wild-type (a synthetic wheat line from a cross between Ldn and *Ae. tauschii* KU-2075) and type II necrosis (a synthetic wheat line from a cross between Ldn and *Ae. tauschii* KU-2025) hexaploid lines. (A, B) Segregation pattern of HRM-24 SNP markers. Green, red and blue curves indicate homozygous individuals with the KU-2075 genotype, homozygous individuals with the KU-2025 genotype and heterozygous individuals, respectively. (C, D) Segregation pattern of HRM-2 SNP markers in DNA samples without addition of 5% KU-2075 DNA. Red and blue curves indicate homozygous and heterozygous individuals, respectively. (E, F) Segregation pattern of HRM-2 SNP markers in DNA samples containing 5% KU-2075 DNA. Green, red and blue curves indicate homozygous individuals with the KU-2025 genotype, homozygous individuals with the KU-2075 genotype and heterozygous individuals, respectively. Two typical genotyping patterns, normalized melting curves (A, C, E) and normalized difference plots (B, D, F), are represented.

of the presence of the A and B genomes. These results implied that application of HRM analysis and barley genome information enabled us to generate SNP markers around the *Net2* region on 2DS. *Net2* is widely distributed in *Ae. tauschii* found in eastern habitats (Mizuno et al., 2010a, 2011), including many accessions with agriculturally important traits (Matsuoka et al., 2008; Takumi et al., 2009b). Type II necrosis inhibits the production of synthetic hexaploid wheat lines through interspecific crosses between tetraploid wheat and *Ae. tauschii* (Mizuno et al., 2011), and thus it should be required to avoid type II necrosis via *Net2* for the efficient production of synthetic wheat. To elucidate molecular nature of

Net2, more SNP markers tightly linked to *Net2* will be required.

To study how many markers can be assigned based on HRM analysis of the diploid D genome, polymorphism in the 24 SNP markers designed in this study was confirmed between two *Ae. tauschii* accessions PI476874 and IG47182, which are phylogenetically closer than KU-2025 and KU-2075 (Mizuno et al., 2010b). A linkage map of *Ae. tauschii* containing 93 SSR loci was previously constructed using 104 F_2 individuals between PI476874 and IG47182 (Koyama et al., 2012). Similarly to synthetic hexaploid wheat, SNP screening and genotyping were performed by HRM analysis with the addition of 10 to

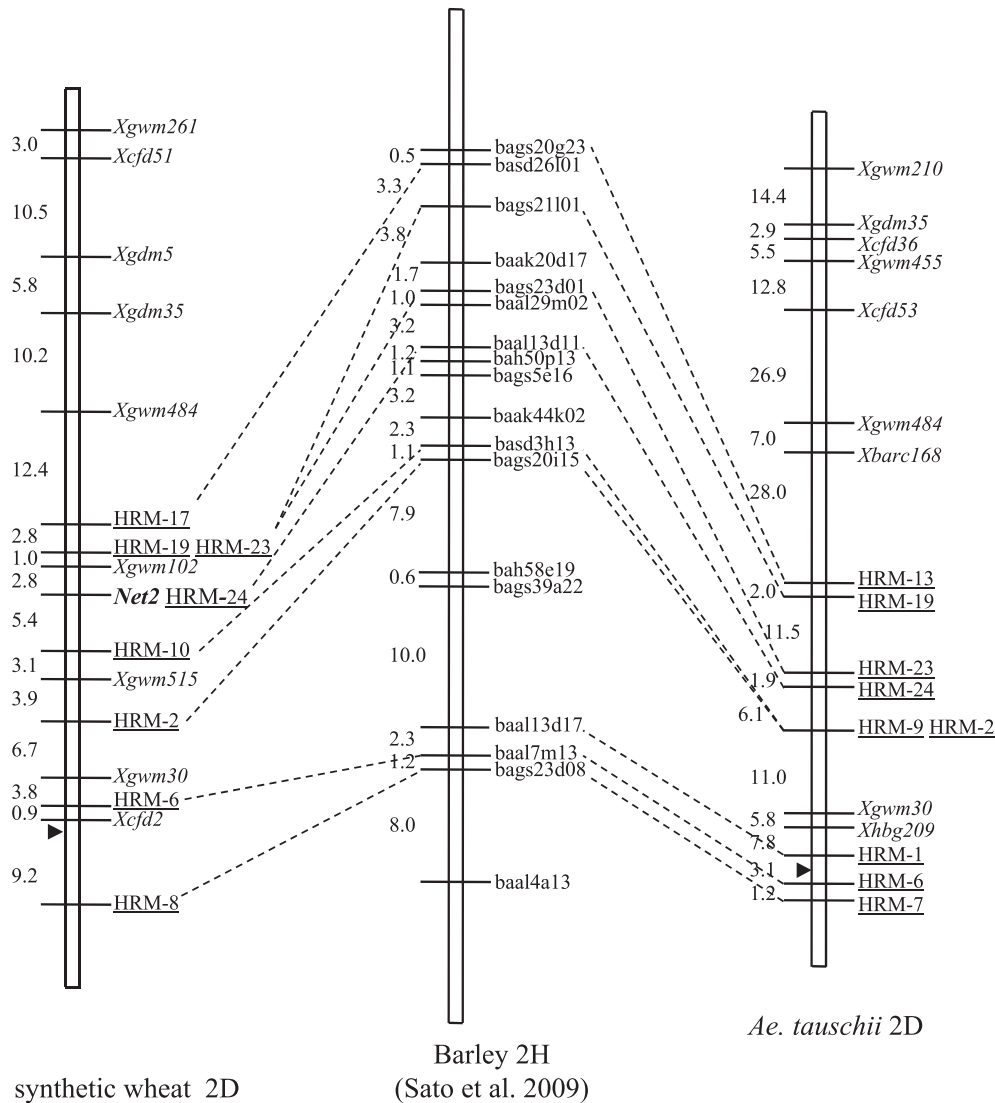


Fig. 2. Comparison of linkage maps for the short arms of group 2 chromosomes among barley, *Ae. tauschii* and synthetic wheat. Genetic distances (cM) are shown on the left and markers are shown on the right. The SNP markers assigned in this study are underlined. Markers derived from orthologous cDNAs in wheat and barley are connected by broken lines. Arrowheads indicate putative positions of the centromeres.

40% PI476874 DNA (v/v) to the template DNA. Nine out of the 24 primer sets (37.5%) showed a polymorphism as a co-dominant marker and were available for genotyping of the 104 F_2 individuals of *Ae. tauschii*. All of the detected SNPs were successfully assigned to chromosome 2D in the same order observed in the barley and synthetic wheat genetic maps (Fig. 2), which implied accurate SNP genotyping via HRM analysis, at least in the D genome of *Ae. tauschii* and synthetic hexaploid wheat.

Our results implied that, although the density of mapped markers was lower in synthetic hexaploid wheat than in its diploid progenitor, genotyping based on HRM analysis was suitable for the hexaploid genetic background. Design of primer sets for the HRM analysis is independent of the recognition sites for restriction

enzymes around the targeted SNPs, which is an advantage of the HRM analysis compared with other SNP-detection systems such as CAPS and dCAPS analyses. Because chromosomal synteny is conserved between barley and wheat, SNP markers between wheat parental accessions for genetic mapping can be developed for target chromosome locations on the barley high-density map. Limited survey sequencing data for wheat genomes through next-generation sequencing is available (Berkman et al., 2011; Vitulo et al., 2011; Lucas et al., 2012), and has proven useful for finding SNPs (You et al., 2011). In addition, recent deep sequencing technologies for wheat mRNA could provide a huge amount of EST and SNP information (Allen et al., 2011). Array-based SNP detection systems are surely as useful for whole-genome geno-

typing in wheat as reported in barley (Close et al., 2009; Sato et al., 2009), but genotyping methods relying on SNPs within a specific chromosomal region might be more effective for generation of target loci-linked markers. The present study showed that HRM analysis using a real-time PCR apparatus recognized SNPs in the D genome of synthetic hexaploid wheat and its diploid progenitor. Moreover, recent high-throughput SNP typing in common wheat was demonstrated by a PCR method based on SNP genotyping called a KASPar assay (KBioscience Ltd, Herts, UK; <http://www.kbioscience.co.jp>), and this method enabled distinguishing genome-specific SNPs in the hexaploid wheat genome (Allen et al., 2011). In conclusion, SNP genotyping based on HRM analysis is a useful tool for development of molecular markers at target loci in wheat.

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