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Molecular characterization of benzoxazinone-deficient mutation in diploid wheat

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Abstract

Benzoxazinones (Bxs) are representative defensive compounds in gramineous plants, including wheat (genus *Triticum*) and its wild relative species (genus *Aegilops*). Bx production was found to be variable among three diploid wheat species with the same A genome as hexaploid wheat (2n = 6x = 42, genomes AABBDD). All accessions of *Triticum monococcum* (2n = 2x = 14, AA) and *Triticum urartu* (2n = 2x = 14, AA) accumulated Bxs, but 18 out of 28 accessions of *Triticum boeoticum* (2n = 2x = 14, AA) were Bx-deficient. Bx-deficient accessions were grouped into two types by genomic PCR analysis of the five Bx biosynthetic loci (*TbBx1–TbBx5*): those retaining all five loci (type I) and those lacking *TbBx3* and *TbBx4* loci (type II). Despite the Bx-deficient phenotype, all five *TbBx* genes were transcribed in the type-I accessions. The Bx deficiency in one accession of type I was due to the disintegration of the *TbBx1*, *TbBx4* and *TbBx5* genes due to insertions or deletions in their coding sequences. The *TbBx2* and *TbBx3* genes of those accessions had the complete sequences of the functional enzymes. In the type-II accessions, the remaining three genes, *TbBx1*, *TbBx2* and *TbBx5*, were all transcribed, with the exception of two accessions in which either *TbBx1* or *TbBx5* was not transcribed. The *TbBx1* coding sequence of the type-II accessions was also disintegrated, like that of the type-I accessions. These findings suggest that the Bx deficiency in *T. boeoticum* first resulted from disintegration of the *TbBx1* coding sequence, followed by transcription failure, disintegration of the coding sequences and elimination of the *TbBx1–TbBx5* genes.

Keywords: *Triticum boeoticum*; Gramineae; Wheat; Secondary metabolism; Biosynthesis; Benzoxazinone; DIBOA; DIMBOA; Mutant

1. Introduction

The benzoxazinones (Bxs), the major secondary metabolites in gramineous plants, including hexaploid wheat (*Triticum aestivum*), rye (*Secale cereale*) and maize (*Zea mays*), are involved in their defense against pathogens and insects (Niemeyer, 1988; Sicker et al., 2000). Two representative Bxs are 2,4-dihydroxy-1,4-benzoxazin-3-one (**1**) (DIBOA) and its 7-methoxy derivative **2** (DIMBOA), which are constitutively present in the vacuole in the form of glucosides (DIBOA-Glc **3** and DIMBOA-Glc **4**). Their amounts reach a maximum soon after germination, and then decrease gradually to constant, low levels. Thus, the Bxs are considered to be important to the defense of plants during the juvenile stage of growth (Nakagawa et al., 1995; Ebisui et al., 1998).

The biosynthetic pathway of Bxs branches off from that of tryptophan at indole-3-glycerol phosphate **5** (Frey et al., 1997, 2000; Melanson et al., 1997; Gierl and Frey, 2001). Five consecutive reactions lead from indole-3-glycerol phosphate **5** to the formation of DIBOA **1** (Fig. 1). The

Abbreviations: Bx, benzoxazinone; CS, Chinese Spring; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; MITE, miniature inverted-repeat transposable element; TIR, terminal inverted repeat; TSD, target site duplication.

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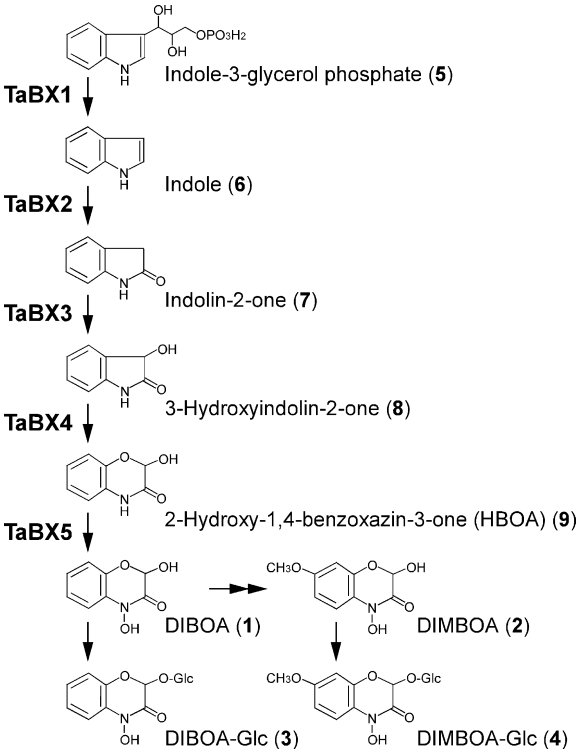

Fig. 1. Bx biosynthetic pathway showing the enzymatic reactions controlled by the *TaBx1–TaBx5* genes of hexaploid wheat.

genes involved have been isolated from maize (*ZmBx1-ZmBx5* genes; Frey et al., 1995, 1997), wild barley (*Hordeum lechleri*, *HlBx1–HlBx5*; Grün et al., 2005), and hexaploid wheat (*TaBx1A–TaBx5A*, *TaBx1B–TaBx5B*, and *TaBx1D–TaBx5D*; Nomura et al., 2002, 2003, 2005).

Hexaploid wheat ($2n = 6x = 42$, genome formula AABBDD) arose from two hybridization events followed by chromosome doubling of three ancestral diploid species ($2n = 2x = 14$) with different genomes A, S(≈B) and D. There are three A-genome diploid species, *Triticum boeoticum*, *Triticum urartu* and *Triticum monococcum*. *T. monococcum* is a domesticated form of the wild species *T. boeoticum*, and *T. urartu* is the donor of the A genome of hexaploid wheat (Dvorák et al., 1988, 1993; Dvorák and Zhang, 1992; Takumi et al., 1993). The donors of the B and D genomes of hexaploid wheat are *Aegilops speltoides* (SS) and *Ae. tauschii* (DD), respectively (Huang et al., 2002a). Nomura et al. (2002, 2003) demonstrated that the five Bx biosynthetic genes are all present in those three diploid progenitors.

Wheat, rye ($2n = 2x = 14$, RR) and barley (*Hordeum vulgare*, $2n = 2x = 14$, HH), which belong to the tribe Triticeae, are considered to be derived from a common ancestor (Huang et al., 2002b). Nevertheless, the occurrence of Bxs is not ubiquitous in Triticeae. Although some wild barley species biosynthesize the Bxs (Barria et al., 1992; Grün et al., 2005), cultivated barley is devoid of all five Bx biosynthetic genes (Gierl and Frey, 2001; Nomura et al., 2003); instead it accumulates another defensive secondary metabolite 'gramine' (Grün et al., 2005). Recently, we have found a Bx-deficient variant of *T. boeoticum* (unpublished data). The Triticeae species seem to have been developing different defense system utilizing secondary metabolites. This raised other questions: whether the deficiency in Bxs is found only in *T. boeoticum* or is also found in the other A-genome species, i.e. *T. monococcum* and *T. urartu*, and what happened to the Bx biosynthetic genes in such variants? In the present study, we first surveyed the Bx production in three A-genome species, *T. boeoticum*, *T. monococcum* and *T. urartu*, and found that some of the *T. boeoticum* accessions were deficient in Bxs. Then we investigated the Bx biosynthetic genes in detail to clarify the cause of Bx deficiency in those accessions.

2. Results

2.1. Variation of Bx production in A-genome diploid wheat species

Bxs were detected in 48-h-old shoots and roots of all accessions of *T. monococcum* (24 accessions) and *T. urartu* (15 accessions). However, Bxs were found only in 10 of the 28 accessions of *T. boeoticum* (Table 1, Fig. 2). The

Table 1
Accessions of A-genome diploid species used in this study. (+) and (−) after the accession numbers indicate the presence and absence of Bxs, respectively

T. boeoticum	*T. urartu*	*T. monococcum*
KU1501 (+)[a]	KU199-1 (+)	KU104-1 (+)
KU1519 (+)	KU199-2 (+)	KU104-2 (+)
KU8417 (+)	KU199-3 (+)	KU104-3 (+)
KU10603 (+)	KU199-4 (+)	KU105 (+)
KU10730 (+)	KU199-5 (+)	KU106 (+)
KU10760 (+)	KU199-6 (+)	KU1404 (+)
KU10782 (+)	KU199-7 (+)	KU1420 (+)
KU10812 (+)	KU199-8 (+)	KU1427 (+)
KU10833 (+)	KU199-9 (+)	KU1428 (+)
KU10859 (+)	KU199-11 (+)	KU1431 (+)
KU102 (−)	KU199-12 (+)	KU1432 (+)
KU8026 (−)	KU199-13 (+)	KU3636 (+)
KU8103 (−)	KU199-14 (+)	KU3637 (+)
KU8116 (−)	KU199-15 (+)	KU3640 (+)
KU8128 (−)	KU199-16 (+)	KU3641A (+)
KU8134 (−)		KU3641B (+)
KU8150 (−)		KU11040 (+)
KU8221 (−)		KU11047 (+)
KU8235 (−)		KU11070 (+)
KU8259 (−)		KU11072 (+)
KU8266 (−)		KU11083 (+)
KU8397 (−)		KU11084 (+)
KU8404 (−)		KU11357 (+)
KU8407 (−)		KT3-5 (+)
KU8412 (−)		
KU10653 (−)		
KU10867 (−)		
KT1-1 (−)[b]		

[a] KU numbers are the accession numbers in the Graduate School of Agriculture, Kyoto University, Japan.
[b] KT numbers are the accession numbers in the Kihara Institute for Biological Research, Yokohama City University, Japan.

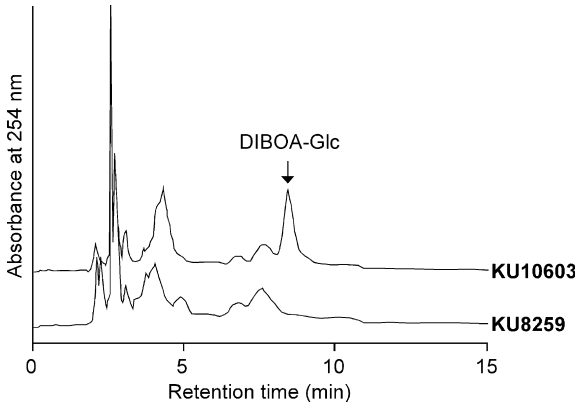

Fig. 2. HPLC chromatograms of shoot extracts from two *T. boeoticum* accessions KU10603 (Bx-accumulating) and KU8259 (Bx-deficient).

detected Bx was DIBOA-Glc **3** in both the shoots and roots.

2.2. TbBx1–TbBx5 loci in Bx-deficient accessions

Genomic PCR revealed that all 10 Bx-accumulating accessions of *T. boeoticum* possessed all five Bx biosynthetic genes (*TbBx1–TbBx5*) (Fig. 3). Nine of the 18 Bx-deficient accessions had all five Bx biosynthetic loci (type I), whereas the remaining nine accessions had the *TbBx1*, *TbBx2* and *TbBx5* loci but lacked the *TbBx3* and *TbBx4* ones (type II) (Fig. 3).

The *TbBx5* PCR product was larger in two accessions, KU1501 and KU8221, than in the other accessions (Fig. 3), but the *TbBx5* RT-PCR product was the same in size for all accessions (Fig. 5). The larger genomic *TbBx5* fragments in the two accessions were presumably attributable to their larger introns.

Southern analysis using as probes the *TaBx3A* and *TaBx4A* cDNAs of a hexaploid wheat cultivar Chinese Spring (CS) demonstrated that two type-I accessions (KU8103 and KU8128) retained the *TbBx3* and *TbBx4* loci but that two type-II accessions (KU8116 and KU102) lacked them (Fig. 4), as speculated from the PCR analysis.

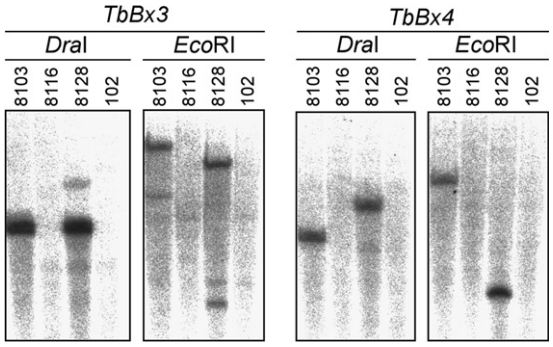

Fig. 4. Southern analysis of four *T. boeoticum* accessions. Note that the *TbBx3* and *TbBx4* loci are absent from KU8116 and KU102.

This result implies that the other type-II accessions are also devoid of the *TbBx3* and *TbBx4* loci.

2.3. TbBx1–TbBx5 transcripts in Bx-deficient accessions

The *TbBx* transcripts were investigated by RT-PCR analysis in two Bx-accumulating accessions, and all type-I and type-II Bx-deficient accessions (Fig. 5). The RT-PCR products of all *TbBx1–TbBx5* genes were detected in all type-I accessions as well as in the two Bx-accumulating accessions. In contrast, no transcripts of *TbBx3* and

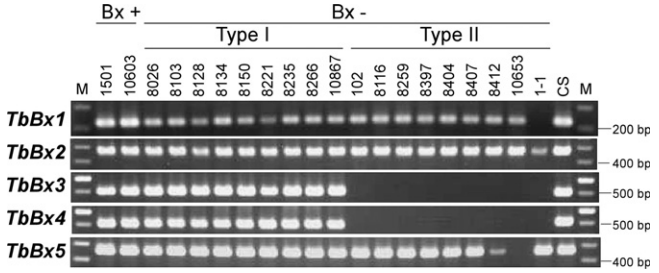

Fig. 5. RT-PCR analysis of the transcripts of the five Bx biosynthetic genes (*TbBx1–TbBx5*) in 20 accessions of *T. boeoticum*, including two Bx-accumulating (Bx+) and 18 Bx-deficient (Bx−) accessions. CS was used as a positive control. *M* = 100-bp DNA ladder marker.

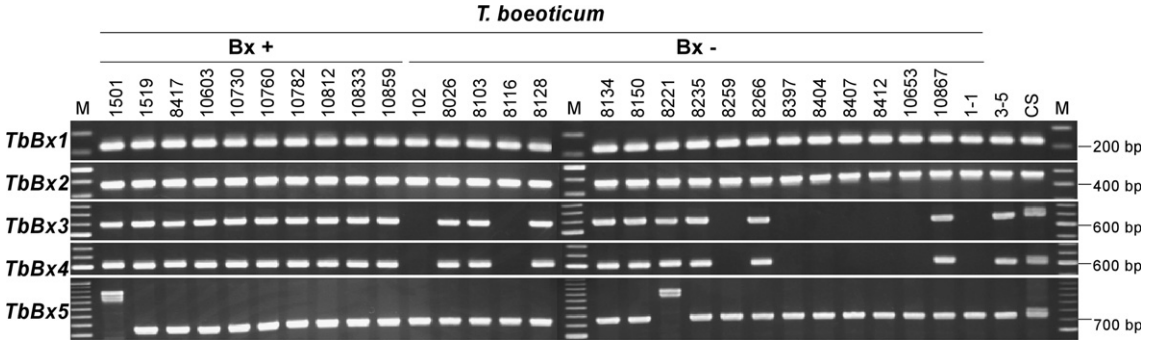

Fig. 3. Genomic PCR analysis of the five Bx biosynthetic loci (*TbBx1–TbBx5*) in 28 accessions of *T. boeoticum*, including 10 Bx-accumulating (Bx+) and 18 Bx-deficient (Bx−) accessions. KT3-5 (3-5) and CS were used as positive controls. *M* = 100-bp DNA ladder marker.

Table 2
TbBx cDNAs isolated from three *T. boeoticum* accessions

Accession	Gene [a]	Open reading frame	Insertion or deletion [b]	GenBank accession no.
KU8026	*TbBx1-1P*	Incomplete	91-bp insertion (688–778)	AB255437
	TbBx1-2P	Incomplete	91-bp insertion (223–313), and 91-bp insertion (779–869)	AB255438
	TbBx1-3P	Incomplete	10-bp insertion (688–697)	AB255439
	TbBx1-4P	Incomplete	55-bp deletion (222/223), and 91-bp insertion (633–723)	AB255440
	TbBx2-1	Complete	None	AB255441
	TbBx3-1	Complete	None	AB255442
	TbBx4-1P	Incomplete	2-bp insertion (73–74)	AB255443
	TbBx5-1P	Incomplete	388-bp insertion (MITE[c]) (95–482), and 3-bp insertion (514–516)	AB255444
	TbBx5-2P	Incomplete	67-bp insertion (95–161), 15-bp deletion (161/162), and 3-bp insertion (178–180)	AB255445
	TbBx5-3P	Incomplete	4-bp insertion (95–98), 25-bp deletion (98/99), and 3-bp insertion (105–107)	AB255446
KU8259[d]	*TbBx1-5P* [e]	Incomplete	91-bp insertion (688–778)	AB255447
	TbBx2-1	Complete	None	–[f]
	TbBx5-4	Complete	3-bp insertion (126–128) (no reading-frame change)	AB255448
KT1-1[g]	*TbBx2-1*	Complete	None	–[f]
	TbBx5-5 [h]	Complete	3-bp insertion (126–128) (no reading-frame change)	AB255449

[a] P stands for pseudogene encoding incomplete protein.
[b] Insertions and deletions of the *TbBx* cDNAs were detected relative to the *TaBx* cDNAs from the A genome of hexaploid wheat. Numbers in parentheses represent the nucleotide numbers from the start codon.
[c] See text and Fig. 6 for details.
[d] *TbBx3* and *TbBx4* loci are absent.
[e] There is a one-nucleotide difference between *TbBx1-1P* and *TbBx1-5P*.
[f] Nucleotide sequence is identical to that of the *TbBx2-1* in KU8026.
[g] The *TbBx1* locus is not transcribed, and the *TbBx3* and *TbBx4* loci are absent.
[h] There is a one-nucleotide difference between *TbBx5-4* and *TbBx5-5*.

TbBx4 were detected in the type-II accessions. Two of the type-II accessions, KU10653 and KT1-1, also lacked the transcripts of *TbBx5* and *TbBx1*, respectively.

2.4. Disintegration of TbBx coding sequences in Bx-deficient accessions

Transcription of the *TbBx1–TbBx5* genes in all type-I accessions suggested that one or more coding sequences of the five *TbBx* genes were disintegrated. In a type-I accession, KU8026, cDNAs covering the entire coding sequences of the *TbBx* genes were isolated and sequenced (Table 2). None of the isolated four *TbBx1* cDNAs (*TbBx1-1P–TbBx1-4P*; P represents pseudogene) encoded the complete enzyme due to insertions or deletions. There were neither insertions nor deletions in the coding regions of the *TbBx2* and *TbBx3* cDNAs (*TbBx2-1* and *TbBx3-1*), showing that they encode functional enzymes. The cDNAs of *TbBx4* and *TbBx5* (*TbBx4-1P*, *TbBx5-1P*, *TbBx5-2P*, and *TbBx5-3P*) also had an insertion or a deletion, resulting in the disintegration of the coding sequences.

The 388-bp insertion found in *TbBx5-1P* had 16-bp terminal inverted repeats (TIRs) flanked by 8-bp target site duplications (TSDs) (Fig. 6). This structural feature suggests that this insertion sequence is a miniature inverted-repeat transposable element (MITE). The 67-bp insertion in *TbBx5-2P* contained one end of the TIR and part of the internal sequence of the MITE, but lacked the TIR and TSD at the 3′ end. The 4-bp insertion in *TbBx5-3P* was part of the TIR.

The coding regions of the *TbBx* cDNAs from the two type-II accessions (KU8259 and KT1-1) were cloned and sequenced to determine whether the remaining three *TbBx* genes (*TbBx1*, *TbBx2*, and *TbBx5*) encode complete enzymes even after the loss of the *TbBx3* and *TbBx4* loci (Table 2). The coding sequence of the *TbBx1-5P* of KU8259 was disintegrated due to the same 91-bp insertion as found in the *TbBx1* sequences of KU8026. On the other hand, the *TbBx2-1* and *TbBx5-4* cDNAs encoded complete enzymes. The *TbBx2-1* and *TbBx5-5* cDNAs of KT1-1 also encoded complete enzymes.

2.5. The 91-bp insertion in the TbBx1 cDNAs

The 91-bp insertion sequences found in the *TbBx1* cDNAs of KU8026 (type I) and KU8259 (type II) were analyzed by genomic- and RT-PCR in all type-I and type-II Bx-deficient accessions, two Bx-accumulating accessions (KU1501 and KU10603) and CS, except for KT1-1, in which the *TbBx1* gene was not transcribed. As shown in Fig. 7a, no difference in the size of the genomic PCR product was observed among these accessions and CS, but the size of the RT-PCR product was approximately 100 bp larger in all Bx-deficient accessions than in Bx-accumulating accessions and CS. Sequencing of the genomic PCR fragments revealed that Bx-accumulating accessions, KU1501 and KU10603, had a 91-bp intron with 'GT' and 'AG' at the 5′- and 3′-ends, respectively, but that all

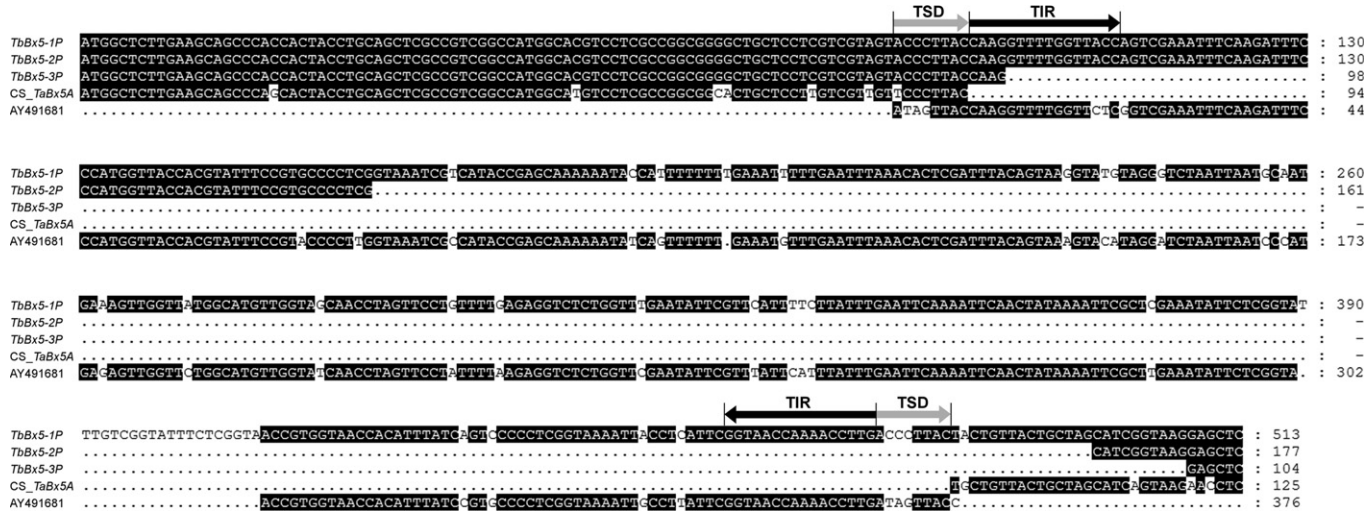

Fig. 6. The nucleotide sequence of the MITE-containing region in the *TbBx5-1P* cDNA isolated from *T. boeoticum* KU8026. It was aligned with those of the other two *TbBx5* cDNAs from KU8026, *TaBx5A* cDNA from CS (GenBank accession no. AB042629, Nomura et al., 2005) and BAC clone 109N23 of *T. monococcum* (AY491681, Chantret et al., 2004). The gray and black arrows indicate 8-bp TSDs and 16-bp TIRs, respectively.

Bx-deficient accessions had 'G to C' transversions at the 3′-ends of the intron, which probably interfered with the splicing (Fig. 7b). These results indicate that the 91-bp insertion found in the *TbBx1* cDNAs was an unspliced intron that was common to the type-I and type-II Bx-deficient accessions.

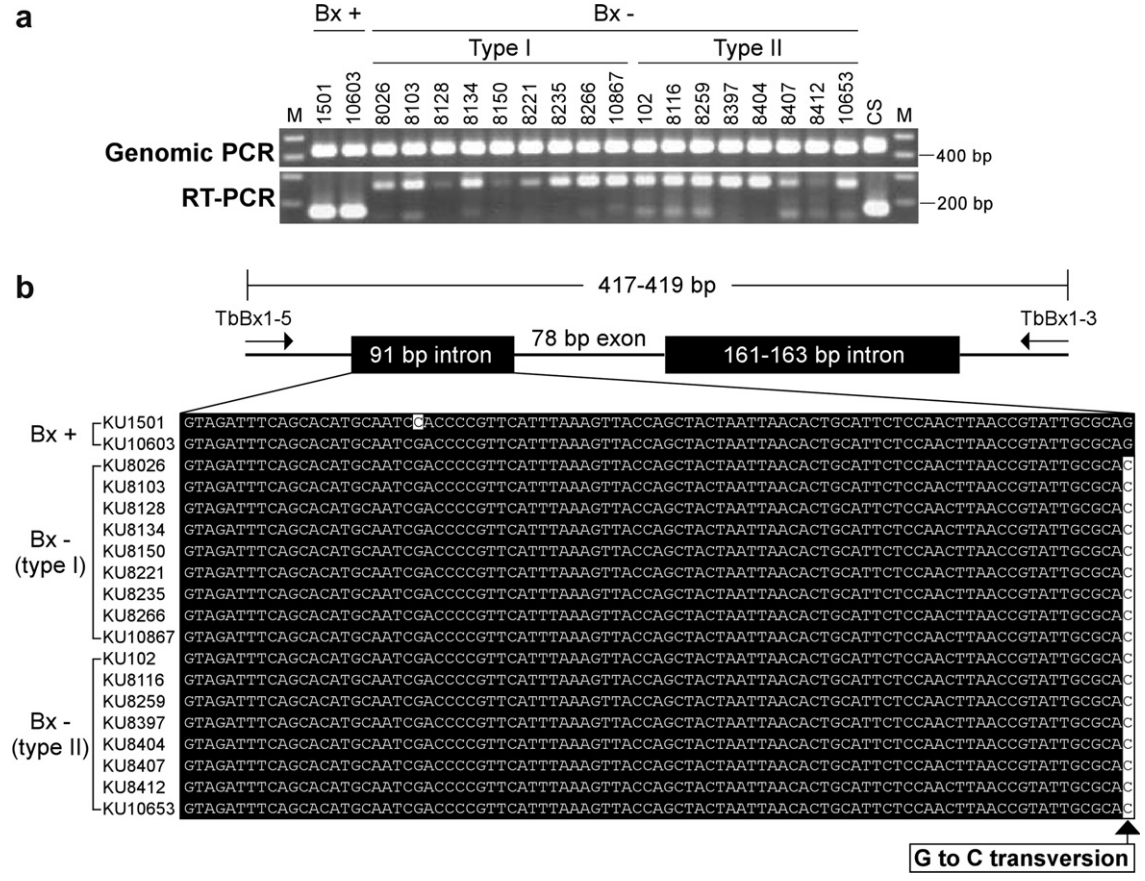

Fig. 7. PCR and sequencing analyses of the *TbBx1* fragment covering the 91-bp insertion in the *TbBx1* cDNAs. (a) Genomic- and RT-PCR analyses of 19 *T. boeoticum* accessions, including two Bx-accumulating (Bx+) and 17 Bx-deficient (Bx−) accessions. Note that unspliced introns were present in all Bx-deficient accessions. CS was used as a positive control. $M = 100$-bp DNA ladder marker. (b) The exon–intron structure of the *TbBx1* genomic fragment amplified with primers TbBx1-5 and TbBx1-3. The fragment length ranges from 417 to 419 bp due to the different length of the second intron, ranging from 161 to 163 bp among the accessions. The alignment of the 91-bp introns of 19 *T. boeoticum* accessions shows a 'G to C' transversion at the 3′ end of the intron in all Bx-deficient accessions.

Table 3
Classification of the Bx biosynthetic loci in *T. boeoticum*

Class	Locus	Transcript	Complete enzyme	Classified locus
1	+[a]	+	+	*TbBx1–TbBx5* in Bx-accumulating accessions, *TbBx2* and *TbBx3* in KU8026, and *TbBx2* and *TbBx5* in KU8259 and KT1-1
2	+	+	–[a]	*TbBx1*, *TbBx4* and *TbBx5* in KU8026, and *TbBx1* in KU8259
3	+	–	–	*TbBx5* in KU10653, and *TbBx1* in KT1-1
4	–	–	–	*TbBx3* and *TbBx4* in type-II Bx-deficient accessions

[a] + and – represent presence and absence, respectively.

3. Discussion

3.1. Process leading to the inactivation of the Bx biosynthetic pathway

The Bx biosynthetic loci in *T. boeoticum* were classified into four groups in terms of their coding region integrity, transcription, and existence (Table 3). Presumably, three independent evolutionary events occurred in the Bx biosynthetic genes in *T. boeoticum*: the loss of the enzyme function due to either insertions or deletions in the coding region (class 2 in Table 3); the loss of transcriptional activity (class 3); and the loss of the loci from the genome (class 4). A possible scenario for the diversification of the Bx biosynthetic genes in *T. boeoticum* is shown in Fig. 8. The differentiation of the type-I and type-II accessions was preceded by the 'G to C' transversion at the splice site of the *TbBx1* intron, which was the first event leading to the Bx deficiency. In the type-I accessions, the *TbBx4* and *TbBx5* genes mutated further, and in type-II accessions *TbBx3* and *TbBx4* loci were eliminated and then either *TbBx1* (in KT1-1) or *TbBx5* (in KU10653) was transcriptionally inactivated.

The presence of multiple Bx-deficient accessions indicates that the Bx-deficiency is not crucial for *T. boeoticum* to survive in the wild. However, no Bx-deficient accessions were found that have mutations in *TbBx2–TbBx5* while retaining the functional *TbBx1* gene. Similarly, one maize Bx-deficient mutant, which had a deletion in *ZmBx1*, was found (Frey et al., 1997; Melanson et al., 1997). Mutated TbBX1 is not considered to be harmful to plant metabolism. The substrate of TbBX1, indole-3-glycerol phosphate **5**, is converted to tryptophan by tryptophan synthase. A key enzyme in the tryptophan pathway is anthranilate synthase, which catalyses a reaction four steps before TbBX1. The flow of the pathway is strictly regulated by the feedback inhibition of anthranilate synthase by tryptophan (Belser et al., 1971; Widholm, 1972). Thus, the decreased demand for indole-3-glycerol phosphate **5** by a *TbBx1* mutation dose not directly disturb the tryptophan pathway.

Nine type-II Bx-deficient accessions had neither the *TbBx3* nor *TbBx4* loci. Their orthologous loci in hexaploid wheat, *TaBx3* and *TaBx4*, are located on the same chromosomal region in each of the homoeologous group-5 chromosomes, 5A, 5B and 5D (Nomura et al., 2003). No recombination was observed between the *TbBx3* and *TbBx4* loci in a linkage analysis using the recombinant inbred lines produced from a cross between *T. boeoticum* and *T. monococcum* (unpublished data). These facts suggest that the *TbBx3* and *TbBx4* loci are closely linked to each other. Most likely the *TbBx3* and *TbBx4* loci were simultaneously eliminated in the type-II accessions.

3.2. Evolution of biosynthetic pathway of plant secondary metabolites

Recently it has been demonstrated that the Bx-producing wild *Hordeum* species are not able to accumulate gramine, another defensive secondary metabolite derived from the tryptophan pathway, and conversely that gramine-accumulating species are deficient in Bxs (Grün et al., 2005). This implies the occurrence of mutual exclusion of one of these

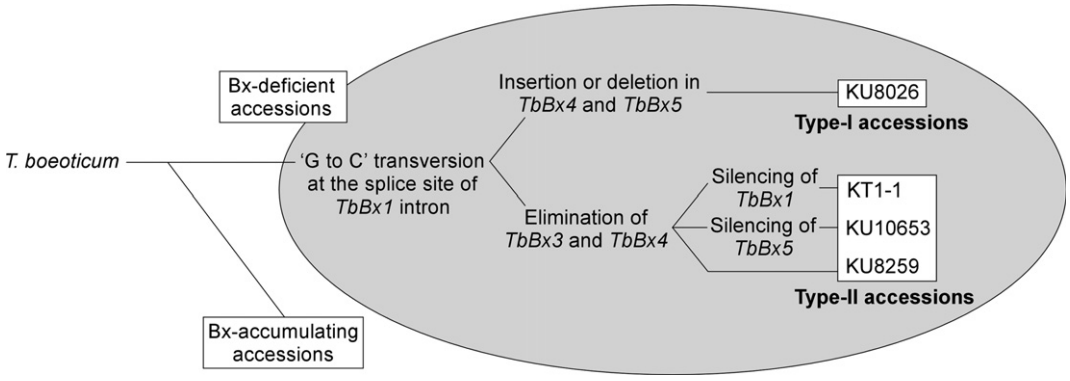

Fig. 8. A schematic representation showing diversification of the five Bx biosynthetic loci (*TbBx1–TbBx5*) in Bx-deficient accessions of *T. boeoticum*.

two secondary metabolites with a common biosynthetic origin. Another example of similar mutual exclusion is that no plant has both types of two red pigments, anthocyanins and betacyanins (Stafford, 2000). The lack of anthocyanins in the betacyanin-producing Caryophyllales plants is attributable to the suppression of the two anthocyanin biosynthetic genes (Shimada et al., 2005). Grün et al. (2005) suggested that a competition for substrates by two secondary metabolic pathways may lead to the elimination of one pathway, which might help to balance primary and secondary metabolism in plants. Alternatively, the suppression of an existing pathway is prerequisite for the acquisition of a new pathway that shares a common precursor because available resources for defensive secondary metabolism are limited. Thus, the Bx-deficient accessions of *T. boeoticum* might have evolved unknown biosynthetic pathways of other defensive secondary metabolites that share the same precursor as that of Bxs. Although only the loss of Bxs was found by the present HPLC analysis in the Bx-deficient accessions, a more detailed analysis might help detect other defense metabolites.

3.3. A MITE sequence in the TbBx5-1P cDNA

None of the three *TbBx5* cDNAs isolated from the Bx-deficient *T. boeoticum* KU8026 encoded a complete enzyme due to insertions or deletions in the coding regions. The 388-bp insertion in *TbBx5-1P* cDNA was identified as a MITE from the presence of TIR and TSD sequences and also its short length (~500 bp), both of which are typical characteristics of MITEs. The insertion of the MITE in the exon is also consistent with the general feature of MITEs that they are preferentially inserted into genic regions (Zhang et al., 2000). A sequence significantly similar to that of the MITE was found in the BAC clone 109N23 of *T. monococcum* (GenBank accession no. AY491681; Chantret et al., 2004) by a BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/) using the 388-bp sequence as a query (Fig. 6). This is reasonable because *T. monococcum* is a domesticated species derived from *T. boeoticum*. We designated this MITE "*Escapee*" (GenBank accession no. AB258455).

The cDNAs *TbBx5-2P* and *TbBx5-3P* were shorter than the *TbBx5-1P* by 336 bp and 409 bp, respectively, due to interstitial deletions including parts of the MITE. The deletions might have resulted from alternative splicing of the *TbBx5-1P* transcript because both deleted sequences had 'GT' and 'AG' at their 5′- and 3′-ends (Fig. 6). Since their 5′-ends are positioned at internal region of the MITE (*TbBx5-2P*) and at TIR (*TbBx5-3P*), the MITE itself might have triggered the alternative splicing. Alternatively, the remaining partial MITE sequences in the *TbBx5-2P* and *TbBx5-3P* cDNAs might be the excision footprints of the MITE. If this is the case, it is likely that the *TbBx5* locus into which the MITE had been inserted was duplicated, and that the insertion was later removed from two of the *TbBx5* loci, leaving the footprints.

4. Conclusions

Bx production was variable among the three A-genome diploid wheat species. *T. monococcum* and *T. urartu* accumulated Bxs, but some accessions of *T. boeoticum* were deficient in Bxs. We established that all Bx-deficient accessions had a 'G to C' transversion at the 3′-end of the intron of the *TbBx1* gene, which would cause the disintegration of the *TbBx1* coding sequence. We also found transcription failure, disintegration of coding sequences and sequence elimination of other Bx biosynthetic genes in some of the Bx-deficient accessions. We hypothesize that the 'G to C' transversion in the *TbBx1* gene was the first event leading to deactivation of the Bx biosynthetic pathway in *T. boeoticum*, followed by other disruptive events in the *TbBx* genes. Recently, the absence of one of the two secondary metabolites with a common biosynthetic origin has been reported in wild barley (Grün et al., 2005) and Caryophyllales plants (Stafford, 2000). This implies that the Bx-deficient accessions of *T. boeoticum* might have evolved unknown biosynthetic pathways of other defensive secondary metabolites that share the same precursor as that of Bxs.

5. Experimental

5.1. Plant materials

A total of 67 accessions of A-genome diploid wheat ($2n = 2x = 14$, AA) were used in this study: 28 accessions of *T. boeoticum*, 24 accessions of *T. monococcum* and 15 accessions of *T. urartu* (Table 1). A hexaploid wheat (*T. aestivum*, $2n = 6x = 42$, AABBDD) cultivar Chinese Spring (CS) was used as a control. All seed stocks used in this study were obtained from the National BioResource Project-Wheat, Japan (http://www.nbrp.jp) except for *T. boeoticum* accession KT1-1 and *T. monococcum* accession KT3-5, which were obtained from the Kihara Institute for Biological Research, Yokohama City University, Japan. Seeds were germinated and grown as described previously (Nomura et al., 2002).

5.2. Extraction and HPLC analysis of Bxs

Bxs were extracted from shoots and roots of 48-h-old seedlings and analyzed by HPLC according to Nomura et al. (2005).

5.3. Genomic PCR analysis for the detection of the TbBx1–TbBx5 loci

Total DNA was isolated from 10-d-old shoots by the CTAB method. PCR was performed in a 20-μl reaction mixture containing 50 ng of template DNA, each primer at 0.5 μM, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 × PCR enhancer solution (Invitrogen) (only for the amplification

of *TbBx1*), 1 × reaction buffer, and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen). After an initial incubation for 2 min at 94 °C, 40 cycles of amplification were performed (30 s at 94 °C and 1 min at 64 °C). The PCR primers for the *TaBx1–TaBx5* orthologs of *T. boeoticum* (designated as *TbBx1–TbBx5* genes) were designed based on the *TaBx1–TaBx5* cDNA sequences of CS wheat (Nomura et al., 2005). Identical nucleotide sequences among the three homoeologs of each *TaBx* cDNA were used as the primer sequences. Forward (F) and reverse (R) primers are as follows: TaBx1-F (5′-CAGCGCTCATCCCGTATATCAC-3′), TaBx1-R (5′-CTCCGGCGTCACCTCCTTG-3′), TaBx2-F (5′-CCGCGGTCGATGGTCTTCGA-3′), TaBx2-R (5′-ATGGTCGTCAATTAGCTTGTCGAG-3′), TaBx3-F (5′-ATAGATCTTCTGCTCTCTCTCAAG-3′), TaBx3-R (5′-CAAATGGCAGGAACCTAAGATC-3′), TaBx4-F (5′-CGAGATCAGCGCCTCCCTGC-3′), TaBx4-R (5′-TTGGTGGAGAAGTGTGGGAGC-3′), TaBx5-F (5′-AAAGGCCGCAACACGCTCTTC-3′), and TaBx5-R (5′-TGCTGAGGTCCTCCTCCGTAAT-3′). After the PCR reaction, aliquots (5 μl) of the mixture were electrophoresed on 2% (w/v) agarose gels and stained with ethidium bromide.

5.4. Southern analysis

Southern analysis of the *TbBx3* and *TbBx4* genes was performed according to Nomura et al. (2003). Aliquots (20 μg) of total DNA were digested with *Dra*I or *Eco*RI. The DNA blots were hybridized with ^{32}P-labeled *TaBx3A* (GenBank accession no. AB042628) and *TaBx4A* (AB124854) cDNA probes.

5.5. RT-PCR analysis for the detection of the TbBx1–TbBx5 transcripts

Total RNA was isolated from 48-h-old shoots using an RNeasy Plant Mini Kit (Qiagen). After DNase treatment, RNA was purified again with an RNeasy column. Single-stranded cDNA was synthesized from 500 ng of the total RNA using oligo-dT primer with of the Thermoscript RT-PCR system (Invitrogen) in a 20-μl reaction mixture. PCR analysis was carried out using 2 μl (equivalent to 50 ng) of the cDNA mixture as a template under the same conditions as described for the genomic PCR. PCR was performed prior to the reverse transcription to confirm that no genomic DNA was contaminating the total RNA samples.

5.6. Cloning of the TbBx1–TbBx5 cDNAs

Using as template the cDNA mixture (50 ng) synthesized as described above, PCR was performed in a 50-μl reaction mixture containing each primer at 0.5 μM, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 1 × reaction buffer, and 1 U of KOD-plus DNA polymerase (Toyobo). After an initial incubation for 2 min at 94 °C, 35 cycles of amplification were performed (30 s at 96 °C, 30 s at 64 °C, and 1 min at 68 °C). The forward (F) and reverse (R) primers were designed at the 5′- and 3′-UTRs of the *TaBx* cDNAs on the A genome of CS wheat (Nomura et al., 2005): TaBx1A-F (5′-CACAAGAAGGTTTCTCAGTCAGC-3′), TaBx1A-R (5′-TTACTGGTGCTGTGTCCATCC-3′), TaBx2A-F (5′-CGATAGCTAGCTCTCTCTTAGCATT-3′), TaBx2A-R (5′-GCACCAAATACCATGATGGCTC-3′), TaBx3A-F (5′-TCCTGATCAGTTGT GTGGACC-3′), TaBx3A-R (5′-TCCTTGCCTTTATTCTTGTGCTC-3′), TaBx4A-F (5′-CTAGTCAGCTGTCT GGACGCC-3′), TaBx4A-R (5′-CATAACGGACAATGGTTAGCTGAC-3′), TaBx5A-F (5′-TTCCTGGTCAG TTGTGGACG-3′), and TaBx5A-R (5′-GGAATCAGGG GCACTACTAGTT-3′).

After the 3′-ends were adenylated by the A-tailing reaction (30 min incubation at 70 °C in 50-μl mixture containing 0.2 mM dATP and 5 U of *Taq* DNA polymerase), the PCR products were cloned into the pT7Blue T-vector (Novagen) for DNA sequence analysis.

5.7. PCR and sequence analyses of the TbBx1 fragments

Total DNA, total RNA and cDNA were prepared as described above. Genomic- and RT-PCR were performed in a 20-μl reaction mixture containing 50 ng of genomic DNA and cDNA, respectively, each primer at 0.5 μM, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 × reaction buffer, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen). After an initial incubation for 2 min at 94 °C, 40 cycles of amplification were performed (30 s at 96 °C, 30 s at 60 °C, and 1 min at 72 °C). To amplify the *TbBx1* fragments containing a 91-bp insertion, primers TbBx1-5 (5′-GAATCACGAAAGCTTCGGAAGG-3′) and TbBx1-3 (5′-CGTGTTCAGGTTTTGATATGCC-3′) were designed from the nucleotide sequences outside of the region (Fig. 7b).

For the sequencing of the genomic *TbBx1* fragment, PCR was performed in a 50-μl reaction mixture containing 50 ng of genomic DNA, 0.5 μM each of the primers TbBx1-5 and TbBx1-3, 0.2 mM dNTPs, 1 mM $MgSO_4$, 1 × reaction buffer, and 1 U of KOD-plus DNA polymerase (Toyobo). After an initial incubation for 2 min at 94 °C, 30 cycles of amplification were performed (30 s at 96 °C, 30 s at 64 °C, and 30 s at 68 °C). Subcloning for sequence analysis was performed as described above.

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