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

Ryusuke Matsuda, Julio C. M. Iehisa and Shigeo Takumi*
Graduate School of Agricultural Science, Kobe University, Rokkodai 1-1, Nada, Kobe 657-8501, Japan

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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-

* Corresponding author. E-mail: takumi@kobe-u.ac.jp

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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 F2 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

| 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              | HRM-2*†                  |
| baal4a13               | AV932776                       | 500                   | 2              | HRM-3*, HRM-4*           |
| baal7m13               | AV933624                       | 830                   | 5              | HRM-5*, HRM-6*†          |
| bags23d08              | AV913763                       | 1100                  | 2              | HRM-7†, HRM-8*           |
| basd3h13               | AV836281                       | 700                   | 1              | HRM-9*, HRM-10*          |
| bah5e19                | BJ481039                       | 450                   | 1              | HRM-11                   |
| bags39a22              | BJ465248                       | 300                   | 1              | HRM-12                   |
| bags20g23              | AV917006                       | 500                   | 15             | HRM-13†, HRM-14*         |
| basd26i01              | AV927083                       | 450                   | 13             | HRM-15, HRM-16, HRM-17*, HRM-18* |
| bags21i01              | AV913331                       | 500                   | 1              | HRM-19†                  |
| basd23f16              | AV926678                       | 1200                  | 1              | HRM-20*                  |
| bags5e16               | AV914236                       | 1100                  | 2              | HRM-21, HRM-22           |
| bags23d01              | AV913757                       | 960                   | 2              | HRM-23†                  |
| baal13d11              | BJ469112                       | 1200                  | 28             | HRM-24†                  |

* 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.
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 Ldon. 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, 2 (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.

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       | ACTGGCAGAGGCTTAAACATAC           | GTGGAGACCCGACTTCAGGAC            | 103              |
| HRM-2       | AACCTAGCCTGGATGTGGGATAGT         | AGATACATCCATTTGGAAGACACGC        | 110              |
| HRM-6       | TACATAGAAGCTTTGGAGGTTTTT         | TCAATGTACAGGTATTCCTCTAGGT        | 120              |
| HRM-7       | TTTTAGCTAATGACCTGTGGGACACT      | TGTTGATCTCCTAGTGGACACTTTACTG     | 107              |
| HRM-8       | TCAGACTACCAATATCAATCACT         | AGTCGTTTTAGAAGGGAACACTGC         | 102              |
| HRM-9       | CCTGAGAAGTCAATCCCTATACCA        | GCGAGACCAGTCATCGTC             | 145              |
| HRM-10      | CTGAGAAGTCAATCCCATCTATAT       | ACTACAGAACCAGTGCCCCATTT         | 112              |
| HRM-13      | GTTATCAACATGGCATTTAAGACTCGT    | GGTCCATATTAGACACGTCAACGTA       | 96               |
| HRM-17      | GAGGTTGGTTGTGCTTCAATATCAT       | ATACATAGTCTCTCCGCTCAGTTGA       | 111              |
| HRM-19      | TGAAGAGGCTGATTTCTACTGTG        | AGAAGGCTATCTAGAAGGATAGAC        | 123              |
| HRM-23      | AACTCTATTAGGAAGGATGTCTTTT       | GCGAGTTGGAGGAGAAAAATTCTCA        | 106              |
| HRM-24      | ATGATCCCTATGGTGAAGATGCTG       | GAGGAAATGACAGCGAGG             | 93               |

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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...
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 F2 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
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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 F2 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-

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.
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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