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ORIGINAL PAPER

Evolution and dispersal of emmer wheat (*Triticum* sp.) from novel haplotypes of *Ppd-1* (photoperiod response) genes and their surrounding DNA sequences

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Abstract The sequence data from 5′ UTR, intronic, coding and 3′ UTR regions of *Ppd-A1* and *Ppd-B1* were investigated for a total of 158 accessions of emmer wheat landraces comprising 19 of wild emmer wheat (*Triticum dicoccoides*), 45 of hulled emmer wheat (*T. dicoccum*) and 94 of free-threshing (FT) emmer wheat (*T. durum* etc.). We detected some novel types of deletions in the coding regions from 22 hulled emmer accessions and 20 FT emmer accessions. Emmer wheat accessions with these deletions could produce predicted proteins likely to lack function. We also observed some novel mutations in *Ppd-B1*. Sixty-seven and forty-one haplotypes were found in *Ppd-A1* and *Ppd-B1*, respectively. Some mutations found in this study have not been known, so they have potential for useful genetic resources for wheat breeding. On the basis of sequence data from the 5′ UTR region, both *Ppd-A1* and *Ppd-B1* haplotypes were divided into two groups (Type AI/AII and Type BI/BII). Types AI and AII of *Ppd-A1* suggested gene flow between wild and hulled emmer. On the other hand, Types BI and BII of *Ppd-B1* suggested gene flow between wild and FT emmer. More than half of hulled emmer accessions were Type AII/BI but few FT emmer accessions were of this type. Therefore, over half of the hulled emmer did not contribute to evolution of FT emmer.

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Introduction

The time of flowering is one of the most important factors of crop adaptation. Therefore, adjusting the flowering time to the environment in areas of cultivation is a very important breeding objective. Wheat (*Triticum* sp.) is one of the oldest and most important cereal crops and was domesticated in the Levant (Southeastern Turkey to Syria) about 10,000 years ago and subsequently spread throughout the world, including in the western hemisphere (Nesbitt and Samuel 1998; Tanno and Willcox 2006; Luo et al. 2007; Dubcovsky and Dvorak 2007). In the process of expansion of its cultivation area, late-maturing varieties were selected under a long growing season while early maturing varieties were selected under a short season for growth (Kato and Yokoyama 1992; Law and Worland 1997).

The flowering traits of wheat depend on three internal factors: photoperiod sensitivity, vernalization requirement and narrow-sense (Kato and Yamagata 1988) earliness; the genetic mechanisms behind these factors are gradually being understood (Yan et al. 2003, 2004, 2006; Beales et al. 2007; Wilhelm et al. 2009). Wheat is a quantitative long-day plant, whose flowering is accelerated by long days. Photoperiod-insensitive varieties flower under both short- and long-day conditions, but photoperiod-sensitive ones show delayed heading or do not head if the day length and number of days fall short of a threshold for floral initiation. In common wheat (*T. aestivum* L.), the major photoperiod response gene (*Ppd-1*) is known as three homologous genes (*Ppd-A1, Ppd-B1, Ppd-D1*) located on

the short arm of chromosome 2 (Worland and Law 1986; Mohler et al. 2004; Tanio and Kato 2007; Wilhelm et al. 2009). Beales et al. (2007) showed that *Ppd-1* is an orthologue of *Ppd-H1* of barley, *Hordeum vulgare* L. *Ppd-H1* was cloned and identified as a member of the *pseudo-response regulator* (PRR) family (Turner et al. 2005). PRR proteins are characterized by a pseudo-receiver domain near the N-terminus and a CCT domain near the C-terminus (Griffiths et al. 2003; Mizuno and Nakamichi 2005; Nakamichi et al. 2007).

In barley, recessive *ppd-H1* mutation is likely to cause loss of function and confers late flowering in long-day conditions (Turner et al. 2005). In common wheat, the semi-dominant photoperiod-insensitive (*Ppd-D1a*) allele, which confers early flowering in short- or long-day conditions, was shown to be associated with a 2,089-bp deletion upstream of the coding region and misexpression of the gene, specifically a loss of its normal circadian regulation (Beales et al. 2007). In the *Ppd-A1* and *Ppd-B1* genes, the likely causal mutations of photoperiod insensitivity were not found by Beales et al. (2007). However, early flowering of a *Ppd-B1* mutant (Mohler et al. 2004; Tanio and Kato 2007) suggests an unknown *Ppd-B1* mutation. Sequencing of the *Ppd-D1* gene from a number of varieties also revealed other polymorphisms and some *Ppd-D1* haplotypes were defined by the combination of its polymorphisms. A previous study showed that some *Ppd-D1* haplotypes affect various agronomic traits, such as days to heading, plant height and 1,000-kernel weight (Guo et al. 2010). Photoperiod insensitivity in emmer wheat (*T. durum*) is well known (Motzo and Giunta 2007). It is also recognized that there is wide variation in heading time among emmer wheat, depending on the area of origin, with this being attributable to differential adaptation (Kato and Yokoyama 1992). In durum wheat (*T. durum*), photoperiod insensitivity was attributed to mutation of the *Ppd-A1* gene (Wilhelm et al. 2009). Two types of deletion upstream of the coding region were found, namely, a 1,027-bp deletion ('GS-100' type) and a 1,117-bp deletion ('GS-105' type) from the *Ppd-A1* gene. These deletions removed a common region, which was also deleted in the *Ppd-D1a* allele (Wilhelm et al. 2009). Therefore, previous studies focused only on these regions, and mutations within the coding region have not been fully investigated (Beales et al. 2007; Wilhelm et al. 2009; Bentley et al. 2010).

At present, 95 % of the world's wheat production comes from common wheat, and the remaining 5 % from durum wheat (Shewry 2009). Hexaploid wheat (AABBDD) including common wheat emerged from interspecific crossing between emmer wheat (AABB) and *Aegilops tauschii* Coss (DD, Kihara 1944; McFadden and Sears 1944). In the process of crossings, only a small part of the genetic resources of emmer wheat has been passed on to hexaploid wheat. Therefore, it is expected that emmer wheat has novel *Ppd-1* haplotypes that are not utilized in common wheat. In this paper, we report on new mutations of *Ppd-A1* and *Ppd-B1* genes, which are likely to be loss-of-function mutations, found in emmer wheat landraces cultivated around the world, as well as the geographical distribution of haplotypes of *Ppd-A1* and *Ppd-B1* genes. We discuss the evolution and expansion of emmer wheat from the combinations between *Ppd-A1* and *Ppd-B1* haplotypes.

Materials and methods

Plant materials

A total of 158 accessions of emmer wheat landraces comprising 19 of wild emmer wheat (*Triticum dicoccoides*), 45 of hulled emmer wheat (*T. dicoccum*) and 94 of free-threshing (FT) emmer wheat (*T. durum*, *T. turgidum*, *T. polonicum*, *T. carthlicum*, *T. turanicum*, *T. abyssinicum*) were used (Online Resource 1). These accessions were maintained at National BioResources Project KOMUGI (Laboratory of Crop Evolution, Graduate School of Agriculture, Kyoto University) and USDA.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from young leaves from each accession by the CTAB method (Escaravage et al. 1998). The extracted DNA was stored in 100 μL of TE buffer at 4 °C. DNA was amplified by PCR using specific primers for *Ppd-A1* and *Ppd-B1*. The PCR amplification involved 50 ng of template DNA, 0.25 μM each primer, 0.2 mM dNTPs, 4 μL of 5 × PrimeSTAR® GXL Buffer (TaKaRa, Japan) and 0.5 U of PrimeSTAR® GXL Polymerase (TaKaRa, Japan) in a total volume of 20 μL. Amplification conditions were 30 cycles of 98 °C for 10 s, 60 °C for 15 s and 68 °C for 5 min. PCR products were cleaned using the AMPure® kit (Bio Medical Science, Tokyo, Japan). The BigDye Terminator v3.1 Cycle Sequencing® kit (Applied Biosystem, Tokyo, Japan) and primers were used for sequencing reactions. Sequencing reaction products were cleaned using CleanSEQ® (Applied Biosystem, Tokyo, Japan) and sequenced using ABI PRISM® 3100 Genetic Analyzer. The primers used for PCR amplification and sequencing, designed for use with primer 3 (Rozen and Skaletsky 2000), were based on sequence data from the DDBJ website (http://www.ddbj.nig.ac.jp).

The PCR primers were designed for the detection of some mutations found in this study. The mutations in *Ppd-A1* haplotypes were an 83-bp insertion downstream of the coding region, an 18-bp deletion from the 2nd exon to

the 2nd intron, a 224-bp deletion from the 4th exon to the 4th intron, a 303-bp deletion from the 5th exon to the 6th exon and a 2-bp deletion of the 7th exon. The mutations in *Ppd-B1* haplotypes were a 914-bp deletion of about 770 bp upstream from the start codon, a 206-bp deletion and a 25-bp unique sequence 360 bp upstream from the start codon, a 206-bp deletion 176 bp upstream from the start codon, and a 15-bp deletion and a 13-bp unique sequence in the 3rd exon. The primers produced a band if these mutations were present. The PCR primers were also designed for the detection of haplotype groups of *Ppd-A1* and *Ppd-B1*. There were two sets of primers to identify haplotype groups of *Ppd-A1*. Each primer pair corresponded to Types AI and AII and produced a band. There was one set of primers to identify haplotype groups of *Ppd-B1*. This primer set generated a 142-bp product from Type BI and a 166-bp product from Type BII. The PCR amplification involved 50 ng of template DNA, 1 μM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 μL of 10 × PCR Buffer (TaKaRa, Japan) and 0.5 U of *Taq* Polymerase (TaKaRa, Japan) in a total volume of 15 μL. Amplification conditions were 96 °C for 2 min followed by 30 cycles of 96 °C for 20 s, 58–62 °C for 20 s and 72 °C for 30 s. PCR products were separated on 1.5–4 % agarose gels in TAE buffer. The PCR primers used in this study are summarized in Table 1.

Table 1 Primers used in this study

Primer name	Primer sequence	Annealing temperature	Use
For *Ppd-A1*			
cd_A_ F1	gcagtctcaatcccttgctc	60	Amplification of coding and 3′ regions
cd_A_R1	gaacggaacgccaaagtaaa		
up_A_F4	tgcaccaatgaatacactcaca	60	Amplification of 5′ region
up_A_R6	acaaccaacgcccaaattc		
Tr_A_type1_F1	tggtccccgtaaattcttagagc	62	Detection of Types AI and AII
Tr_A_type2_F1	acatgaggagcaaaatccatgac		
Tr_A_common_R1	cctctcagctcaactcctctgac		
18 bp_del_F1	tgcaaggacatccccgat	59	Detection of a 18-bp deletion from 2nd exon to 2nd intron
18 bp_del_R1	actactgtggaagaacagaacagac		
224 bp_del_F3	gaagtgccattcagactcagataaa	59	Detection of a 224-bp deletion from 4th exon to 4th intron
224 bp_del_R1	gtccgttaggtaacgaagagag		
303 bp_del_F2	cttacatctgtgagaagtatctgcatc	60	Detection of a 303-bp deletion from 5th exon to 6th exon
303 bp_del_R3	cagatcagcagctcgaacaattac		
83 bp_in_F1	ggctgatgaaaaatgggtaac	58	Detection of a 83-bp insertion of 3′ region
83 bp_in_R1	caagaatcagctgtctaaatagtactcc		
2 bp_del_F1	gccgccgtgaacaagttg	63	Detection of a 2-bp deletion of 7th exon
2 bp_del_R1	ggtaacgcacctgcaaaatgag		
For *Ppd-B1*			
cd_B_F6	cactcgtctgctctgttcctg	60	Amplification of coding and 3′ regions
cd_B_R3	tctgcaggcatgttctatgg		
up_B_F7	tccgccaacagtaggtgttc	60	Amplification of 5′ region
up_B_R4	ggtagtagctgagacagagtatgagtaagg		
Tr_B_Type1_F1	cacacacacacgcggactac	62	Detection of Types BI and BII
Tr_B_common_R2	Cagagcagacgagtgacagacag		
917bp_del_F1	aaggaaagaccattccaccg	62	Detection of a 917-bp deletion of 5′ region
Tr_B_common_R2	cagagcagacgagtgacagacag		
206 bp_del_25_F2	gattggggatcgaatcaacg	62	Detection of a 206-bp deletion and 25-bp unique sequence of 5′ region
206 bp_del_25_R1	cctgactccaagaggaaacatg		
206 bp_del_F1	cgtcctcctcctcctcaacc	62	Detection of a 206-bp deletion of 5′ region
206 bp_del_R2	aggaggcgcgcataatagac		
15 bp_del_13_F1	caagccgataatggtagtggag	60	Detection of a 15-bp deletion and 13-bp unique sequence of 3rd exon
15 bp_del_13_R1	caactttgttttagtacccagtaccatac		

RNA was extracted from each accession with deletion of both exon and intron using NucleoSpin® RNA Plant kit (TaKaRa, Japan). DNA was removed by digestion with DNAseI prior to reverse transcription. cDNA was synthesized using PrimeSCript® II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) using the manufacturer's protocol with total RNA as template and OligodT as primers. Synthesized cDNA were used as templates for PCR amplification and also sequenced.

Data analyses

Sequences were manually inspected with BioEdit ver. 7.0.9 (Hall 1999) and the alignments were generated with Clustal W (Thompson et al. 1994). The total number of polymorphic sites (S), the nucleotide diversity (π, Nei 1987; Nei and Miller 1990), the number of haplotypes (h) and the haplotype diversity (Hd, Nei 1987) were calculated with DnaSP ver. 5.1 (Librado and Rozas 2009). The sequence data from the 5′ UTR, intronic, coding and 3′ UTR regions of *Ppd-A1* and *Ppd-B1* were analyzed for phylogenetic relationships by the neighbor-joining (NJ) method (Saitou and Nei 1987) using MEGA ver. 4.0.2 (Tamura et al. 2007). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and all positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The percentage of replicate trees in which the associated haplotypes clustered together was calculated in the bootstrap test (1,000 replicates). GenBank sequencing accessions are: Ppd-A1, AB691782–AB691938 and AB693038. Ppd-B1, AB692786–AB692942 and AB693039.

Results

Haplotypes and genetic diversity of *Ppd-A1*

We obtained 5,823–7,076 bp of sequence data from 5′ UTR, intronic, coding and 3′ UTR regions of *Ppd-A1*. Sixty-seven haplotypes were found in *Ppd-A1* and its surrounding region (Table 2). The most common haplotype was haplotype A15 (Hap-A15), which was found in 21 accessions. Forty-three haplotypes were found from one each accession. Twenty-three haplotypes were found from hulled emmer (45 accessions), 34 haplotypes were found from free-threshing (FT) emmer (94 accessions) and 14 haplotypes were found from wild emmer (19 accessions). Two haplotypes, Hap-A10 and Hap-A15, were shared by hulled and FT emmer accessions. Two haplotypes, Hap-A30 and Hap-A43, were shared by FT and wild emmer accessions.

One FT emmer accessions from Yemen (Hap-A40) had the GS-105-type deletion (a 1,117-bp deletion upstream of the coding region, Fig. 1a) that was reported by Wilhelm et al. (2009). There was an 83-bp insertion downstream of the coding region in nine wild emmer accessions and one hulled emmer accession from Portugal (Hap-A4, A56, A57, A58, A60, A61, A63, A64, A65 and A66, Fig. 1a). We found an 18-bp deletion from the 2nd exon to the 2nd intron in three hulled emmer accessions from Belarus, Turkey and Iran (Hap-A6 and Hap-A20, Fig. 1a) and a 224-bp deletion from the 4th exon to the 4th intron in two hulled emmer accessions from Spain and Poland (Hap-A14, Fig. 1a). We detected a 303-bp deletion from the 5th exon to the 6th exon in 17 hulled emmer accessions and five FT emmer accessions (Hap-A1, A3, A10, A11, A16, A21, A22, A23, A26, A29, A37 and A39, Fig. 1a). This 303-bp deletion was the same as reported in *T. aestivum* cv. Cappelle-Desprez (Beales et al. 2007). A 2-bp deletion of the 7th exon was found in 15 FT emmer accessions (Hap-A31, A33, A34, A35 and A45, Fig. 1a). This 2-bp deletion caused a frameshift mutation within the CCT domain and produced a new C-terminus for the protein that had 46 extra amino acids. We obtained the sequences of cDNA derived from accessions with deletions including both intron and exon regions. These accessions had alternative stop codons after the deletions and could not produce normal proteins.

Fourteen single nucleotide polymorphisms (SNPs) caused amino acid changes compared with the sequence of *Ppd-A1* of Chinese spring (DQ885753). Three changes were included in the PRR domain: a Var to Met change (Hap-A7, A8, A12 and A13), an Ile to Var change (Hap-A4) and a Glu to Asp change (Hap-A2, A4, A5, A7, A8, A9, A12, A13, A17, A18, A19, A27, A28, A30, A32, A36, A40, A41, A43, A44, A46, A47, A48, A49, A51, A52, A53, A54, A55, A56, A57, A58, A59, A60, A61, A62, A63, A64, A65, A66 and A67). One change was included in the CCT domain, an Arg to His change (Hap-A7, A8, A12 and A13).

The genetic relationships among haplotypes of *Ppd-A1* are shown by an NJ tree (Fig. 2). *Ppd-A1* haplotypes were divided into two groups (Type AI and Type AII) based on about 200-bp sequences, which were around 1 kbp upstream from the start codon and included insertion/deletion mutations (Figs. 1a–c, 2). An accession from Oman, PI244061, was devoid of the distinct parts of Types AI and AII, but classified into Type AII from the phylogenetic tree. We found some accessions that were devoid of 100 bp of MITE-like sequences in Type AII (Hap-A7, A8, A12 and A13, Type AIIa, Fig. 1b, c). Wild, hulled and FT emmer accessions were included in both Types AI and AII (Tables 2, 3). The accessions that have 18-bp, 224-bp or 2-bp deletions were grouped into Type AI. The

Table 2 Overview of haplotype of Ppd-A1

Haplotype	Number of accessions			Type	Characteristic change[a]	Country
	Hulled	FT	Wild			
Hap_A1	3			AI	303 bp del	Russia, Spain
Hap_A2	2			AII	–	India, Oman
Hap_A3	1			AII	303 bp del	France
Hap_A4	1			AI	84 bp in	Portugal
Hap_A5	8			AII	–	Afghanistan, Denmark, Ethiopia, India, Ukraine
Hap_A6	1			AI	–	Belarus
Hap_A7	1			AIIa	–	Morocco
Hap_A8	2			AIIa	–	Germany, Hungary
Hap_A9	1			AII	–	Saudi Arabia
Hap_A10	3	1		AI	303 bp del	Greece, Italy, Portugal
Hap_A11	7			AII	303 bp del	Ancient Palestine, Former USSR, Romania, Turkey
Hap_A12	1			AIIa	–	Iran
Hap_A13	1			AIIa	–	Georgia
Hap_A14	2			AI	224 bp del	Spain, Poland
Hap_A15	1	20		AI	–	Azerbaijan, Cyprus, Czech Republic, Egypt, France, Greece, India, Italy, Jordan, Lebanon, Malta, Oman, Spain, Switzerland, Turkey
Hap_A16	1			AI	303 bp del	Former Yugoslavia
Hap_A17	1			AI	18 bp del	Former Yugoslavia
Hap_A18	1			AI	18 bp del	Montenegro
Hap_A19	1			AI	–	Bosnia and Herzegovina
Hap_A20	2			AI	–	Iran, Turkey
Hap_A21	2			AI	303 bp del	Iran
Hap_A22	1			AI	303 bp del	Spain
Hap_A23	1			AII	303 bp del	Turkey
Hap_A24		5		AI	–	Algeria, Italy, Jordan, Spain
Hap_A25		1		AI	–	Tunisia
Hap_A26		1		AI	303 bp del	Morocco
Hap_A27		14		AI	–	Eritrea, Ethiopia
Hap_A28		2		AI	–	Israel, Tunisia
Hap_A29		1		AI	303 bp del	Pakistan
Hap_A30		2	6	AI	–	Iraq, Israel, Libya, Turkey
Hap_A31		6		AI	2 bp del	Armenia, Azerbaijan, Denmark, Hungary, Italy, Romania
Hap_A32		8		AI	–	Afghanistan, Cyprus, Egypt, Ethiopia, Iran, Iraq, Italy
Hap_A33		2		AI	2 bp del	Bulgaria, Moldova
Hap_A34		1		AI	2 bp del	India
Hap_A35		4		AI	2 bp del	Iran, Lebanon, Turkey
Hap_A36		2		AI	–	Saudi Arabia, Turkey
Hap_A37		1		AI	303 bp del	United Kingdom
Hap_A38		1		AI	–	Portugal
Hap_A39		1		AII	303 bp del	Serbia
Hap_A40		1		AII	GS-105 del	Yemen
Hap_A41		1		AI	–	Poland
Hap_A42		2		AII	–	Macedonia
Hap_A43		1	1	AII	–	Montenegro, Syria
Hap_A44		1		AI	–	Oman
Hap_A45		2		AI	2 bp del	Egypt
Hap_A46		1		AI	–	Jordan

Table 2 continued

Haplotype	Number of accessions			Type	Characteristic change[a]	Country
	Hulled	FT	Wild			
Hap_A47		1		AI	–	Ethiopia
Hap_A48		2		AI	–	Ethiopia
Hap_A49		1		AI	–	Iraq
Hap_A50		1		AI	–	Iraq
Hap_A51		1		AI	–	Ethiopia
Hap_A52		3		AI	–	Georgia, Iran
Hap_A53		1		AI	–	Iraq
Hap_A54		1		AI	–	Ethiopia
Hap_A55		1		AI	–	Ethiopia
Hap_A56			1	AI	84 bp in	Iran
Hap_A57			1	AI	84 bp in	Iran
Hap_A58			1	AI	84 bp in	Iran
Hap_A59			1	AI	–	Iraq
Hap_A60			1	AII	84 bp in	Israel
Hap_A61			1	AI	84 bp in	Israel
Hap_A62			1	AI	–	Israel
Hap_A63			1	AII	84 bp in	Israel
Hap_A64			1	AII	84 bp in	Turkey
Hap_A65			1	AII	84 bp in	Turkey
Hap_A66			1	AII	84 bp in	Turkey
Hap_A67			1	AI	–	Turkey

[a] Characteristic change was shown in Fig. 1

accessions that have 303-bp deletion or 83-bp insertion were grouped into both Types AI and AII. These distributions are shown in Fig. 3a, b.

The values of nucleotide diversity (π) of *Ppd-A1* and its surrounding region were 0.00426 from wild emmer accessions, 0.00344 from hulled accessions and 0.00131 from FT emmer accessions (Table 4). When the values of π were calculated for 5′ UTR, intronic, coding and 3′ UTR regions separately, they ranged from 0.00104 (5′ UTR region of FT emmer accessions) to 0.00649 (5′ UTR region of wild emmer accessions, Fig. 6).

Haplotypes and genetic diversity of *Ppd-B1*

We obtained 5,651–6,566 bp of sequence data from 5′ UTR, intronic, coding and 3′ UTR regions of *Ppd-B1*. Forty-one haplotypes were found in *Ppd-B1* and its surrounding region (Table 5). The most common haplotype was B38 (Hap-B38), which was found in 38 accessions. Twenty-six haplotypes (Hap-B3, B4, B7, B8, B9, B12, B16, B18, B19, B20, B21, B22, B24, B27, B28, B29, B30, B32, B33, B34, B35, B36, B37, B38, B39 and B41) were found in only one accession. Thirteen haplotypes were found in hulled emmer (45 accessions), 20 haplotypes were found in free-threshing (FT) emmer (94 accessions) and 13 haplotypes were found in wild emmer (19 accessions). One haplotype, Hap-B2, was shared by wild, hulled and FT emmer accessions. Three haplotypes, Hap-B1, Hap-B10 and Hap-B13, were shared by hulled and FT emmer accessions.

There was a 914-bp deletion of about 770 bp upstream from the start codon in one FT emmer accession from India (Hap-B16, Fig. 4a), a 206-bp deletion and a 25-bp unique sequence 360 bp upstream from the start codon in one wild emmer accession from Israel (Hap-B36, Fig. 4a) and a 206-bp deletion 176 bp upstream from the start codon in four hulled emmer accessions from Germany, Greece, Georgia and Turkey (Hap-B6, Fig. 4a). A 3-bp insertion in the 1st exon was found in two hulled emmer accessions from the areas of Ancient Palestine and the former USSR, 36 FT emmer accessions and four wild emmer accessions (Hap- B10, B14, B16, B18, B19, B21, B25, B26, B30, B33, B37 and B38, Fig. 4a). These deletions found in 5′ UTR regions removed a common region that were also deleted in the *Ppd-D1a* and *Ppd-A1a* alleles (Beales et al. 2007; Wilhelm et al. 2009, Fig. 4a). We detected a 15-bp deletion and a 13-bp unique sequence in the 3rd exon in one FT emmer accession from Ethiopia (Hap-B27, Fig. 4a). This deletion and the unique sequence caused amino acid changes in the 10 terminal amino acids of the PRR domain.

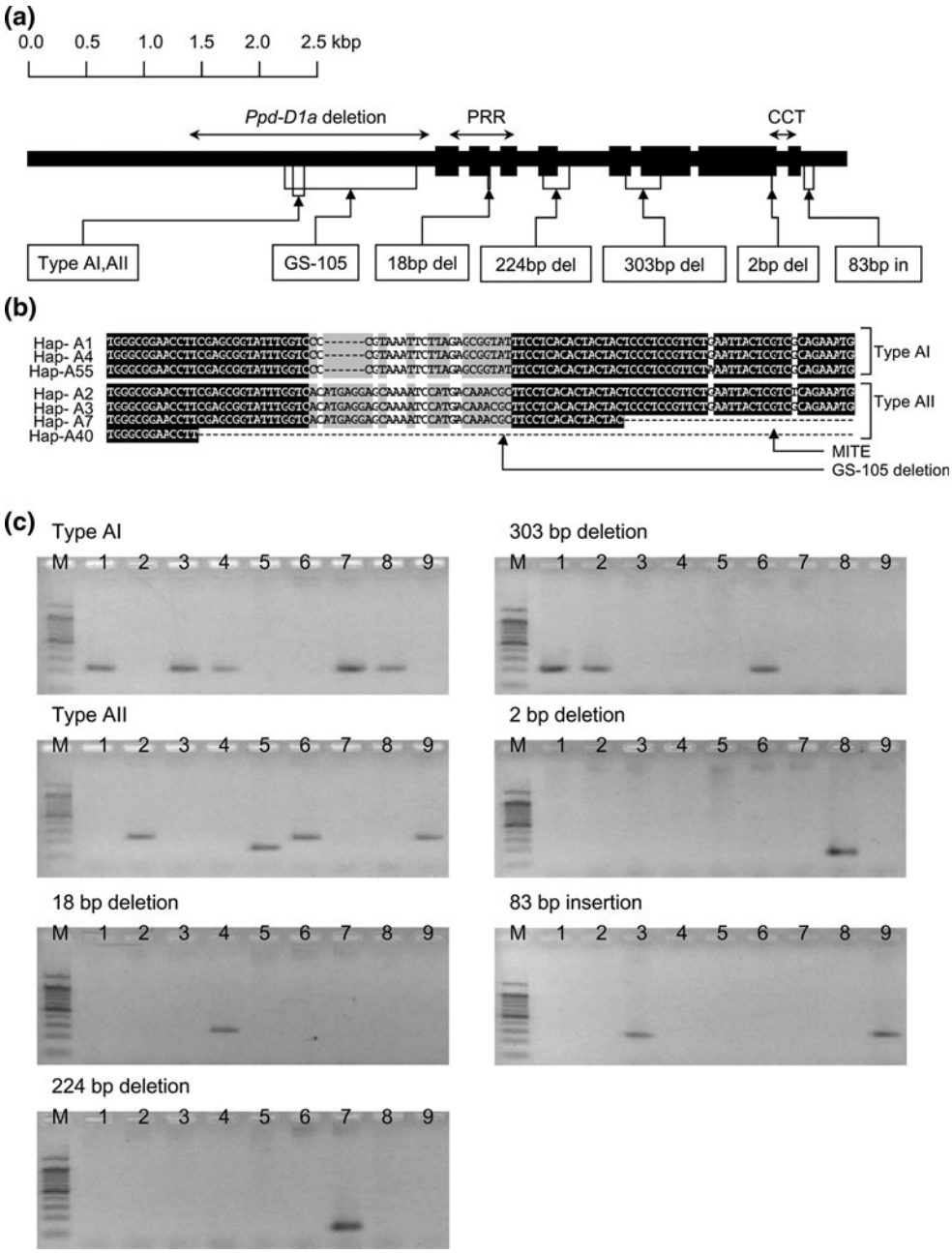

Fig. 1 Mutations of *Ppd-A1* gene. **a** Sequence features of *Ppd-A1* of emmer wheat. *Tall rectangles* represent coding regions and *short rectangles* represent introns, 5′ UTR and 3′ UTR regions. The region corresponding to a 2,089-bp deletion of *Ppd-D1a*, Pseudo-receiver domain and CCT domain are shown as *arrows* above *rectangles*. Only insertion/deletion mutations that are mentioned in this study are shown. **b** The unique sequences of Types AI and AII. **c** Reverced images of PCR marker products of *Ppd-A1*. *M* 100 bp DNA markers, *1* Hap-A1 (Cirt306534), *2* Hap-A3 (Cirt12213), *3* Hap-A4 (PI56234), *4* Hap-A6 (PI94618), *5* Hap-A7 (PI94633), *6* Hap-A11 (PI355496), *7* Hap-A14 (PI 286061), *8* Hap-A31 (PI64684), *9* Hap-A60 (KU-198)

A 15-bp deletion was found in the 7th exon in one wild emmer accession from Israel (Hap-B38, Fig. 4a). This 15-bp deletion caused a change from nine repeats of Ala to four repeats of it. We detected a 9-bp deletion, with a decreased number of CTG repeats, in the 7th exon in four hulled emmer accessions from Bosnia and Herzegovina, Montenegro and the former Yugoslavia (Hap-A11 and Hap-B12, Fig. 4a). This decrease in the repeat number caused a change from six repeats of Ala to three repeats.

Eleven SNPs caused amino acid changes compared with the sequence of *Ppd-B1* of Chinese spring (DQ885757). Two changes were present in the PRR domain: a Var to Phe change (Hap-B7 and Hap-B8) and an Asn to Asp change (all accessions had the change). One change was present in the CCT domain, an Arg to Trp change (Hap-B3).

The genetic relationships among haplotypes of *Ppd-B1* are shown by an NJ tree (Fig. 5). *Ppd-B1* haplotypes were also divided into two groups (Type BI and Type BII) based on about 30-bp sequences, which were located around 600 bp upstream from the start codon and included insertion/deletion mutations (Figs. 4a–c, 5). The wild, hulled and FT emmer accessions were included in both Types BI and BII (Tables 3, 5). The accessions that have the 3-bp insertion in the 1st exon were grouped into both Types BI and BII. These distributions are shown in Fig. 3c, d.

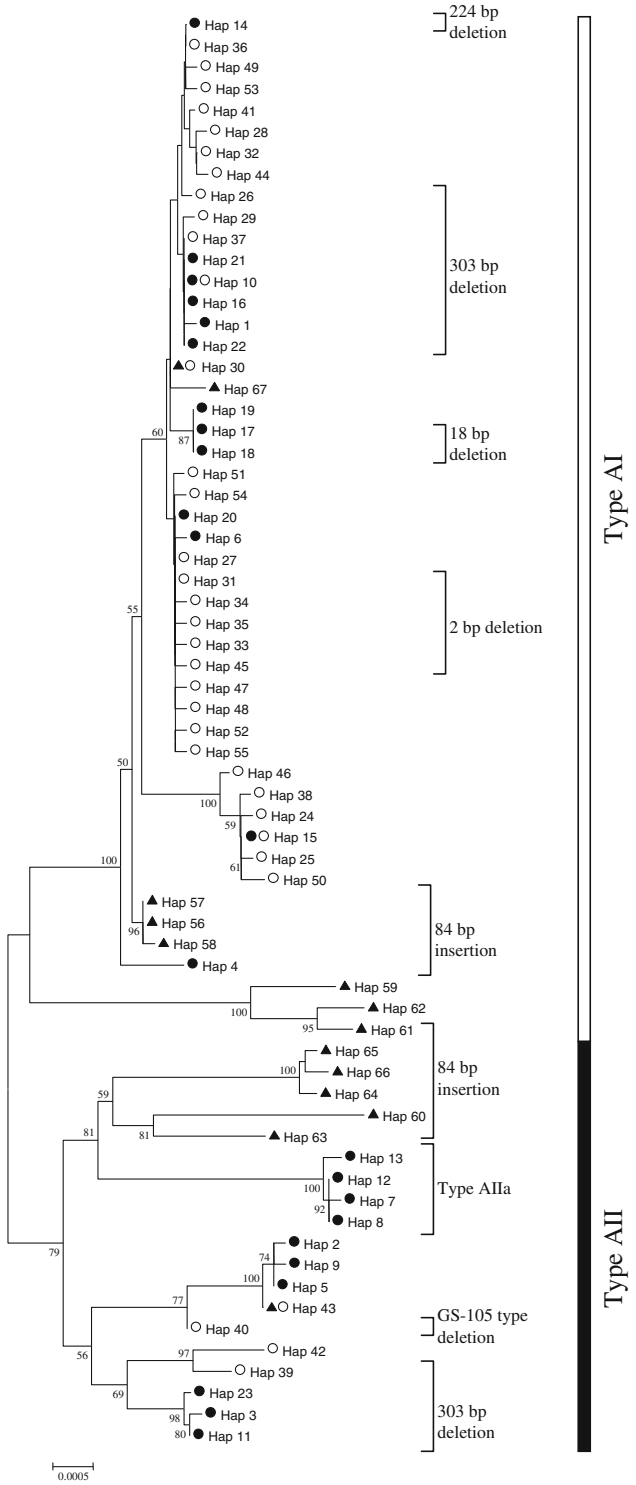

Fig. 2 Neighbor-joining phylogenetic tree built with full sequences of *Ppd-A1* haplotypes. Bootstrap values (1,000 replicates, more than 50) are shown next to the branches. The *black circles*, *white circles* and *black triangles* correspond to domesticated hulled emmer, domesticated free-threshing emmer and wild emmer, respectively. *Right side bar* shows two types of *Ppd-A1*

Table 3 The number of accessions shown by combination of types of *Ppd-A1* and *Ppd-B1*

	n	AI/BI	AI/BII	AII/BI	AII/BII
Wild	19	10	3	4	2
Hulled	45	20	0	23	2
FT	94	54	35	4	1
Total	158	84	38	31	5

The values of π of *Ppd-B1* and its surrounding region were 0.00219 from wild emmer accessions, 0.00055 from hulled emmer accessions and 0.00201 from FT emmer accessions (Table 4). When the value of π was calculated for the 5′ UTR, intronic, coding and 3′ UTR regions separately, the values ranged from 0 (3′ UTR region of all accessions) to 0.00363 (5′ UTR region of wild emmer accessions, Fig. 6).

Discussion

Haplotype variation and evolution of *Ppd-A1*

In this study, *Ppd-A1* haplotypes were grouped into Types AI and AII on the basis of their sequences, which were around 1 kbp upstream from the start codon (Tables 2, 3; Figs. 1a, b, 2). The hulled emmer accessions of Type AI were distributed north from the Mediterranean Sea through Europe and to the Middle East (Fig. 3a). On the other hand, the hulled emmer accessions of Type AII were distributed across a wider range, from Europe to Africa and India, than the hulled emmer accessions of Type AI (Fig. 3b). Both Types AI and AII were confirmed in wild emmer accessions, so we considered that the differentiation occurred before domestication. This may indicate that emmer wheat was domesticated more than once. The wild emmer accessions of Type AI were distributed in almost all the distribution area range from Turkey, Iran, Iraq and Israel. The wild emmer accessions of Type AII were distributed in Israel, Syria and Turkey (Fig. 3b). Luo et al. (2007) and Ozkan et al. (2011) showed that wild emmer was divided into two groups (northern and southern wild emmer) and hulled emmer was domesticated from northern wild emmer lines. We thought that Types AI and AII wild emmer could correspond to northern and southern wild emmer. Therefore, the hulled emmer of Type AII was not domesticated from the wild emmer of Type AII, but rather arose by introgression between the wild emmer of Type AII and the hulled emmer that was introduced to southern Levant

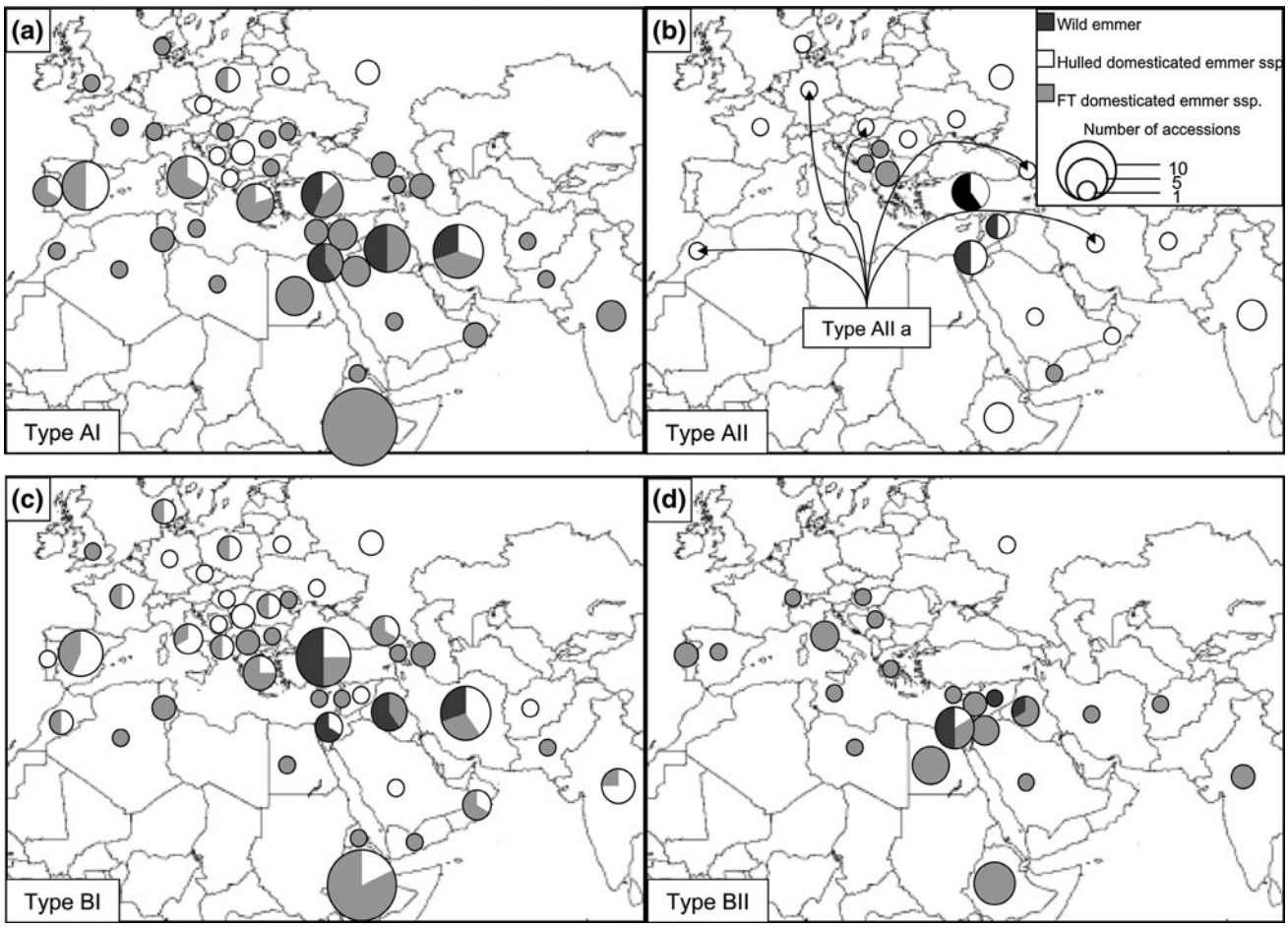

Fig. 3 Geographical distribution of *Ppd-A1* and *Ppd-B1* haplotypes. **a** *Ppd-A1* of Type AI, **b** *Ppd-A1* of Type AII, **c** *Ppd-B1* of Type BI, **d** *Ppd-B1* of Type BII

Table 4 Sequence statistics

	n	Gene	S	π	h	Hd
All	159	*Ppd-A1*	164	0.00282	67	0.9628
		Ppd-B1	138	0.00177	41	0.8930
Wild	19	*Ppd-A1*	110	0.00426	14	0.9123
		Ppd-B1	87	0.00219	13	0.9240
Hulled	45	*Ppd-A1*	80	0.00344	23	0.9394
		Ppd-B1	36	0.00055	13	0.8707
FT	94	*Ppd-A1*	68	0.00131	34	0.9199
		Ppd-B1	62	0.00201	20	0.8453

n Number of accessions, *S* total number of polymorphic sites, π nucleotide diversity calculated on individual sites, *h* number of haplotypes, *Hd* haplotype diversity

region where Type AII wild emmer grows. Because the hulled emmer accessions were distributed across a wide range, this introgression and establishment of Type AII hulled emmer occurred at an early stage of the spread of hulled emmer wheat. The introgressions between hulled and wild emmer in Israel have already been reported (Luo et al. 2007). Type AI hulled emmer was found only to the north of the Mediterranean. This suggests that it spread from Turkey to the east and west without going through Syria and Israel, where the Type AII wild emmer grows. In this study, we focused on domesticated emmer and used only 19 wild emmer accessions. To clarify the evolution of emmer, more research for wild emmer is needed.

Less than half (44.4 %) of hulled emmer accessions were of Type AI, but most (94.7 %) FT emmer accessions were of Type AI (Table 3). This difference suggested that the majority of the *Ppd-A1* genes of FT emmer cultivated today are derived from the *Ppd-A1* gene of Type AI hulled

Table 5 Overview of haplotype of *Ppd-B1*

Haplotype	Number of accessions			Type	Characteristic change[a]	Country
	Hulled	FT	Wild			
Hap_B1	10	14		BI	–	Egypt, Eritrea, Ethiopia, France, Italy, Morocco, Romania, Russia, Spain, Syria, Tunisia, Turkey
Hap_B2	11	26	1	BI	–	Afghanistan, Algeria, Ancient Palestine, Armenia, Azerbaijan, Cyprus, Czech Republic, Denmark, Ethiopia, Former USSR, Georgia, India, Iraq, Italy, Lebanon, Malta, Montenegro, Oman, Pakistan, Poland, Portugal, Saudi Arabia, Spain, Turkey, Ukraine, United Kingdom
Hap_B3	1			BI	–	Portugal
Hap_B4	1			BI	–	Belarus
Hap_B5	6			BI	–	Hungary, Iran, Morocco
Hap_B6	4			BI	206 bp del	Georgia, Germany, Greece, Turkey
Hap_B7	1			BI	–	Spain
Hap_B8	1			BI	–	Poland
Hap_B9	1			BI	–	Italy
Hap_B10	2	22		BII	3 bp del	Afghanistan, Ancient Palestine, Egypt, Ethiopia, Former USSR, France, Greece, India, Iran, Iraq, Israel, Italy, Jordan, Lebanon, Libya, Portugal, Saudi Arabia, Tunisia
Hap_B11	3			BI	9 bp del	Former Yugoslavia, Montenegro
Hap_B12	1			BI	9 bp del	Bosnia and Herzegovina
Hap_B13	3	1		BI	–	India, Oman, Yemen
Hap_B14		5		BII	3 bp del	Azerbaijan, Hungary, Italy, Serbia, Switzerland
Hap_B15		4		BI	–	Bulgaria, Macedonia, Moldova
Hap_B16		1		BII	914 bp del and 3 bp del	India
Hap_B17		3		BI	–	Iran, Iraq, Turkey
Hap_B18		1		BII	3 bp del	Cyprus
Hap_B19		1		BII	3 bp del	Israel
Hap_B20		1		BI	–	Oman
Hap_B21		1		BII	3 bp del	Jordan
Hap_B22		1		BI	–	Ethiopia
Hap_B23		4		BII	3 bp del	Ethiopia
Hap_B24		1		BII	–	Ethiopia
Hap_B25		3		BI	3 bp del	Egypt, Greece, Iran
Hap_B26		2		BI	3 bp del	Greece
Hap_B27		1		BI	15 bp del + 13 bp	Ethiopia
Hap_B28		1		BI	–	Ethiopia
Hap_B29		1		BI	–	Iran
Hap_B30			1	BII	3 bp del	Syria
Hap_B31			3	BI	–	Iran
Hap_B32			1	BI	–	Iraq

Table 5 continued

Haplotype	Number of accessions			Type	Characteristic change[a]	Country
	Hulled	FT	Wild			
Hap_B33			1	BII	3 bp del	Iraq
Hap_B34			1	BI	–	Iraq
Hap_B35			1	BII	–	Israel
Hap_B36			1	BII	206 bp del + 25 bp	Israel
Hap_B37			1	BII	3 bp del	Israel
Hap_B38			1	BI	15 bp del and 3 bp del	Israel
Hap_B39			1	BI	–	Turkey
Hap_B40			5	BI	–	Turkey
Hap_B41			1	BI	–	Turkey

[a] Characteristic change was shown in Fig. 4

emmer, and that gene flow between hulled and FT emmer has been limited. Such limitation in flow is consistent with a previous study that investigated other genes (Takenaka et al. 2010). The FT emmer accessions of Type AII were only found in former Yugoslavian countries (except PI244061); however, hulled emmer accessions of Type AII were not found in this region (Fig. 3b). This suggests that the FT emmer accessions of Type AII evolved from hulled emmer of Type AII, which were in other regions but introduced into this region.

FT emmer accessions of Type AIIa, which were devoid of MITE-like sequences and had 20 specific SNPs including nonsynonymous mutations in PRR and CCT domains, were rare but widely distributed variant (found in Iran, Georgia, Hungary, Germany and Morocco, Fig. 3b). The same deletion of MITE-like sequences could not occur independently, so these accessions had relationships between each other. This distribution may suggest a trace of the introduction of hulled emmer wheat from the Fertile Crescent to Europe and North Africa, via the northern shore of the Black Sea and through the Strait of Gibraltar. We need more researches for FT emmer accessions of Type AIIa.

The accessions with the 18-bp deletion from the 2nd exon to the 2nd intron, or the 224-bp deletion from the 4th exon to the 4th intron were only found among hulled emmer accessions of Type AI (Fig. 2) and were found in separate regions (Table 2). This suggests that these deletions probably occurred in the early stage of domestication and remained in each region. The accessions that had the 2-bp deletion in the 7th exon were only found among FT emmer accessions of Type AI and were present in discontinuous regions from Denmark to India (Table 2). This suggests that the deletion occurred early in the evolution from hulled emmer to FT emmer and spread to distant areas. The 303-bp deletion, from the 5th exon to the 6th exon, was found in both hulled and FT emmer accessions and both Types AI and AII (Fig. 2). This deletion probably occurred in hulled emmer of Type AI or Type AII, and has been shared between the two types by recombination. The Type AI FT emmer accessions with this 303-bp deletion were found in separate regions (Europe and India, Table 2), so these FT emmer probably evolved independently from hulled emmer in each region. On the other hand, a phylogenetic tree showed that the Type AII FT emmer accessions with the 303-bp deletion were probably the result of gene flow from Type AII hulled emmer with this deletion (Fig. 2). This 303-bp deletion was the same deletion as reported in *T. aestivum* cv. Cappelle-Desprez (Beales et al. 2007), so the accessions that had this deletion were involved in the origin of common wheat.

Haplotype variation and evolution of *Ppd-B1*

In this study, *Ppd-B1* haplotypes were divided into two groups, Types BI and BII, on the basis of their sequences at around 600 bp upstream from the start codon (Tables 3, 5; Figs. 4a–c, 5). Most hulled emmer accessions were of Type BI (95.6 %, Fig. 3c) and hulled emmer accessions of Type BII numbered only two, from the areas of Ancient Palestine (PI 352367) and the former USSR (PI 355497, Fig. 3d). We also found the wild emmer accessions of Type BII, so Type BII hulled emmer might be derived from them. However, there were very few hulled emmer accessions, and hulled emmer and FT emmer accessions shared the same haplotypes (Hap-B10, Fig. 5). Therefore, we assumed that the hulled emmer of Type BII did not evolve from wild emmer of Type BII, but is a result of gene flow from Type BII FT emmer instead. Since the wild and FT emmer accessions of Type BII showed a close relationship (Fig. 5), the FT emmer of Type BII probably arose from introgression between FT and wild emmer. The accessions that had the 3-bp insertion at the 1st exon were found in the wild and FT emmer accessions of Type BI and the wild,

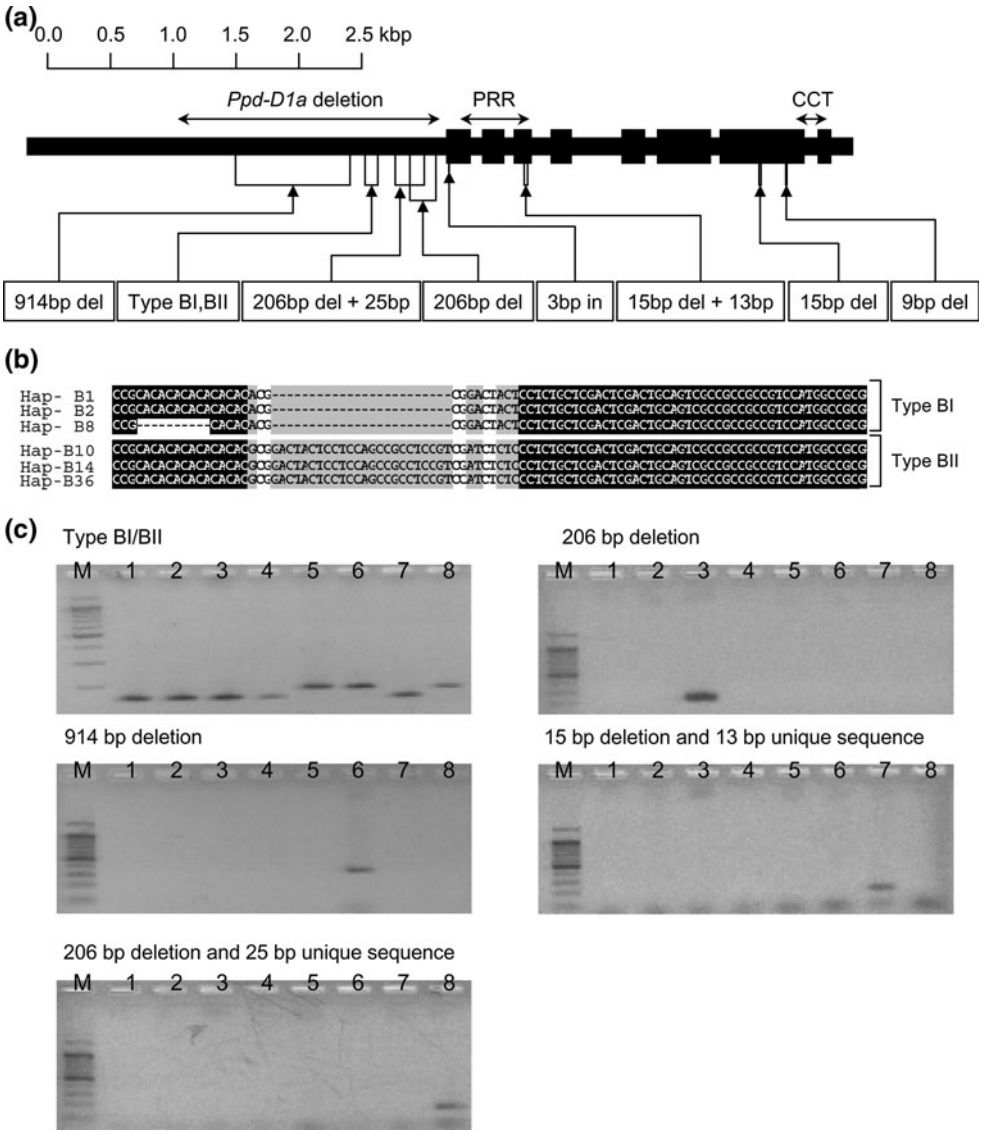

Fig. 4 Mutations of *Ppd-B1* gene. **a** Sequence features of *Ppd-B1* of emmer wheat. *Tall rectangles* represent coding regions and *short rectangles* represent introns, 5′ UTR and 3′ UTR regions. The region corresponding to a 2,089-bp deletion of *Ppd-D1a*, pseudo-receiver domain and CCT domain are shown *arrows* above *rectangles*. Only insertion/deletion mutations that are mentioned in this study are shown. **b** The unique sequences of Types BI and BII. **c** Reverced images of PCR marker products of *Ppd-B1*. *M* 100 bp DNA marker, *1* Hap-B1 (PI11650), *2* Hap-B2 (PI94671), *3* Hap-B6 (PI254189), *4* Hap-B8 (PI286061), *5* Hap-B14 (PI57189), *6* Hap-B16 (PI115515), *7* Hap-B27 (KU-9049), *8* Hap-B36 (KU-14404)

hulled and FT emmer accessions of Type BII (Fig. 5). This suggested that there had been introgression among them. The hulled emmer accessions that had the 9-bp deletion (decrease of CTG repeats) in the 7th exon were found only in the former Yugoslavian countries. In these regions, we found the FT emmer accessions of Type AII, so the unique emmer races would remain there.

Combination of the types of *Ppd-1* and evolution of emmer wheat

In this study, we found that both *Ppd-A1* and *Ppd-B1* were classified into two groups. As discussed above, Types AI and AII of *Ppd-A1* suggested gene flow between wild and hulled emmer. On the other hand, Types BI and BII of *Ppd-B1* suggested gene flow between wild and FT emmer. We could not find distinct evidence of gene flow between wild and FT emmer from *Ppd-A1* and between wild and hulled emmer from *Ppd-B1*; therefore, it is suggested that each type of gene flow among wild and domesticated emmer was limited.

Overall, 44.4 % of hulled emmer accessions and 57.4 % of FT emmer accessions were of Type AI/BI (Table 3). These types of domesticated emmer probably had genetic relationships with each other and spread to various areas without genetic interaction with wild emmer. Overall, 51.1 % of hulled emmer accessions were of Type AII/BI but only 4.3 % of FT emmer accessions were of this type (Table 3). This suggests that over half of the hulled emmer did not contribute to the evolution of FT emmer, and Type AII/BI hulled emmer have new genetic resources that have not been utilized yet. The results showed that 37.2 % of FT emmer accessions were of Type AI/BII, but we could not find the same type of hulled emmer accessions (Table 3).

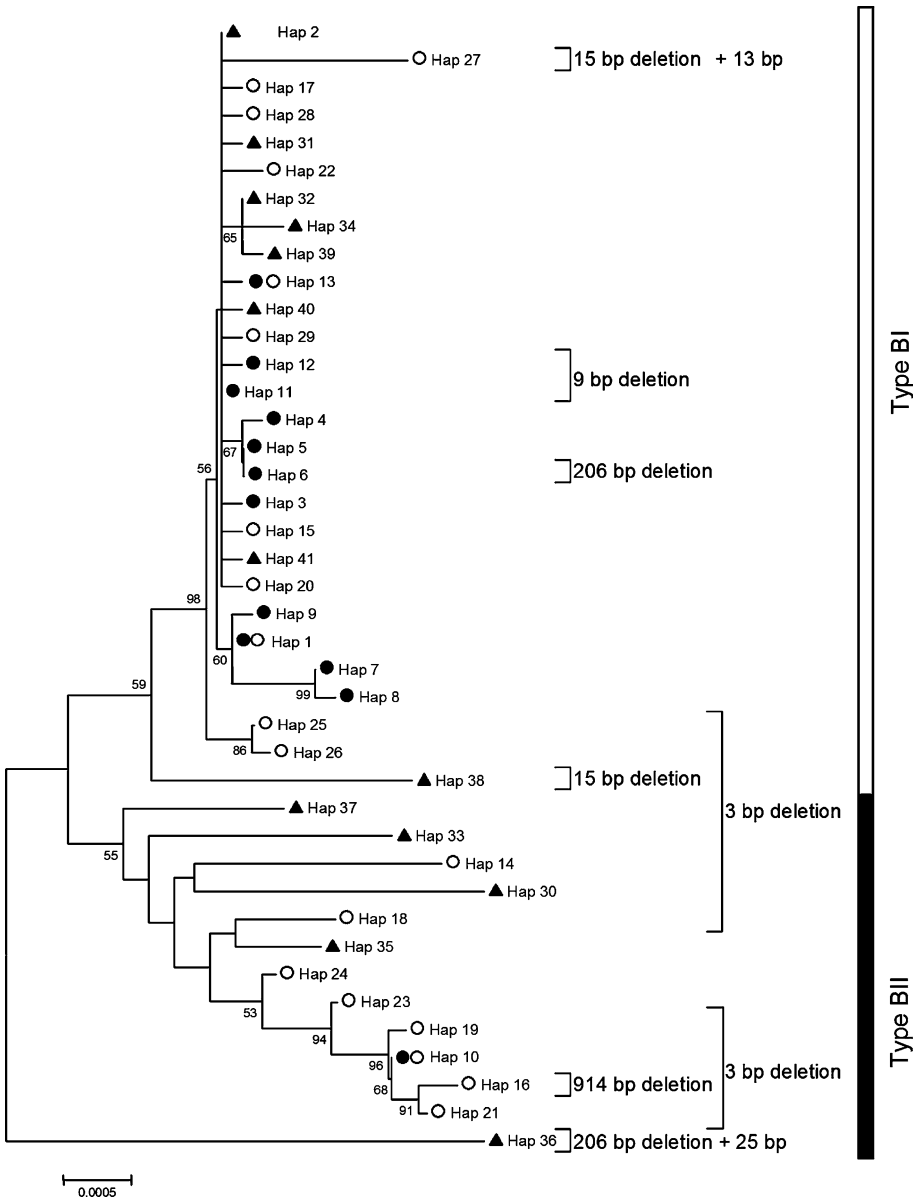

Fig. 5 Neighbor-joining phylogenetic tree built with full sequences of *Ppd-B1* haplotypes. Bootstrap values (1,000 replicates, more than 50) are shown next to the branches. The *black circles*, *white circles* and *black triangles* correspond to domesticated hulled emmer, domesticated free-threshing emmer and wild emmer, respectively. *Right side bar* shows two types of *Ppd-B1*

This type of FT emmer probably did not evolve from the same type of hulled emmer but arose from gene flow from wild emmer. Type AII/BII was very rare and only 4.4 % of hulled emmer and 1.1 % of FT emmer were of this type (Table 3). Each accession that was grouped into Type AII/BII was found sporadically; therefore, this type might have arisen from independent crossing. We need more research on the origin of this unique type of emmer race.

Nucleotide diversity of *Ppd-1*

The values of π of *Ppd-A1* and its surrounding region were the lowest for wild and hulled emmer accessions in intron and exon regions, and for FT emmer accessions in the 5′ UTR region (Fig. 6). This difference was probably caused by the fact that the FT emmer accessions of Type AII were few in number, only 5 accessions in total. For whole sequences, the values of π decreased in the following order: wild emmer accessions ($\pi = 0.00426$), hulled emmer accessions ($\pi = 0.00344$) and FT emmer accessions ($\pi = 0.00131$). This decrease suggests a bottleneck effect in the evolution of stages from wild emmer to hulled domesticated emmer and hulled emmer to FT emmer. However, when the values of π were compared for only intron and exon regions, the value of the hulled emmer accessions was the highest ($\pi = 0.00222$). This indicates that the hulled emmer could probably accumulate mutations easier than wild and FT emmer accessions owing to null mutations, such as the 18-bp, 224-bp and 303-bp deletions.

The values of π of *Ppd-B1* and its surrounding region were the highest for wild, hulled and FT emmer accessions

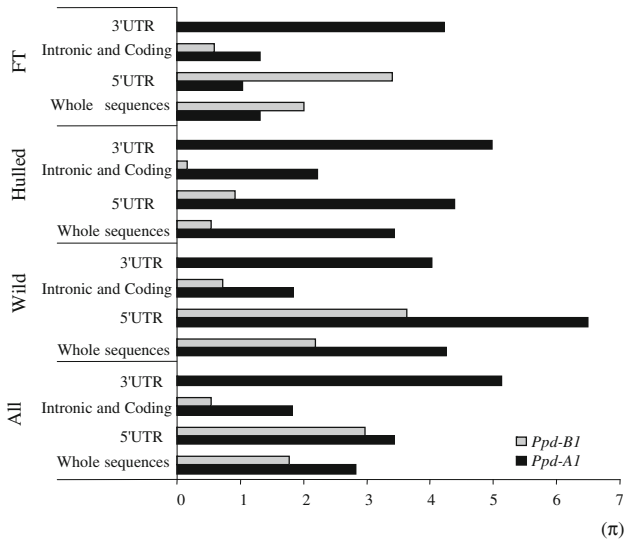

Fig. 6 Nucleotide diversity (π) of *Ppd-A1* and *Ppd-B1*, with values expressed in terms of 10^{-3}, in the wild emmer accessions, domesticated hulled emmer accessions, domesticated free-threshing emmer accessions and all accessions. Nucleotide diversity was estimated from whole sequences, and 5′ UTR, intronic, coding and 3′ UTR regions separately

in the 5′ UTR region (Fig. 6). For the whole sequences, the values of π decreased in the following order: wild emmer accessions ($\pi = 0.00209$), FT emmer accessions ($\pi = 0.00201$) and hulled emmer accessions ($\pi = 0.00055$). Even when the values of π were compared for only intron and exon regions, they also decreased in the same order: wild emmer accessions ($\pi = 0.00073$), FT emmer accessions ($\pi = 0.00059$) and hulled emmer accessions ($\pi = 0.00017$). The decrease of the value from wild emmer to hulled emmer suggests the bottleneck effect of domestication. On the other hand, the increase of the value from hulled emmer accessions to FT emmer accessions is probably the result of gene flow from wild emmer.

New haplotypes and photoperiod sensitivity

In emmer wheat, a photoperiod-insensitive (*Ppd-A1a*) allele was associated with a deletion upstream of the coding region, including regulatory elements, and a loss of its normal expression with a circadian rhythm. On the other hand, a photoperiod-insensitive allele of *Ppd-B1* was not found and *Ppd-B1* gene was normally expressed (Beales et al. 2007; Wilhelm et al. 2009). This indicates that a mutation *Ppd-A1* allele, which cannot be expressed with a circadian rhythm, is dominant over the normal *Ppd-B1* allele and shows a photoperiod-insensitive (Wilhelm et al. 2009). In this study, we found some new type of mutations in *Ppd-1* gene, which had deletions at cording regions. A 303-bp deletion from the 5th exon to the 6th exon of *Ppd-A1* was the same deletion as found in *T. aestivum* L. cv. Cappelle-Desprez. The deletion was not considered a direct cause of photoperiod insensitivity because Cappelle-Desprez is photoperiod-sensitive (Beales et al. 2007). Some datasets on agronomic traits including days to flowering and days to anthesis were reported from USDA and some accessions used in this study were included in these datasets (http://www.ars-grin.gov/cgi-bin/npgs/html/desc.pl?65003). According to these datasets, we could not point to a clear link between photoperiod insensitivity and the deletions found in this study. Therefore, *Ppd-A1* alleles with other deletions (18 bp, 224 bp and 2 bp) that likely showed null alleles were also not direct causes of photoperiod insensitivity. We could not find the mutations that likely damaged the functions of the gene in *Ppd-B1*, excluding KU-9049 (KU-9049 had normal *Ppd-A1* allele). These indicated that mutation *Ppd-1* alleles which lost their functions were recessive for normal *Ppd-1* alleles and the photoperiod sensitivity was maintained. We could reaffirm that mutant *Ppd-1* alleles that cannot be expressed with a circadian rhythm are dominant alleles over normal *Ppd-1* alleles and mutant *Ppd-1* alleles that lost their functions are recessive alleles. The recessive mutation found by us may have no effect for photoperiod sensitivity because emmer wheat is alloploid and has two homologous *Ppd-1* genes. However, we could find such mutations from domesticated emmer and not from wild one. Therefore, mutant *Ppd-1* alleles may play somewhat adaptive roles in domesticated emmer. Null mutants are very useful for studying the functions of genes. In this study, we found four *Ppd-A1* and one *Ppd-B1* alleles that likely lost their functions. These mutations will have great potential for studying the functions of *Ppd-1* gene.

Both *Ppd-A1a* and *Ppd-D1a* alleles have deletions in upstream of the cording regions (Beales et al. 2007; Wilhelm et al. 2009). From *Ppd-B1* gene, we found three types of deletions in same region (a 914-bp deletion, a 206-bp deletion and a 206-bp deletion and a 25-bp unique sequence, Fig. 4a). In this study, we could not check the effect of these deletions on the photoperiod sensitivity.

Emmer wheat landraces from Ethiopia and India are known to mature early (Pecetti et al. 1992; Pecetti and Damania 1996) and some accessions from these regions, as used in this study, also mature earlier than accessions from other regions. However, we could not find unique *Ppd-1* haplotypes in them. In these accessions, there is a possibility that the photoperiod sensitivity is controlled by different genes (Sourdille et al. 2000; Kuchel et al. 2006) or that the narrow-sense earliness is affected more strongly than the photoperiod sensitivity controlled by the *Ppd-1* gene. We need more research on these issues.

A previous study showed that *Ppd-D1* haplotypes that carry mutations outside of the transcriptional regulatory

region do not show clear photoperiod insensitivity but affect various agronomic traits including days to flowering (Guo et al. 2010). Therefore, the new haplotypes found in this study are not immediate causes of photoperiod insensitivity but are important as genetic resources for the fine tuning of genotypes to environmental conditions.

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