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ORIGINAL PAPER

Mapping diploid wheat homologues of *Arabidopsis* seed ABA signaling genes and QTLs for seed dormancy

Shingo Nakamura · Takao Komatsuda · Hideho Miura

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Abstract Abscisic acid (ABA) sensitivity in embryos is one of the key factors in the seed dormancy of wheat. Many ABA signaling genes have been isolated in *Arabidopsis*, while only a few wheat homologues have been identified. In the present study, diploid wheat homologues to *Arabidopsis* ABA signaling genes were identified by *in silico* analysis, and mapped them using a population of diploid wheat recombinant inbred lines derived from a cross between *Triticum monococcum* (Tm) and *T. boeoticum* (Tb). Four diploid wheat homologues, *TmVP1*, *TmABF*, *TmABI8* and *TmERA1* were located on chromosome $3A^m$ and *TmERA3* was on the centromere region of chromosome $5A^m$. In two consecutive year trials, one major QTL on the long arm of $5A^m$, two minor QTLs on the long arm of $3A^m$ and one minor QTL on the long arm of $4A^m$ were detected. The $5A^m$ QTL explained 20–27% of the phenotypic variations and the other three QTLs each accounted for approximately 10% of the phenotypic variations. Map positions of the loci of *TmABF* and *TmABI8* matched the LOD peaks of the two QTLs on $3A^m$, indicating that these two homologues are possible candidate genes for seed dormancy QTLs. Moreover, we have found two SNPs result in amino acid substitutions in *TmABF* between Tb and Tm. Comparison of the marker positions of QTLs for seed dormancy of barley revealed that the largest QTL on $5A^m$ may be orthologous to the barley seed dormancy QTL, SD1, whereas there seems no orthologous QTL to the corresponding barley SD2 locus.

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S. Nakamura (✉)
Wheat and Barley Biotechnology Research Team,
National Institute of Crop Science, Tsukuba,
305-8518 Ibaraki, Japan
e-mail: shingo@affrc.go.jp

T. Komatsuda
Plant Genome Research Unit,
National Institute of Agrobiological Sciences,
Tsukuba, 305-8602 Ibaraki, Japan

H. Miura
Department of Crop Science,
Obihiro University of Agriculture and Veterinary Medicine,
Obihiro, 080-8555 Hokkaido, Japan

Introduction

Seed dormancy is an important trait of the wheat breeding program in order to prevent pre-harvest sprouting (PHS), which has detrimental effects on the quality of wheat flour. For malting barley, a moderate level of seed dormancy is desirable because efficient germination of the grain is required in order to simultaneously accelerate the production of high levels of enzyme activity. However, it is difficult for wheat and barley breeders to manipulate seed dormancy in practical breeding programs due to complex inheritance and large environmental effects. Significant efforts have been made to detect quantitative trait loci (QTLs) in hexaploid wheat and barley. Although QTLs for seed dormancy have been frequently found in similar chromosomal locations in various crosses of hexaploid wheat and barley, suggesting similar alleles controlling seed dormancy, there are no QTLs for which the function and mechanism are known.

On the other hand, physiological studies have shown that the sensitivity of the plant hormone, abscisic acid (ABA) in

embryos is one of the key factors for seed dormancy of wheat and barley (Walker-Simmons 1987; Romagosa et al. 2001). The mechanism of ABA sensitivity in seeds has mainly been studied in *Arabidopsis*. To date, molecular genetic studies on ABA signal transduction in *Arabidopsis* have found a number of genes, among which ABA-insensitive (ABI) or enhanced response to ABA (ERA) *Arabidopsis* mutants have revealed 6 ABI and 2 ERA genes that function in seed ABA signal transduction pathways (Finkelstein et al. 2002). For example, *ABI3* encodes a B3-type transcription factor that plays an important role in the acquisition of seed dormancy (Giraudat et al. 1992). This is also an orthologous gene to maize *Viviparous 1* (*VP1*) (McCarty 1995). *ABI5* encodes a bZIP transcription factor (Finkelstein and Lynch 2000), which is involved in regulation of seed maturation and germination (Finkelstein and Lynch 2000; Carles et al. 2002), and retardation of shoot growth (Lopez-Molina et al. 2002; Bensmihen et al. 2004). The expression of *ABI3* and *ABI5* is cross-regulated in ABA signaling of seeds (Söderman et al. 2000). *ABI8* encodes a protein of unknown function that is also involved in the inhibition of germination, but functions in a separate pathway from *ABI3* and *ABI5* (Brocard-Gifford et al. 2004). *ERA1* encodes a beta-subunit of protein farnesyl transferase (Culter et al. 1996). *ERA3* is also known as *EIN2*, which functions in the ethylene signaling pathway, encoding a membrane protein that shows similarity to the Nramp family of metal ion transporters (Ghassemian et al. 2000; Alonso et al. 2000). Mutants of *ERA1* and *ERA3* show hypersensitivity to ABA during seed germination and have high levels of seed dormancy; therefore, it is of interest to examine whether wheat homologues of these *Arabidopsis* genes are involved in seed dormancy of wheat.

Until now, however, only a few wheat homologues have been identified. *TaVP1* and *TaABF* have been identified as homologues of *ABI3* (Nakamura and Toyama 2001; McKibbin et al. 2002) and *ABI5* (Johnson et al. 2002), respectively. Wheat homologues corresponding to the rest of the *Arabidopsis* ABA signaling genes have not been examined. Recent completions of genome sequencing of *Arabidopsis* (Initiative 2000) and rice (IRGSP 2005) have made it possible to identify homologues between the two species by database search. In addition, the number of registered wheat ESTs in the public database has risen to almost a million (http://www.ncbi.nlm.nih.gov/UniGene). These advances facilitate the identification of wheat homologues of *Arabidopsis* ABA signaling genes by *in silico* analysis.

The A^m-genome of diploid wheat (*Triticum monococcum* L.) has the same origin as the A-genome of hexaploid, on which several seed dormancy QTLs have been identified (Zanetti et al. 2000; Kato et al. 2001; Groos et al. 2002; Osa et al. 2003; Kulwal et al. 2004; Mares et al. 2005; Mori et al. 2005; Torada et al. 2005). The homoeologous linkage groups between *T. monococcum* and barley are remarkably conserved (Dubcovsky et al. 1996). In addition, compared to hexaploid wheat, diploid wheat has a great advantage for genetic mapping because of its high levels of polymorphism, especially between domesticated *T. monococcum* and its wild-type *T. boeoticum* (Liu and Tsunewaki 1991; Castagna et al. 1994; Le Corre and Bernard 1995; Dubcovsky et al. 1996). In addition, wild-type *T. boeoticum* shows a higher level of seed dormancy than *T. monococcum*. Taking this advantage into account, we could more effectively map wheat genes which are potentially related to seed dormancy.

The aims of the present study are the identification of diploid wheat homologues of *Arabidopsis* ABA signaling genes, and mapping them on diploid wheat genetic maps for comparison of the map positions with those of QTLs for seed dormancy to genetically identify possible candidate genes. Positional comparison of diploid wheat QTLs with previously reported QTLs for seed dormancy in barley was also carried out to identify orthologous QTLs.

Materials and methods

Mapping population

A diploid mapping population of recombinant inbred lines (115 RILs), derived from a cross, *Triticum monococcum* L. (Accession KT3-5) × *T. boeoticum* L. Boiss (Accession KT1-1) was used in the present study. This population was generated at the Kihara Institute of Biology, Yokohama City University, Japan using the single-seed descent method. The original genotyping data of the diploid wheat population is available on the website http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp.

Identification of wheat ESTs

In the first step, corresponding rice homologues of *Arabidopsis* ABA signaling genes were identified by Basic Local Alignment Search Tool (BLAST) analyses (Altschul et al. 1990) at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) using the full-length amino acid sequence of *Arabidopsis* genes *ABI1* (At4g26080), *ABI5* (At2g36270), *ABI8* (At3g08550), *ERA1* (At5g40280), and *ERA3* (At5g03280). Rice homologues were identified as *OsABI1* (AP003332), *OsABI5* (AP003287), *OsABI8* (AP002481), *OsERA1* (AP003218) and *OsERA3* (XP469414). The amino acid sequence identities of the predicted rice proteins to those of *Arabidopsis* were 49% for *ABI1*, 39% for *ABI5*, 57% for *ABI8*, 45% for *ERA1* and 35% for *ERA3*. Subsequently, wheat ESTs showing high similarity to those of

rice genes were identified by BLAST search as described above.

Isolation of cDNA and genomic DNA sequences

Full or partial wheat cDNA sequences were isolated by PCR from a cDNA library constructed from mature embryos of a wheat variety, Minaminokomugi, as described previously (Nakamura and Toyama 2001). Diploid wheat cDNA sequences were isolated by PCR from first strand cDNA synthesized from total RNA extracted from mature embryos of Tb and Tm. The total RNA was extracted using Trizol reagents (Invitrogen, USA) according to the manufacturers' manual. The first strand cDNA synthesis was carried out using SuperScript First-Strand Synthesis System (Invitrogen, USA). Genomic DNA was isolated from leaves using DNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturer's protocol. Full or partial genomic DNA sequences were isolated by PCR. The PCR primers for isolation of the cDNA and genomic DNA sequences are summarized in Table 1. The isolated sequences were determined by 3100 Avant Genetic Analyzer using Bigdye version 3.0 terminator reagents (Applied Biosystems, USA). The determined sequences were analyzed by DNASIS Pro sequence analysis software version 2.0 (Hitachi Software, Japan) and Sequencher version 4.1 (Gene Codes Corporation, USA). Amino acid sequence alignments were constructed by ClustalW ver.1.83 (Thompson et al. 1994) at the website of the DNA databank of Japan (http://www.ddbj.nig.ac.jp/) and the results are presented using BOXSHADE (http://www.ch.embnet.org/). Phylogenetic trees were drawn by Tree View (Page 1996).

Table 1 Primers for isolation of cDNA and genomic DNA

Primer name	Primer sequence (5′→3′)
T7	GTAATACGACTCACTATAGGGC
T3	AATTAACCCTCACTAAAGGG
TaABI1-F1	GGAGGAGATGGAGGACGTG
TaABI1-R1	CAAGCAYTCAGCAGCTGCTT
TmABI1-F1	GGAGGAGATGGAGGACGTG
TmABI1-R1	TCTCGGGTGCTACAGGATCT
TmABI1-F2	AATGGGCATGGTAGATCCTG
TmABI1-R2	TCTGGAGAGCAAGCTTCGAC
TmVP1-F1	GGCGCTTGTCGTGCCTGCAG
TmVP1-R1	TCAGATGCTCACSGCCATCTGG
TaABF-F1	ATGGCATCGGAGATGAGCAA
TaABF-R1	CTTTCAGACGAGCGTTCTCC
TmABF-R1	ACTAGCACGGCTATGGAAGT
TmABF-R2	TCACCAGATGCAGCTGCCGC
TaABI8-F1	TCCACCAACCTGCCACCGACT
TaABI8-R1	TCATGACAATGCGCTTTCCATGA
TmABI8-F1	GACACCGCTATAACCCCCAA
TmABI8-R1	TGTGAAACACCAATGACCTTGTG
TmABI8-R2	CAACCCCAGCCCTATCTGTA
TmERA1-F1	TGACCTCGAGAACGATATTGT
TmERA1-R1	AGCACCTGATTTGTCCTTCATT
TmERA3-F1	CTGGATCTTTCCCTTGTGGA
TmERA3-R1	AAGATGGCAGCTGAAGTGGT

Construction of genetic maps including diploid wheat homologues

The cleaved-length amplified polymorphic sequence (CAPS) and the degenerated cleaved-length polymorphism (dCAPS) methods were used for genotyping. Single nucleotide polymorphisms (SNPs) were identified by sequencing each parent's gene. dCAPS primers were designed for SNPs by using dCAPS finder 2.0 available on the website http://www.helix.wustl.edu/dcaps/dcaps.html (Neff et al. 2002). Primer sequences, restriction enzymes and SNP locations are summarized in Table 2. The PCR condition for dCAPS markers was 1 cycle of 94°C for 1 min and 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 15 s. Genetic maps were constructed by JoinMap, version 3.0 (Biometris, Wageningen, The Netherlands, http://www.joinmap.nl) with Kosambi function (Kosambi 1944). The chromosomal locations of the rice homologues were determined using the data on the IRGSP website http://www.rgp.dna.affrc.go.jp/IRGSP/index.html.

SNP markers closely linked to barley SD1 and SD2 loci

Wheat ESTs were searched with the barley STS sequence (Accession number BQ548063) of the *ABC302* marker that is closely linked to the SD1 locus (Han et al. 1999). A wheat EST (Accession number CJ580018) was found to show the highest score with 95% identity as a nucleotide sequence with the barley sequence. We made a pair of primers for the CAPS method on the basis of the EST sequence for amplification of the diploid wheat sequences, as shown in Table 3. The amplified sequences from Tm and Tb genomic DNA have a SNP that makes an additional *Afa*I restriction site in Tm. The SNP was used to genotype RILs and map the *ABC302* marker on diploid wheat.

Li et al. (2004) reported synteny between the barley 5HL terminal region where SD2 QTL resides and the rice chromosome 3 telomere region. Rice genes LOC Os03g63720 and LOC Os03g64210 were located at this region. We found barley ESTs (CA031190 and BQ766221) corresponding to rice genes by BLAST and SNPs between the parent lines Azumamugi and Kanto Nakate Gold. Using SNPs we made CAPS and dCAPS markers designated as *Hv5E15* for LOC Os03g63720 and as *Hv5E16* for LOC

Table 2 dCAPS markers for mapping the diploid wheat homologues

Marker name	SNP/position (Tm→Tb)	Accession number	Primer sequence (5′→3′)	Fragment size (bp)	Restriction enzyme
TmVP1	A→G/1272	AB239678	TGATCACTGCATCCCTGTGT	81	*Bam*HI
			TTCCCATCATATGTATTCATA**G**GA**T**		
TmABF	T→C/1668	AB238933	TATAGTAATTTACTAGTAGTACCT	73	*Hha*I
			TTCACTTGCTTGACCAGATAA**G**C		
TmABI8	A→G/815	AB239132	GATTATAGCGACTGCAAGTTTCT	86	*Hha*I
			TGGCTCATTGGTGCACGGAT**G**C		
TmERA1	G→T/755	AB239133	CCTAGGCTACTTGGCATGCTCA**T**A	124	*Nde*I
			AGACAAATACCAAACACACAGATACA		
TmERA3	C→T/508	AB239134	GGAAATGTATCCGTGGCGAA	80	*Nde*I
			AAAGAAATCCTATGTACCGAA**C**A**T**A		

Bold letters indicate altered sequences to create restriction endonuclease recoginition sites

Table 3 Markers linked to the barley SD1 and SD2 loci

Marker name	Primer sequence (5′→3′)	Fragment size (approx. bp)	Restriction enzyme
MWG813	TCAAGCTGCTGGAGAAGCAC	300	*AfaI*
	GCAACACTTTCAAACGACCA		
Hv5E15	ATTTTGGACACAAGCAAGGC	1,000	*XhoI*
	CGGCAAGACCCCTCTCTATA		
Hv5E16	TGGCTTCGTTCTTGTTCTCA	140	*TaqI*
	CGTAAGAGATAGGAAAAACAGGTCG		
ABC302	CTGGGGCTTTCGATGCTGT	1,000	*Afa*I
	CTGAGTCTTCGCCATGCATA		
5E15	ATTTTGGACACAAGCAAGGC	850	*Afa*I
	CGGCAAGACCCCTCTCTATA		
5E16	GCTCTCCTTCAGGACATTGC	700	*Xsp*I
	TCACAGCAACTCCACCAGAG		

Os03g64210. We mapped them together with the *MWG813* marker as a CAPS marker using 99 RILs derived from a cross, Azumamugi × Kanto Nakate Gold (Mano et al. 2001). The original map data are available on the website GrainGenes (http://www.wheat.pw.usda.gov/GG2/index.shtml). The primers are summarized in Table 3. Wheat ESTs corresponding to these rice and barley genes were searched by BLAST, and a corresponding wheat EST (Accession number BI479160) for rice LOC Os03g63720 and a wheat EST (BF483796) for rice LOC Os03g64210 were found. Then we made a pair of primers for each EST on the basis of the EST sequences for amplification of the diploid wheat sequences, as shown in Table 3. The amplified sequences from Tm and Tb genomic DNA using each pair of primers have SNPs that made extra *Afa*I and *Xsp*I restriction sites, respectively, in Tb. Using the SNPs we made CAPS markers and tentatively designated as *5E15* and *5E16* (Table 3).

Evaluation of seed dormancy

The parental *T. monococcum* L. (Tm) and *T. boeoticum* L. (Tb) are spring and winter wheat, respectively. Due to allelism between Tm and Tb at the *Vrn-A^{m}1* and *Vrn-A^{m}2* loci on chromosome 5A^m (Shindo et al. 2002), approximately half of the RILs developed between the parents required vernalization for heading. Seed dormancy was evaluated by growing RILs in a controlled environment in two years, 2003 and 2004. In order to promote heading, the winter-type RILs and parental Tb were pregerminated and then vernalized at 3.5°C for five weeks. Subsequently, the spring-type RILs and parental Tm were planted, and all RILs were grown in a greenhouse. After flowering, they were transferred to growth chambers (day/night temperature; 22/16°C, with 16 h day length). They were harvested at 40 days′ post-anthesis to coincide with physical maturity, and air-dried for one week.

The degree of seed dormancy for each RIL was evaluated by duplicate germination tests using 30 seeds per RIL. The seeds were sown on filter paper wetted with distilled water in 9-cm Petri dishes. The dishes were incubated in the dark at 20°C. The number of germinated seeds was counted at daily intervals for 5 days and expressed as a weighted germination index (Walker-Simmons 1988). This index gives the maximum weight of seeds that germinate rapidly and is calculated from the following formula:

Germination Index (GI) = (5 × N1 + 4 × N2 + 3 × N3 + 2 × N4 + N5)/(total days of test × total seeds), where N1 to N5 are the number of seeds that germinated from day 1 to 5. The maximum index is 1.0 if all seeds germinate by day 1 whilst lower indices are indicative of an increasing level of seed dormancy or reduced germinability. The results are presented as the mean germination index of two replicates.

QTLs for seed dormancy were determined in the diploid wheat mapping population by the simple interval mapping method using the MapQTL 5 (Biometris, Wageningen, The Netherlands, http://www.joinmap.nl). A log-likelihood (LOD) score threshold of 2.0 was used to identify regions containing putative loci associated with seed dormancy.

Results

Identification of wheat ESTs homologous to *Arabidopsis* ABA signaling genes

One of the highest sequence similarities to *OsABI1* was found with wheat EST (BE445496), which has 84% nucleotide sequence identity. *TaABF* (AF519803) has the highest sequence similarity to *OsABI5* showing 71% nucleotide sequence identity. For *OsABI8*, three wheat EST clones (BM137049, BQ752685 and CD893289) were identified that show 89, 83 and 68% nucleotide sequence identities to rice homologues, respectively. For *OsERA1*, four wheat ESTs (BJ269074, BQ788955, CK155782 and BJ319195) showed identities of 74, 79, 63 and 72%. For *OsERA3*, two wheat EST sequences (AJ612302 and BQ842061) showed identities of 71 and 54%. Using these identified EST sequences, PCR primers were designed to isolate 5′ or 3′ cDNA and genomic DNA fragments (Table 1).

Identification of diploid wheat homologues of *Arabidopsis* ABA signaling genes

TmABI1, a diploid wheat homologue of *ABI1*: A gene-specific TaABI1-R1 primer was designed based on the sequence of EST BE445496. Using this primer and T7 primer specific to T7 promoter on the vectors of a cDNA library, a cDNA fragment was amplified by PCR from the cDNA library. It was confirmed by sequence analysis that the amplified cDNA fragment was a 5′ region of *TaABI1* that includes the start codon. The full-length *TaABI1* sequence (Accession number AB238930) was isolated from the cDNA library by PCR using TaABI1-F2 primer that includes the sequence for the start codon and T3 primer that corresponds to the T3 promoter on the vector. The length of the obtained cDNA sequence was 1440 bp encoding for 479 amino acid residues. The deduced amino acid sequence of *TaABI1* shows 82% identity to the deduced sequence of *OsABI1*. Subsequently, the genomic DNA fragments (Accession number: AB238931) of the gene were also isolated from Tm by PCR using two pairs of primers (TmABI1-F1 and TmABI1-R1) and (TmABI1-F2 and TmABI1-R2). The two sequences were determined, and were combined to complete the genomic sequence of *TmABI1*. Comparison of the genomic sequences and cDNA sequences revealed 4 exons and 3 introns spanning 4,044 bp. The locations of the introns were well conserved among *Arabidopsis*, rice and wheat.

According to Schweghofer et al. (2004), *Arabidopsis* protein phosphatase 2C (PP2C) genes related to ABA signaling are classified to Group A, which consists of nine members. We examined the phylogenetic relation between *TmABI1*, *TaABI1, OsABI1* and nine PP2C genes. This analysis showed that *TmABI1*, *TaABI1* and *OsABI1* were related to *ABI1*, but were more closely related to *HAB1* and *HAB2* (Saez et al. 2004) (supplemental data Fig. 6A). This result suggested that *TmABI1* was isolated as a diploid wheat *ABI1* homologue, but its true *Arabidopsis* homologues are *HAB1* and *HAB2*.

TmVP1, a diploid wheat homologue of *ABI3*, was isolated based on the previously reported *TaVP1* information (Nakamura and Toyama 2001; McKibbin et al. 2002). We cloned diploid wheat genomic DNA fragments corresponding to *TaVP1* amplified by PCR using TmVP1-F1 and TmVP1-R1 primers and sequenced them.

TmABF, a diploid wheat homologue of *ABI5*. Blast search revealed that *TaABF* has the highest similarity to *OsABI5*. Therefore, TaABF-R1 primer was designed based on the sequence of *TaABF* to isolate the *ABI5* wheat homologue from the cDNA library. A 5′ partial fragment of *TaABF* was amplified by PCR using this primer and T7 primer. Full-length *TaABF* (Accession number AB238932) was isolated from the cDNA library by PCR using TaABF-F1 primer and T3 primer. Johnson et al. (2002) reported two forms of *TaABF*, *TaABFA* and *TaABFB*. Their amino acid sequence identity is 95%, although the sequence of *TaABFA* is incomplete at the 5′ end. The amino acid sequence of full-length *TaABF* cloned in this study is completely identical to *TaABFA*, and shows 67% amino acid sequence identity to *OsABI5*. *TmABF* genomic DNA sequences from Tm (Accession number AB238933) were

isolated by PCR using TaABF-F1 and TmABF-R1 primers and determined. Comparison of genomic DNA sequences and cDNA sequences revealed 4 exons and 3 introns spanning 1,541 bp for *TmABF*. The locations of introns were identical among *Arabidopsis*, rice and wheat.

Arabidopsis bZip transcription factors related to ABA signaling are classified into Group A, which is composed of seven members (Jakoby et al. 2002). Phylogenetic analysis showed that *TmABF*, *TaABF* and *OsABI5* are more closely related to *ABI5* among the members of Group A (supplemental data Fig. 6B).

TmABI8, a diploid wheat homologue of *ABI8*. The deduced amino acid sequences from ESTs BQ752685 and CD893289 indicate that they include initiation and stop codons; therefore, TaABI8-F1 and TaABI8-R1 primers corresponding to these regions were designed and used to isolate full-length wheat *ABI8* homologue (*TaABI8*) from Zenkoujikomugi embryo total RNA. The isolated sequence (Accession number AB238935) is 1,620 bp encoding 539 amino acid residues. The amino acid sequence identity between *OsABI8* and *TaABI8* is 76%. Based on this sequence information, the Tm partial genomic DNA sequence including an intron (Accession number AB239132) was isolated by PCR using TmABI8-F1 and TmABI8-R1 primers and determined. The location of the intron is identical to the predicted location in *OsABI8*.

TmERA1, a diploid wheat homologue of *ERA1*. Three wheat ESTs (BJ269074, BJ319195 and CK155782) make a contig that constitutes the full-length wheat *ERA1* homologue (*TaERA1*). The identity between deduced amino acid sequences from *OsERA1* and *TaERA1* is 69%. A partial genomic DNA sequence spanning three introns (Accession number AB239133) was isolated from Tm by PCR using TmERA1-F1 and TmERA1-R1 primers and determined. The locations of the introns were consistent with the predicted locations in *OsERA1*.

TmERA3, a diploid wheat homologue of *ERA3*. The predicted amino acid sequence from EST AJ612302 is 67% identical to the corresponding amino acid sequence of *OsERA3*. TmERA3-F1 and TmERA3-R1 primers were designed around a presumed intron as predicted based on the rice sequence. The isolated genomic DNA sequence (Accession number AB239134) contains the predicted intron.

Alignments of amino acid sequences of *TmABI1*, *TmVP1*, *TmABF*, *TmABI8*, *TmERA1* and *TmERA3* with those of the corresponding *Arabidopsis*, rice and wheat ESTs or isolated homologues are shown as supplemental data (Fig. 5).

Genetic mapping of the diploid wheat homologues and markers closely linked to barley SD1 and SD2 loci.

For each of the examined diploid wheat homologues, at least one SNP was identified between parental Tm and Tb except for *TmABI1*. The descriptions of the SNPs are summarized in Table 2. All SNPs in Table 2 were located in intron or 3′ untranslated sequences.

In order to map the genes, dCAPS markers were designed for the SNPs (Table 2). The dCAPS products run on agarose gels are shown in Fig. 7 (supplemental data).

Using these dCAPS markers, the homologues were mapped on diploid wheat. Four homologues, *TmABI8, TmERA1, TmABF* and *TmVP1* were mapped at 22, 29, 44, 56 cM from the top of the short arm of $3A^m$ and *TmERA3* was mapped at 9 cM from the top of the short arm of $5A^m$. The gene orders of the rice homologues on rice chromosome 1 and the wheat homologues on $3A^m$ were identical (Fig. 1), indicating a highly conserved synteny relationship between rice chromosome 1 and $3A^m$L.

The *ABC302* marker (Table 3) was mapped on the diploid wheat linkage map to determine the diploid wheat synteny locus for the barley QTL SD1. The *ABC302* marker was located 20 cM from the top of $5A^m$ (Fig. 1).

As expected from the synteny relation, *Hv5E15* and *Hv5E16* markers were mapped on the terminal region of 5HL (Fig. 1). They are located 0 and 1 cM from the *MWG813* marker (Fig. 1). Subsequently, the two corresponding diploid wheat CAPS markers *5E15* and *5E16* (Table 3) were mapped on the diploid wheat linkage map to locate the diploid wheat synteny region of the barley QTL SD2. The *5E15* and *5E16* were mapped at 66 cM and 78 cM from the top of $4A^m$, respectively (Fig. 1). The CAPS products run on agarose gels are also shown in Fig. 7 (supplemental data).

Mapping QTLs for seed dormancy

There was a remarkable difference in dormancy between parental Tm and Tb. Tm had little or no seed dormancy as its GI was almost 1.0 in both 2003 and 2004 trials, while Tb showed high dormancy as its GI was less than 0.1 (Fig. 2). The distributions of GI for RILs ranged from 0.087 to 1.0 in 2003 and from 0.007 to 1.0 in 2004. Around half and one quarter of RILs gave a GI of more than 0.9 in 2003 and 2004 trials, respectively (Fig. 2).

Four QTLs for seed dormancy which showed more than 2.0 of LOD scores were consistently detected in two consecutive year trials (Table 4). A relatively major QTL was mapped to the long arm of $5A^m$. The RFLP markers *Xcdo1326c* and *ABC302* flanked this QTL, within a 10-cM interval (Fig. 3c). This major QTL on 5AL explained approximately 20–27% of the phenotypic variation and the Tb allele contributed to higher dormancy. Two minor QTLs were identified on chromosome $3A^m$L (Fig 3a), one in the *TmABI8* region and another in the *TmABF* region. As shown in Fig. 3b, a minor QTL was mapped at the locus of *Xrz141* on $4A^m$, These three minor QTLs accounted for

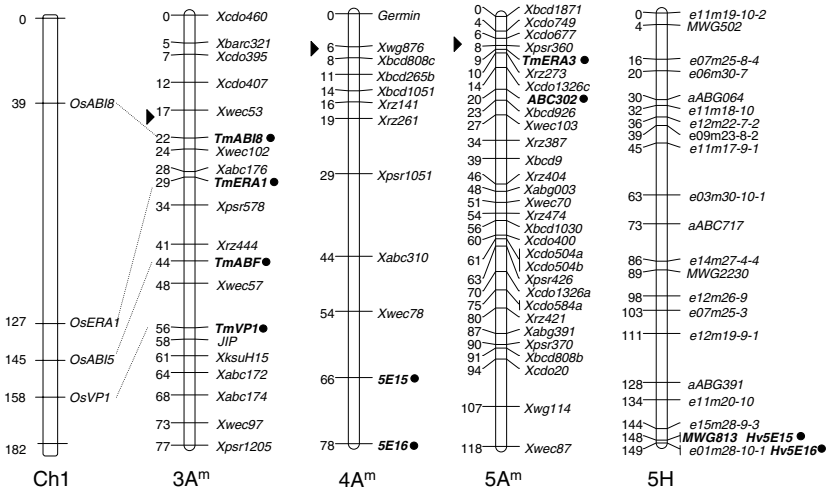

Fig. 1 Genetic maps of ABA signaling homologues of diploid wheat, and the markers corresponding to SD1 and SD2 loci. The marker order is shown on the *right*, while genetic distances (centiMorgan (*cM*) scale) are on the *left*. The approximate positions of centromeres are indicated by *closed triangles*. Mapped genes are indicated by *bold letters* and *attached closed circles*. To facilitate the comparison of synteny relations, rice chromosome 1 (*Ch1*) is shown with corresponding rice genes according to the IRGSP website (http://www.rgp.dna.affrc.go.jp/IRGSP/index.html)

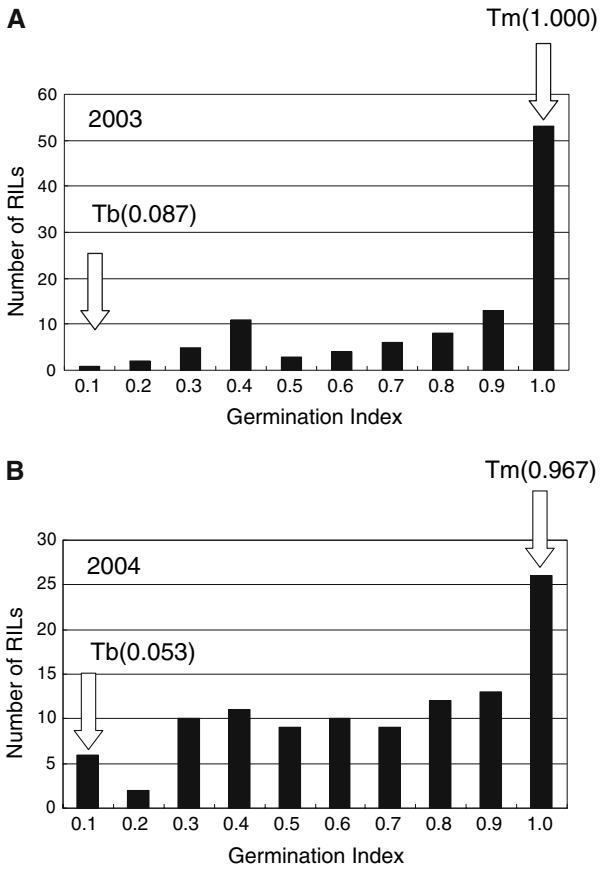

Fig. 2 Frequency distribution of the number of RILs for seed germination index (GI): **a** 2003, **b** 2004. GI for 109 and 108 RILs were measured in 2003 and 2004, respectively

about 10% of the phenotypic variation, respectively, and again the Tb allele at each locus was responsible for increasing seed dormancy.

Identification of SNPs in *TmABF* and *TmABI8* between Tb and Tm

TmABF and *TmABI8* cDNA sequences (each Accession number AB286054 and AB279762) were isolated by PCR using TaABF-F1 and TmABF-R2 primers, and TaABI8-F1 and TmABI8-R2 primers (Table 1), respectively, and determined. From comparison of the sequences between Tb and Tm, we have identified two SNPs in *TmABF* cDNA sequences. They change the 266th nucleic acid base cytosin (C) in Tm to thymine (T) in Tb, and the 283th guanine (G) in Tm to C in Tb, as shown in Fig. 4a. These SNPs were also found in the genomic sequences of *TmABF*. The SNPs result in amino acid substitutions of the 89th alanine (A) in Tm to valine (V) in Tb, and the 95th glycine (G) in Tm to arginine (R) in Tb (Fig. 4b). On the other hand, no SNPs have been found in the coding sequences of *TmABI8*.

Discussion

Recent progress of the increasing number of wheat ESTs registered in the public database and the accomplishment of entire sequences of *Arabidopsis* and rice genomes enables us to more systematically identify wheat homologues to

Table 4 LOD scores and explained phenotypic variations (R^2) at the QTLs for seed dormancy

Marker interval	Chromosome	Year	LOD score	R^2
TmABI8/Xwec102	$3A^m$	2003	2.53	0.110
		2004	2.18	0.092
Xrz444/TmABF	$3A^m$	2003	2.30	0.105
		2004	2.11	0.091
Xrz261/Xrz141	$4A^m$	2003	2.11	0.094
		2004	2.14	0.093
Xcdo1326c/ABC302	$5A^m$	2003	6.17	0.268
		2004	4.56	0.204

Arabidopsis genes by *in silico* analysis. Exploiting this situation, we attempted to identify rice and wheat genes homologous to *Arabidopsis* ABA signaling genes by sequence similarity. Eventually, we identified diploid wheat homologues to *ABI1*, *ABI3*, *ABI5*, *ABI8*, *ERA1* and *ERA3*, although phylogenetic analysis suggested that the *ABI1* homologue is diploid wheat *HAB1* homologue. To determine the homologues to which *TmABI1/HAB1* is really attributed, functional analysis of the gene is needed. We tried to map them on the diploid wheat linkage map, and we successfully mapped five of the homologues except for the *ABI1/HAB1* homologue. In hexaploid wheat, so far only *TaVP1* has been mapped on the long arm of group 3 chromosomes (Bailey et al. 1999; Osa et al. 2003). Although we could not map the *ABI1/HAB1* homologue due to no SNP between the parental lines, the barley homologue was mapped on 3H (data not shown), suggesting that the diploid wheat *ABI1/HAB1* homologue would be located on $3A^m$.

We have identified four QTLs for seed dormancy in the diploid wheat mapping population (Table 4). To determine whether the mapped diploid wheat homologues are positional candidate genes for seed dormancy, their map locations were compared with QTL loci. Consequently, the two QTLs on $3A^m$ were co-located with *TmABF* and *TmABI8* (Fig. 3a); therefore, they are thought to be positional candidate genes for seed dormancy. Their *Arabidopsis* orthologues, *ABI5* and *ABI8* mutants, were screened by their defects in ABA sensitivity at germination. Although *ABI5* is involved in shoot growth arrest (Lopez-Molina et al. 2002; Bensmihen et al. 2004) and *ABI8* affects the retardation of radicle emergence at germination (Brocard-Gifford et al. 2004), the mutations do not affect the level of seed dormancy in *Arabidopsis* (Finkelstein et al. 2002). However, to compare the functions of their homologues, we may need to consider differences in the developmental stages of mature embryos between wheat and *Arabidopsis*. Wheat mature embryos arrest their tissue differentiation at more advanced stages (Rogers and Quatrano 1983) compared to *Arabidopsis* mature seeds (West and Harada

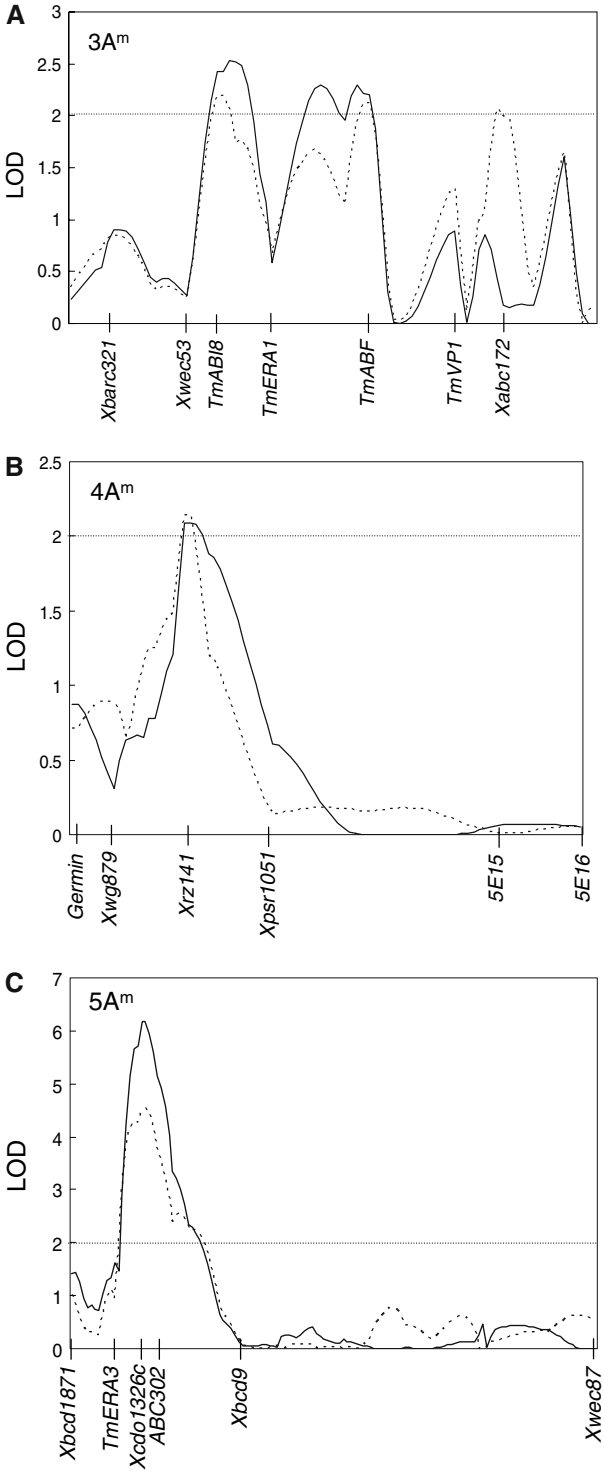

Fig. 3 QTL likelihood curves of LOD scores showing the locations of QTL for seed dormancy in the 2003 (*solid line*) and 2004 (*dotted line*) trials: **a** $3A^m$, **b** $4A^m$, **c** $5A^m$

1993), meaning that developmentally arrested shoots and roots in mature wheat embryos are more developed than those of *Arabidopsis* mature seeds. This indicates that wheat homologues could have roles in the growth retardation of young shoots and roots in wheat mature embryos at

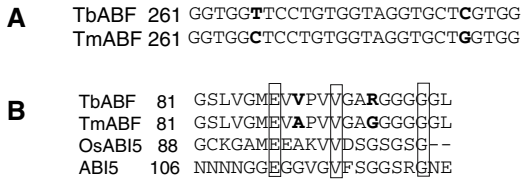

Fig. 4 SNPs and the substituted amino acid residues of *TmABF* between Tb and Tm. Alignments of the nucleic acid sequences (A) and amino acid sequences (B) of *TbABF*, *TmABF*, *OsABI5* and *ABI5* by Clustal W program are shown. *Bold letters* indicate SNPs and the corresponding substituted amino acid residues. The *number* shows the position of nucleic acid and amino acid residues from the start codon. *Open rectangular* shows conserved amino acid residues

germination. Our genetic studies have identified *TmABF* and *TmABI8* as positional candidate genes for QTLs on $3A^m$. Moreover, we found two SNPs which alter amino acid residues between *Tb* and *TmABF*. One changes an amino acid residue G in Tb to R in Tm. Glycine (G) is a nonpolar and neutral amino acid, whereas arginine (R) is a polar and strongly basic amino acid, the properties of the substituted amino acids being distinctively different. ABI5 family members have several conserved domains (Finkelstein et al. 2002). These substitutions appear to not occur in highly conserved domains of *TmABF* (Fig. 4), however, these substitutions might have a minor effect on the activity of *TmABF*. Recently, Bentsink et al. (2006) have identified *Delay Of Germination 1* (*DOG1*) gene whose expression levels are involved in the control of seed dormancy. They found ABA-responsive element (ABRE) in the promoter region. Since ABI5 family members bind to ABRE to regulate ABRE-containing genes (Finkelstein et al. 2002), if diploid wheat has *DOG1* homologues, the activity of *TmABF* might be related to the control of seed dormancy through regulation of their expressions. On the other hand, the coding sequences of *TmABI8* are identical between Tb and Tm; however, we could not exclude the possibility that there may be some differences in their promoter sequences. Thus, further molecular and biochemical analyses are necessary to confirm their involvement in seed dormancy.

So far, a number of QTLs for seed dormancy of hexaploid wheat have been identified, some of which are located around *TaVP1* loci in group 3 chromosomes (Groose et al. 2002; Mares et al. 2002). Since *TmABF* is mapped 12 cM apart from *TmVP1*, the QTLs on group 3 chromosomes might be related to the QTL at *TmABF* on $3A^m$; however, due to the lack of enough common markers between the linkage maps, it is hard to compare their locations with those of our QTLs in detail.

The QTL on $4A^m$ may seem to correspond to the major QTL on 4AL which Mares et al. (2005) and Torada et al. (2005) reported. The QTL on 4AL is located between *Xgwm397* and *Xgwm637*. From the linkage map constructed by Korzun et al. (1999) using durum wheat, the region between *Xgwm397* and *Xgwm637* is included by the region between *Xwg876* and *Xpsr1051*, which is located at 6 and 29 cM from the top of $4A^m$ (Fig. 1) where the QTL on $4A^m$ is located (Fig. 3b). Therefore, the marker positions indicate that the QTL on $4A^m$ is orthologous to the QTL on 4AL, although the QTL effect on $4A^m$ is small.

The QTL on $5A^m$ also might correspond to the QTL on 5A which Groos et al. (2002) detected. The QTL on 5A was located within an approximate 30 cM interval between RFLP markers *Xgwm205* to *Xgwm156* in which *Xbcd1871* was located, and the *Xbcd1871* was mapped on the distal end of the short arm of chromosome $5A^m$ (Fig. 1). Somers et al. (2004) reported that *Xgwm205* and *Xgwm156* are located 33 cM and 72 cM from the top of 5A, and the region covered by the markers extends from the short arm of 5A over the centromere into the approximately 10 cM proximal region of the long arm of 5A. This result indicates that the QTL detected on $5A^m$ might be orthologous to the QTL on 5A.

In diploid wheat QTL mapping, we are able to detect recessive QTLs. In contrast, since hexaploid wheat generally has at least three sets of orthologous genes on its A, B and D genomes, in hexaploid wheat QTL mapping, it is highly probable that the effect of a recessive allele on one of the three sets of genomes is masked by the other two sets of alleles. Therefore, if these QTLs detected on diploid wheat are recessive, it is not surprising that there are no detections on the corresponding QTLs of hexaploid wheat.

From the results of comparing diploid wheat and barley maps by Dubcovsky et al. (1996), the region around the centromere in $5A^m$ corresponds to the centromeric region of 5H of barley. Barley seed dormancy QTL SD1 resides on the long arm of 5H near the centromere (Han et al. 1999). SD1 had the largest effect on seed dormancy in the Steptoe x Morex cross, has the most consistent effect in various environments, and is mapped in the interval between *Ale* and *ABC302* (Han et al. 1999). In the present study, *ABC302* was located around the LOD peak of the QTL on $5A^m$ (Fig. 3c). This marker position indicates that the $5A^m$ QTL is orthologous to SD1.

SD2 is the second largest QTL for barely seed dormancy in the Steptoe x Morex cross (Oberthur et al. 1995; Gao et al. 2003). Seed dormancy QTL SD2 resides at the most terminal region of the long arm of 5H which has a homoeologous relation to $5A^m$. In diploid wheat, a reciprocal translocation is thought to occur between $4A^mL$ and $5A^mL$ (Devos et al. 1995). Since we found a minor QTL for seed dormancy on $4A^mL$, we examined the relationships between the QTL and SD2 syntenic locus using two CAPS markers, *5E15* and *5E16*, flanking the barley SD2 locus. As expected, they were mapped at the distal region of $4A^m$; however, the mapped loci were located outside of the QTL

on $4A^m$ (Fig. 3b). Therefore, the QTL on $4A^m$ seems to not have an orthologous relationship to barley SD2.

Previously, Kato et al. (2001) described the orthologous relationship between seed dormancy QTLs on 4AL of hexaploid wheat and barley SD4 by comparing wheat and barley maps. As mentioned above, we have also found a presumable orthologous relationship between QTLs in diploid wheat and barley by comparing orthologous marker positions. These results suggest that common causal genes between the two genera *Triticum* and *Hordeum* have effects on their levels of seed dormancy. In general, genetic and biochemical analyses of a given trait in diploid plants are much simpler than those in polyploidy plants. Thus, further analysis of diploid wheat and barley QTLs for seed dormancy would contribute to studies on seed dormancy of hexaploid wheat.

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