Dispersed Benzoxazinone Gene Cluster: Molecular Characterization and Chromosomal Localization of Glucosyltransferase and Glucosidase Genes in Wheat and Rye[1[W]]

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Benzoxazinones (Bxs) are major defensive secondary metabolites in wheat (*Triticum aestivum*), rye (*Secale cereale*), and maize (*Zea mays*). Here, we identified full sets of homeologous and paralogous genes encoding Bx glucosyltransferase (GT) and Bx-glucoside glucosidase (Glu) in hexaploid wheat ($2n = 6x = 42$; AABBDD). Four *GT* loci (*TaGTa–TaGTd*) were mapped on chromosomes 7A, 7B (two loci), and 7D, whereas four *glu1* loci (*Taglu1a–Taglu1d*) were on chromosomes 2A, 2B (two loci), and 2D. Transcript levels differed greatly among the four loci; B-genome loci of both *TaGT* and *Taglu1* genes were preferentially transcribed. Catalytic properties of the enzyme encoded by each homeolog/paralog also differed despite high levels of identity among amino acid sequences. The predominant contribution of the B genome to GT and Glu reactions was revealed, as observed previously for the five Bx biosynthetic genes, *TaBx1* to *TaBx5*, which are separately located on homeologous groups 4 and 5 chromosomes. In rye, where the *ScBx1* to *ScBx5* genes are dispersed to chromosomes 7R and 5R, *ScGT* and *Scglu* were located separately on chromosomes 4R and 2R, respectively. The dispersal of Bx-pathway loci to four distinct chromosomes in hexaploid wheat and rye suggests that the clustering of Bx-pathway genes, as found in maize, is not essential for coordinated transcription. On the other hand, barley (*Hordeum vulgare*) was found to lack the orthologous *GT* and *glu* loci like the *Bx1* to *Bx5* loci despite its close phylogenetic relationship with wheat and rye. These results contribute to our understanding of the evolutionary processes that the Bx-pathway loci have undergone in grasses.

Benzoxazinones (Bxs) are one of the better studied classes of plant secondary metabolites in terms of their distribution, biological activities, as well as their biosynthesis from biochemical and molecular genetic aspects (for review, see Niemeyer, 1988, 2009; Sicker et al., 2000; Frey et al., 2009). Bxs are produced in many species of Poaceae, including the major agricultural crops wheat (*Triticum aestivum*), maize (*Zea mays*), and rye (*Secale cereale*). Reported functions include defense against microbial attack or herbivore predation as well as allelopathic agents. DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) and its C7 methoxy derivative DIMBOA are the predominant forms of Bxs in plants. They are stored in the vacuole as 2-*O*-β-D-glucopyranosides (Bx-Glcs), designated DIBOA-Glc and DIMBOA-Glc.

DIBOA is biosynthesized in five sequential reactions starting with indole-3-glycerol phosphate derived from the Trp pathway (Fig. 1; Frey et al., 1997, 2000; Melanson et al., 1997). The genes involved have been isolated in maize (*ZmBx1–ZmBx5*; Frey et al., 1995, 1997), wild barley (*Hordeum lechleri*; *HlBx1–HlBx5*; Grün et al., 2005), wild diploid wheat (*Triticum boeoticum*; *TbBx1–TbBx5*; Nomura et al., 2007a), and hexaploid wheat (*TaBx1A–TaBx5A*, *TaBx1B–TaBx5B*, *TaBx1D–TaBx5D*; Nomura et al., 2002, 2003, 2005). Of the five biosynthetic genes, *Bx2* to *Bx5* encode cytochrome P450 monooxygenases of the CYP71C subfamily. In maize, two genes (*ZmBx8* and *ZmBx9*), each encoding a UDP-Glc:Bx glucosyltransferase (GT), have been identified (von Rad et al., 2001). It has long been unclear whether the conversion of DIBOA to DIMBOA occurs on the aglycone or the glucoside, but recently it was shown that ZmBX6, a 2-oxoglutarate-dependent dioxygenase, accepts DIBOA-Glc but not DIBOA as a substrate. This indicates that the 2-*O*-glucosylation of DIBOA by the GTs precedes the 7-hydroxylation, which is followed by 7-*O*-methylation of TRIBOA-Glc by the *O*-methyltransferase ZmBX7 to form DIMBOA-Glc (Frey et al., 2003; Jonczyk et al., 2008; Fig. 1). Although genes orthologous to *ZmBx6* to *ZmBx9* have not yet been identified in other plants, the same reactions likely occur. In wheat, Bx-GT has been char-

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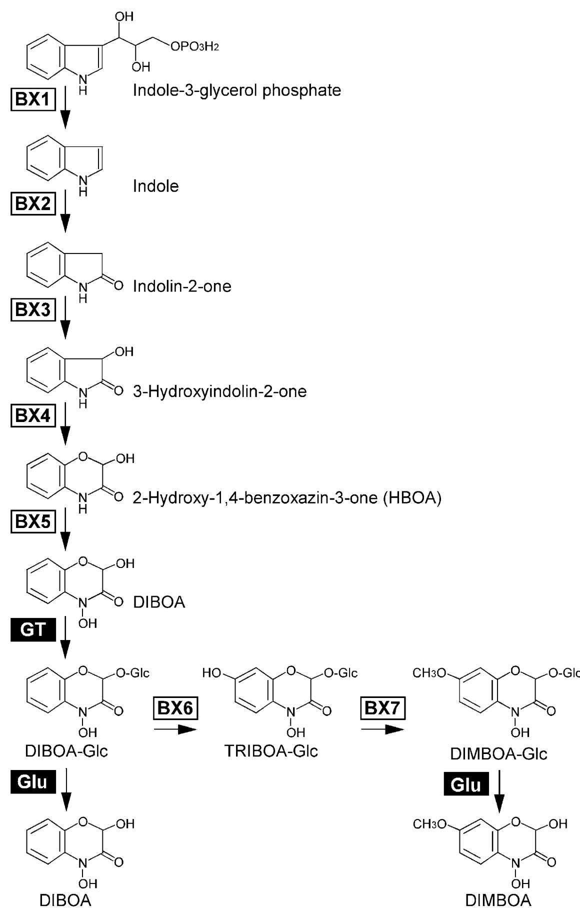

Figure 1. The Bx biosynthetic pathway and deglucosylation by β-glucosidase (Glu). The enzyme catalyzing each reaction is shown in a box. Black boxes indicate glucosyltransferase (GT) and Glu examined in this study. BX6 and BX7, which catalyze sequential 7-hydroxylation and 7-*O*-methylation of DIBOA-Glc to DIMBOA-Glc, have been identified in maize but not in wheat or rye. The GTs we identified have been designated TaGTs and ScGT in wheat and rye, respectively. The corresponding GTs in maize have been designated BX8 and BX9.

acterized enzymatically (Sue et al., 2000a). The Bx-Glcs, which have reduced toxicity compared with the aglycones, are stored in the vacuole, and the toxic aglycones are released by a specific β-glucosidase (Glu) existing in the plastid when cells are disrupted by wounding and/or infection (Fig. 1). The Bx-Glc Glus have been identified from maize (Cicek and Esen, 1999; Czjzek et al., 2000; Verdoucq et al., 2003), wheat (Sue et al., 2000c, 2005, 2006), and rye (Sue et al., 2000b; Nikus et al., 2003).

Common wheat is hexaploid with the genome constitution AABBDD ($2n = 6x = 42$), which originated through successive chromosome doubling of hybrids involving three ancestral diploid species ($2n = 2x = 14$): the A genome came from *Triticum urartu* (AA), the B genome from *Aegilops speltoides* (SS) or another species classified in the genus *Aegilops* (Sitopsis section), and the D genome from *Aegilops tauschii* (Huang et al., 2002; Feldman and Levy, 2005; Salse et al., 2008b). Allopolyploidization leads to the generation of duplicated homeologous genes (homeologs). Consequently, the hexaploid wheat genome contains triplicated homeologs for most genes derived from the diploid progenitors, but elimination and/or amplification also occur for some homeologs (Ozkan et al., 2001). In addition, even though triplicated homeologs are retained through allopolyploidization, they do not always function equally due to biased transcription, including homeolog-specific silencing, and nonfunctionalization of a specific homeolog caused by structural alteration (Bottley et al., 2006; Shitsukawa et al., 2007; Akhunova et al., 2010). Previously, three sets of the five *TaBx* genes were all identified in hexaploid wheat, and their chromosomal locations were determined (Nomura et al., 2002, 2003, 2005). *TaBx1* and *TaBx2* homeologs were located in the same chromosomal bin on homeologous group 4 chromosomes (4A, 4B, and 4D), while *TaBx3* to *TaBx5* homeologs existed in the same chromosomal bin on group 5 chromosomes (5A, 5B, and 5D). Transcription of *TaBx1* to *TaBx5* is coordinated, but levels vary depending on the genome, where B-genome homeologs are transcribed preferentially (Nomura et al., 2005). In addition to transcript levels, enzymatic activities also vary with genome despite extremely high sequence identity. Based on the differences in transcript levels and enzymatic activities among the three homeologs of *TaBx1* to *TaBx5*, it has been suggested that the B genome contributes most to Bx biosynthesis in hexaploid wheat (Nomura et al., 2005). Moreover, it was also proposed that differential transcription of the three homeologs in hexaploid wheat originated during diploidy and was retained through polyploidization.

In rye ($2n = 2x = 14$; RR), the *TaBx1* to *TaBx5* orthologs, *ScBx1* to *ScBx5*, were also shown to be located on two distinct chromosomes, 7R (*ScBx1* and *ScBx2*) and 5R (*ScBx3*–*ScBx5*; Nomura et al., 2003). In cultivated barley (*Hordeum vulgare*; $2n = 2x = 14$; HH), however, none of the five *Bx* loci was present (Nomura et al., 2003) despite close relationships between species in the tribe Triticeae, which evolved from a common ancestor, share the same basic chromosome number, and have highly similar gene sequences (Devos and Gale, 1997; Huang et al., 2002). In contrast to wheat and rye, maize genes *ZmBx1* to *ZmBx5* form a cluster on the short arm of chromosome 4 (Frey et al., 1995, 1997). In addition, genes *ZmBx6* to *ZmBx8* are also included in the cluster (von Rad et al., 2001; Jonczyk et al., 2008), whereas *ZmBx9*, a highly identical homolog of *ZmBx8*, is situated on chromosome 1 (von Rad et al., 2001), and *Zmglu1* and *Zmglu2* are on chromosome 10 (http://www.maizesequence.org/index.html). In general, genes for most metabolic pathways are not clustered in plants, but evidence that genes for secondary metabolic pathways are clustered has recently emerged: the maize *ZmBx* genes, the diploid oat (*Avena strigosa*) avenacin biosynthetic genes (Papadopoulou et al., 1999; Qi et al., 2004), the rice

(*Oryza sativa*) momilactone (Wilderman et al., 2004; Shimura et al., 2007) and phytocassane (Swaminathan et al., 2009) biosynthetic genes, and the Arabidopsis (*Arabidopsis thaliana*) thalianol pathway genes (Field and Osbourn, 2008). Gene clustering is thought to facilitate not only the inheritance of beneficial gene combinations but also the coordinated transcription of pathway genes by enabling localized changes in chromatin structure (Wegel et al., 2009). It has been demonstrated that Bx-Glc levels peak soon after germination and then decrease to a constant level in wheat (Nomura et al., 2005), maize (Ebisui et al., 1998), and rye (Sue et al., 2000b). At the same time, enzymatic activities of GT and Glu also occur concomitantly with the accumulation profiles of Bxs (Ebisui et al., 1998, 2001; Sue et al., 2000a, 2000b, 2000c). Consistent with this, transcript levels of all Bx-pathway genes increase transiently in seedlings and decrease to a lower constant level as plants grow (Frey et al., 1995; von Rad et al., 2001; Nomura et al., 2005; Sue et al., 2006; Jonczyk et al., 2008).

To complete the elucidation of the Bx biosynthetic system in hexaploid wheat, the mechanisms of (1) Bx-pathway gene coexpression in each of the three genomes and (2) differential transcription and catalytic activity among the three genomes need to be determined. Moreover, considering that the Bx biosynthetic genes identified so far are clustered in maize, but not in wheat and rye, the Bx pathway is an excellent model to investigate the biological and molecular genetic significance of gene clusters for secondary pathways in plants. In wheat and rye, however, molecular characterization of the *GT* and *glu* genes, and the genes involved in the conversion of DIBOA-Glc to DIMBOA-Glc, has not yet been completed. In this study, we focused on the *GT* and *glu* genes in hexaploid wheat and rye. Through cDNA isolation, chromosome assignment, and transcriptional and enzymatic characterizations, we demonstrate the differential contribution of the three genomes of hexaploid wheat to the GT and Glu reactions and propose an evolutionary process for the Bx-pathway loci in grasses, in particular in the tribe Triticeae including the genera *Triticum*, *Aegilops*, *Secale*, and *Hordeum*. We also discuss a molecular basis for the coexpression of *Bx* genes and their biased expression among the three genomes of hexaploid wheat as well as the significance of gene cluster formation in secondary metabolite biosynthesis.

RESULTS

Isolation of *GT* and *glu* cDNAs from Hexaploid Wheat and Rye

Screening of the cDNA library prepared from young seedlings of hexaploid wheat (cv Chinese Spring [CS]) using the maize *ZmBx8* cDNA as a probe and additional reverse transcription (RT)-PCR resulted in the isolation of four *TaGT* cDNAs, *TaGTa* to *TaGTd* (GenBank accession nos. AB547237–AB547240). The cDNA library prepared from young rye seedlings was screened using *TaGTa* cDNA as a probe to obtain *ScGT* cDNA (GenBank accession no. AB548283), the *TaGT* ortholog of rye. TaGTa to TaGTd shared 96.3% to 98.5% identity with each other at the amino acid level (Supplemental Table S1; Supplemental Fig. S1) and had high identity (91.6%–94.3%) to ScGT. Amino acid identities of the TaGTs to the maize ortholog ZmBX8 were 68.2% to 68.8%, slightly higher than those to ZmBX9 (66.2%–67.0%; Supplemental Table S1). Phylogenetic analysis showed a close relationship among the GTs of wheat, rye, and maize (Fig. 2A).

We previously isolated three *Taglu1* cDNAs (*Taglu1a*–*Taglu1c*) from hexaploid wheat (Sue et al., 2006). As described below, however, they were localized to chromosomes 2B and 2D. Since most genes are localized in the three genomes in hexaploid wheat, the presence of another homeolog in the A genome was expected. PCR of genomic DNA followed by RT-PCR resulted in the isolation of the novel *Taglu1d* cDNA (GenBank accession no. AB548284). *Taglu1d* encoded a polypeptide of 564 amino acids with a plastid-targeting transit peptide similar to those found in TaGlu1a to TaGlu1c. Amino acid sequences of the four TaGlu1s shared 91.8% to 95.1% identity with each other and approximately 92% and 60%, respectively, with their orthologs in rye (ScGlu; AAG00614) and maize (ZmGlu1 and ZmGlu2; AAA65946 and AAD09850, respectively; Supplemental Table S2; Supplemental Fig. S2). Phylogenetic analysis of plant family 1 glycoside hydrolases (Fig. 2B) showed that the TaGlu1s and ScGlu are closely related to each other and also to ZmGlu1 and ZmGlu2, as well as to dhurrinases in sorghum (*Sorghum bicolor*; AAC49177 and AAK49119) and β-glucosidase in oat (CAA55196).

BLAST searches against the maize sequence database (http://www.maizesequence.org/blast) using *TaGT* and *Taglu1* sequences as queries detected *ZmBx8*/*ZmBx9* and *Zmglu1*/*Zmglu2*, respectively, as sequences of best matches. In addition, searches against the wheat sequence database (http://www.nbrp.jp/) detected only the *TaGT* and *Taglu1* sequences identified, including sequences having interspecific single nucleotide polymorphisms, with significant E-values and coverage rates. These results indicate that the four cDNAs for each of the *TaGT* and *Taglu1* genes cover their functionally expressing loci in CS wheat.

Chromosomal Assignment of *GT* and *glu* Genes in Hexaploid Wheat and Rye

Genomic PCR of the aneuploid lines of CS wheat using primers specific to *TaGTa*, *TaGTb*, *TaGTc*, or *TaGTd* amplified no PCR products from N7B-T7A, N7B-T7A, N7A-T7B, and N7D-T7B, respectively (Fig. 3A), indicating that the *TaGTa*, *TaGTb*, *TaGTc*, and *TaGTd* loci are located on chromosomes 7B, 7B, 7A, and 7D, respectively, in hexaploid wheat. Chromosomal locations of the four *Taglu1* loci were deter-

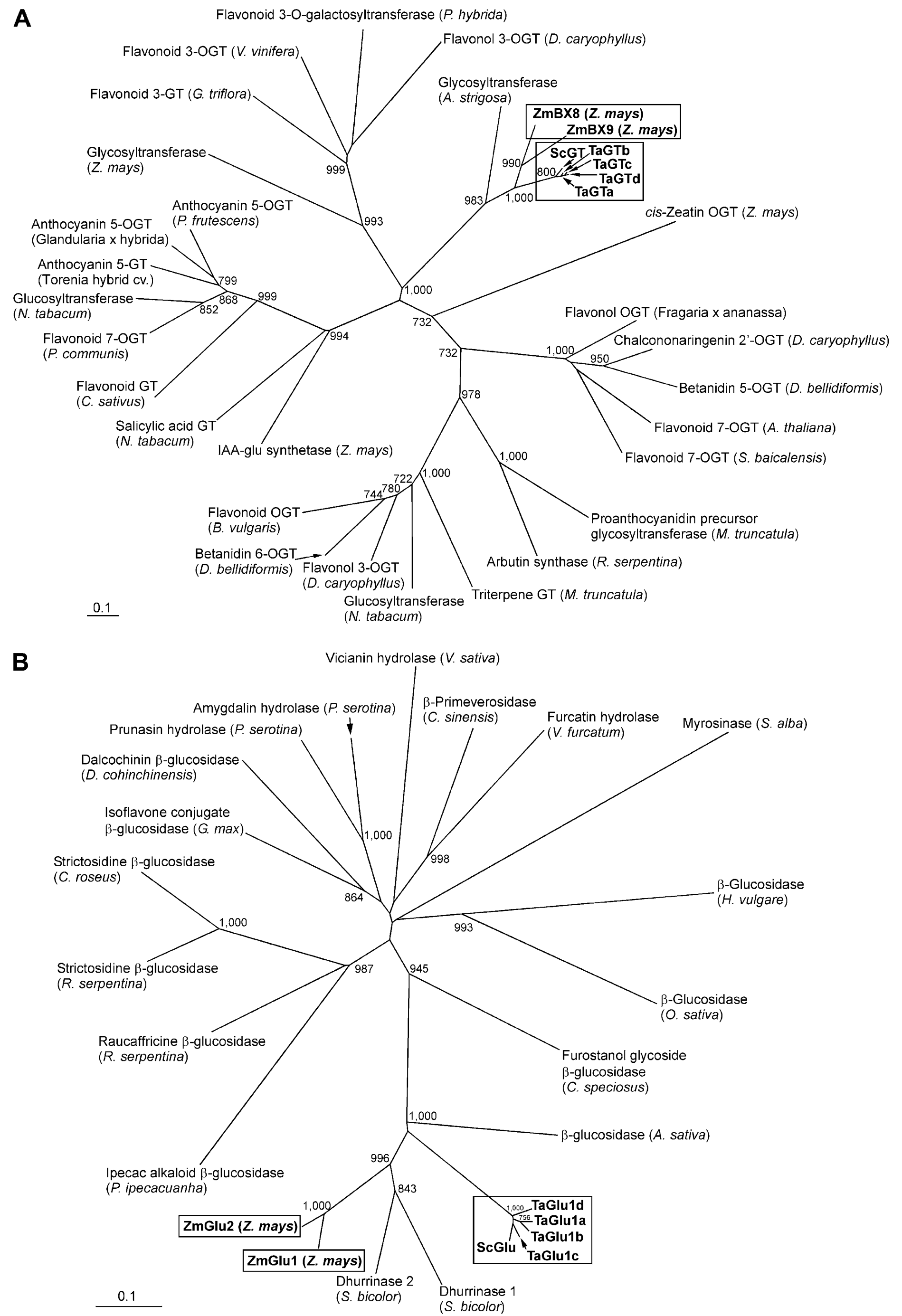

Figure 2. Unrooted phylogenetic trees of GTs (A) and Glus (B). Full-length amino acid sequences were aligned using ClustalW. The phylogenetic tree was built on calculations using the neighbor-joining method (Saitou and Nei, 1987) with bootstrap analysis of 1,000 replicates and was visualized with Treeview. Numbers at each node are bootstrap values (over 70%) per 1,000 trials. Scale bars indicate substitutions per site. GTs and Glus of Bx glucosylation and deglucosylation, respectively, in wheat, rye, and maize are shown within a box in each tree. Accession numbers of proteins used for constructing the trees are listed in Supplemental Materials and Methods S1.

mined using the same procedure. Specific PCR products for each of the four *Taglu1*s were missing in N2B-T2D, N2B-T2D, N2D-T2A, and Dt2AS, respectively (Fig. 3B). A *Taglu1c*-specific product was also absent in Dt2DS. These results showed that the *Taglu1a*, *Taglu1b*, *Taglu1c*, and *Taglu1d* loci are located on chromosomes 2B, 2B, 2DL (where L represents long arm), and 2AL, respectively. These results indicate that four loci of each of the *TaGT* and *Taglu1* genes are composed of three homeologs (one homeolog on each genome) and one paralog on the B genome, but it remains unclear which of the two loci on the B genome is the original locus or the paralogous locus that arose by duplication of the original locus for both *TaGT* and *Taglu1* genes.

Chromosomal locations of the rye orthologs, *ScGT* and *Scglu*, were assigned by specific PCR of the wheat (CS)-rye (cv Imperial) chromosome addition lines. *ScGT*-specific amplification gave a PCR band in the CS/Imperial amphidiploid, which possesses whole

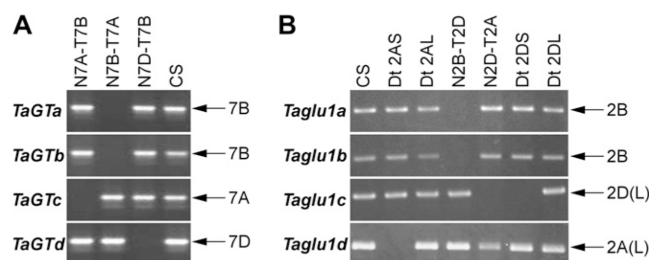

Figure 3. Genomic PCR of nullisomic-tetrasomic (N-T) and ditelosomic (Dt) lines of CS for chromosomal assignment of *TaGT* (A) and *Taglu1* (B) loci in hexaploid wheat. PCR was performed with primer sets that specifically amplify each of the four loci of *TaGT* and *Taglu1* genes. Results of CS aneuploid lines of homeologous group 7 and group 2 chromosomes are represented for *TaGT* (A) and *Taglu1* (B). The chromosome on which each locus is located is indicated on the right. For *Taglu1c* and *Taglu1d*, chromosomal arm location was determined to be the long arm (L) using Dt lines.

rye chromosomes in the CS wheat genetic background, and in the 4R chromosome addition line (Fig. 4). This showed that the *ScGT* gene is located on chromosome 4R in rye. *Scglu*-specific amplification was detected in the 2R chromosome addition line as well as in the CS/Imperial amphidiploid (Fig. 4), indicating that the *Scglu* gene is located on chromosome 2R.

Catalytic Activities of TaGT and TaGlu1 Enzymes

Kinetic parameters of each of the TaGTa to TaGTd enzymes were determined using purified recombinant enzymes (Table I). All four TaGT enzymes showed higher reaction efficiencies (k_{cat}/K_m) for DIMBOA than for DIBOA, from 1.9-fold for TaGTd to 3.3-fold for TaGTa. K_m and k_{cat} values differed among the four enzymes within a 3-fold range for both DIBOA and DIMBOA. TaGTa exhibited the highest reaction efficiencies, where the k_{cat}/K_m values of TaGTa were approximately two and three times higher than those of TaGTb to TaGTd for DIBOA and DIMBOA, respectively.

Newly isolated TaGlu1d was expressed in *Escherichia coli* after truncation of its plastid-targeting transit peptide, as described previously for TaGlu1a to TaGlu1c (Sue et al., 2006). Kinetic parameters of TaGlu1d for DIBOA-Glc and DIMBOA-Glc were determined and compared with those of TaGlu1a to TaGlu1c (Sue et al., 2006). As shown in Table II, K_m and k_{cat} values differed among the four enzymes. For DIBOA-Glc, TaGlu1d showed the highest reaction efficiency (313 s^{-1} mM^{-1}), which was 9-fold higher than that of TaGlu1a (34.6 s^{-1} mM^{-1}). For DIMBOA-Glc, TaGlu1b exhibited the highest reaction efficiency (4,141 s^{-1} mM^{-1}), which was 10-fold higher than that of TaGlu1d. Notably, TaGlu1a to TaGlu1c preferentially accepted DIMBOA-Glc, where the reaction efficiencies of TaGlu1a, TaGlu1b, and TaGlu1c for DIMBOA-Glc were 27-, 28-, and 15-fold, respectively, higher than those for DIBOA-Glc. In contrast, the reaction efficiency of TaGlu1d for DIMBOA-Glc was only 1.3-fold higher than that for DIBOA-Glc, showing higher reactivity with DIBOA-Glc than the other TaGlu1s. A similar catalytic property was observed in the rye glucosidase (ScGlu; Sue et al., 2006). This is attributable to the mature enzyme amino acid residues Gly-464 and Ser-465 shared by TaGlu1d and ScGlu, which are involved in distinguishing DIBOA-Glc from DIMBOA-Glc (Sue et al., 2006). The counterpart residues in the TaGlu1a to TaGlu1c enzymes that preferentially hydrolyzed DIMBOA-Glc were Ser-464 and Leu-465 (Supplemental Fig. S2).

Transcript Profiles of *TaGT* and *Taglu1* Genes in Hexaploid and Tetraploid Wheat

We first examined changes in the transcript levels of the *TaGT* and *Taglu1* genes in young shoots of hexaploid wheat by northern-blot analysis. The levels of *TaGT* and *Taglu1* transcripts peaked 48 h after seeding and then decreased to lower levels (Fig. 5A). This pattern correlated well with those of *TaBx1* to *TaBx5* genes as well as those for Bx content (Nomura et al., 2005). Since northern analysis cannot distinguish transcripts from each of the four homeologous and paralogous loci due to their high identities, we performed locus-specific quantitative (q)RT-PCR analysis to compare transcript levels of four loci of the *TaGT* and the *Taglu1* genes in hexaploid wheat shoots 48 h (high Bx content) and 96 h (low Bx content) after seeding; Bx contents (total of DIBOA-Glc and DIMBOA-Glc) in 48- and 96-h-old shoots are 15.7 and 5.7 μmol g^{-1} fresh weight, respectively (Nomura et al., 2005). *TaGTa* located on the B genome was transcribed at the highest ratio in both 48-h-old (63.5%) and 96-h-old (75.5%) shoots (Fig. 5B). Transcripts of the D-genome homeolog *TaGTd* were detected at a ratio comparable to that of the *TaGTb* on the B genome in 48-h-old shoots but decreased to the lower ratio (3.0%) in 96-h-old shoots. The A-genome homeolog *TaGTc* was transcribed at the lowest ratio in both 48-h-old (1.7%) and 96-h-old (0.8%) shoots.

Similarly, among the four *Taglu1* loci, *Taglu1a* and *Taglu1b* on the B genome were preferentially tran-

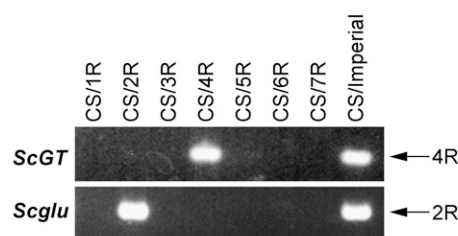

Figure 4. Genomic PCR of wheat (CS)-rye (Imperial) chromosome addition lines for chromosome assignment of *ScGT* and *Scglu* genes in rye. PCR was performed with primer sets that specifically amplify *ScGT* and *Scglu* but not their orthologs in CS wheat. CS/Imperial has whole sets of rye chromosomes, and CS/1R-CS/7R each has a pair of respective rye chromosomes in the wheat genetic background. Chromosomes corresponding to PCR products derived from the rye genome are indicated on the right.

Table I. *Kinetic parameters of TaGT enzymes*

Enzyme	DIBOA			DIMBOA		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	s^{-1} mM^{-1}	μM	s^{-1}	s^{-1} mM^{-1}
TaGTa	14.4	11.2	778	11.3	29.4	2,600
TaGTb	14.4	5.1	354	23.8	19.6	824
TaGTc	27.8	8.8	317	13.7	11.1	810
TaGTd	21.2	9.0	425	16.6	13.3	801

scribed. Transcript ratios of *Taglu1a* and *Taglu1b* in 48-h-old shoots were 55.0% and 41.2%, respectively, and those in 96-h-old shoots were 84.3% and 15.4% (Fig. 5C). The sum of ratios of the two B-genome locus transcripts reached 96.2% and 99.7% in 48- and 96-h-old shoots, respectively. In contrast, the D-genome homeolog *Taglu1c* and the A-genome homeolog *Taglu1d* were transcribed at low ratios (Fig. 5C).

To see if the transcript levels of A- and B-genome loci are affected by the D genome, we investigated the profiles in Tetra-CS, which is a tetraploid wheat generated from hexaploid CS; thus, its A and B genomes are identical to those of CS. Changes in the *TaGT* and *Taglu1* transcripts between 48- and 96-h-old shoots, where the Bx contents are 18.4 and 10.9 μmol g^{-1} fresh weight, respectively (Nomura et al., 2005), were substantially the same as observed in hexaploid CS wheat (Fig. 5A). Locus-specific qRT-PCR revealed, as observed in CS, that the majority of *TaGT* transcripts were contributed by the B-genome loci, *TaGTa* and *TaGTb* (i.e. 78% and 20%, respectively, in both 48- and 96-h-old shoots; Fig. 5D). In contrast, the transcript ratio of the *TaGTc* homeolog located on the A genome was only 1.8% and 1.2% of the total in 48- and 96-h-old shoots, respectively. For the three *Taglu1* loci, transcript profiles were similar to those in CS (Fig. 5E). The sum of ratios of the transcripts from the two B-genome loci (*Taglu1a* and *Taglu1b*) reached 93.3% and 99.0% in 48- and 96-h-old shoots, respectively.

Transcript Profiles of *TaGT* and *Taglu1* Orthologs in Diploid Progenitors of Hexaploid Wheat

To determine whether biased transcription among the three genomes of hexaploid wheat (Fig. 5) is a function of polyploidization, transcript levels of *TaGT* and *Taglu1* orthologs in the three diploid progenitors of hexaploid wheat were examined by northern analysis (Fig. 6). Blots of RNA isolated from 48- and 96-h-old shoots of each diploid progenitor were probed with orthologous sequences from hexaploid wheat. For both the *TaGT* and *Taglu1* probes, strongest hybridization signals were detected in *A. speltoides* (SS), the B-genome donor to hexaploid wheat. In contrast, only faint signals were detected in *T. urartu* (AA) and *A. tauschii* (DD). The patterns correlated well with the Bx contents: 1.2 (48 h) and 0.3 (96 h) μmol g^{-1} fresh weight in *T. urartu*, 20.8 (48 h) and 9.3 (96 h) μmol g^{-1} fresh weight in *A. speltoides*, and 6.9 (48 h) and 0.7 (96 h) μmol g^{-1} fresh weight in *A. tauschii* (Nomura et al., 2005). These results suggested that the preferential transcription of B-genome *TaGT* and *Taglu1* loci in hexaploid wheat originated in a diploid progenitor and was not caused by polyploidization.

Southern Analysis to Search for *TaGT* and *Taglu1* Orthologs in Barley

Since wheat and barley are closely related, their orthologous genes usually share about 95% identity at the nucleotide level. Therefore, wheat orthologs can be detected in barley DNA by Southern hybridization under stringent hybridization/washing conditions (Nomura et al., 2003). Southern analysis of DNA from two barley cultivars, however, revealed no hybridization when probed with wheat *TaGTa* or *Taglu1a* (Fig. 7). When the same DNA blot was probed with barley *HvACT* cDNA, orthologs of which are present in hexaploid wheat (Nomura et al., 2007b), clear hybridization bands were detected in both barley cultivars and in CS wheat, thus validating the negative result using wheat *TaGTa* and *Taglu1a* probes. In addition, we performed BLAST searches against the barley full-length cDNAs and ESTs (Barley DB; http://www.shigen.nig.ac.jp/barley). No barley sequences exhibiting significant identity with the *TaGT* and *Taglu1*

Table II. *Kinetic parameters of TaGlu1 enzymes*

Enzyme	DIBOA-Glc			DIMBOA-Glc		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	s^{-1} mM^{-1}	mM	s^{-1}	s^{-1} mM^{-1}
TaGlu1a[a]	1.40	48.8	34.6	0.36	338	939
TaGlu1b[a]	1.44	214	149	0.29	1,201	4,141
TaGlu1c[a]	1.05	137	131	0.39	773	1,982
TaGlu1d[b]	1.83	572	313	0.79	330	418

[a]Data taken from Sue et al. (2006). [b]This study.

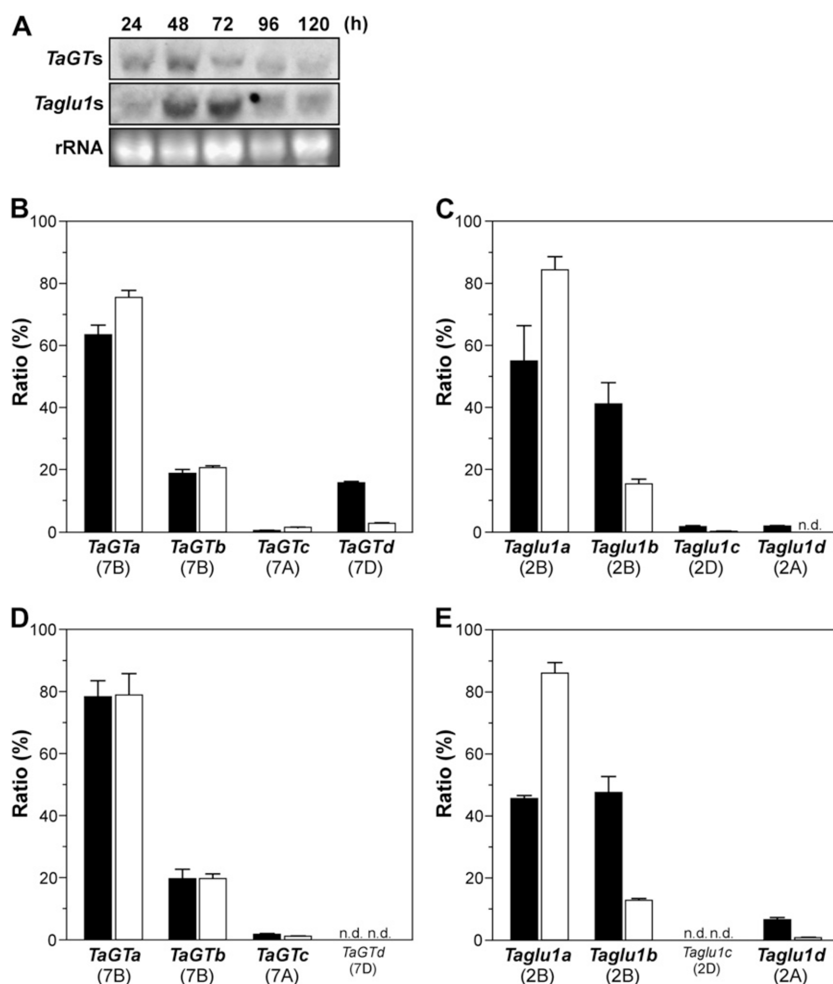

Figure 5. Transcript levels of *TaGT* and *Taglu1* genes in CS and Tetra-CS. A, Northern-blot analysis of 24- to 120-h-old CS shoots. Ethidium bromide-stained rRNA was used as a loading control. B to E, Transcript ratios of four loci in 48-h-old (black bars) and 96-h-old (white bars) shoots analyzed by qRT-PCR. B, *TaGT*s in CS. C, *Taglu1*s in CS. D, *TaGT*s in Tetra-CS. E, *Taglu1*s in Tetra-CS. The chromosomal location of each locus is shown in parentheses. Note that data for each growth stage (B–E) are presented as the transcript ratio of each locus to the sum of four loci. The absence of transcripts of *TaGTd* and *Taglu1c* in Tetra-CS (D and E, respectively) is due to lack of the D genome in Tetra-CS. Data are expressed as means of triplicate experiments with SD. n.d., Not detected.

coding regions were found, an outcome consistent with the Southern-blot result. We conclude that neither *TaGT* nor *Taglu1* orthologs exist in cultivated barley.

DISCUSSION

Dispersed Chromosomal Locations of Bx-Pathway Genes in Wheat and Rye

Of the two *GT* genes in the Bx pathway in maize (*ZmBx8* and *ZmBx9*), *ZmBx8* is only 44 kb apart from *ZmBx1* (Frey et al., 2009), which is 2.5 kb from *ZmBx2*. Therefore, we expected that the *TaGT* loci would map to the homeologous group 4 chromosomes on which the *TaBx1* and *TaBx2* genes are situated (Nomura et al., 2003). However, we found the *TaGT* loci on homeologous group 7 chromosomes 7A, 7B, and 7D (Fig. 8; Supplemental Table S3). It has been reported that segmental translocations occurred between groups 4, 5, and 7 chromosomes during the evolution of wheat, but the events involved only 4A, 5A, and 7B (Liu et al., 1992). Therefore, these translocations cannot explain the split location of the *TaGT* locus from *TaBx1* and *TaBx2* in all three genomes. It is more likely that the split location of the *TaGT* locus occurred prior to the divergence of the three diploid progenitors of hexaploid wheat. This scenario is supported by the fact that the *ScGT* gene was mapped to chromosome 4R, which is different from the *ScBx1* and *ScBx2* genes on chromosome 7R (Nomura et al., 2003) in rye (Fig. 8; Supplemental Table S3). The locations of *TaGT* loci on homeologous group 7 chromosomes and *ScGT* on chromosome 4R are consistent with the chromosomal synteny between wheat and rye (Devos et al., 1993). Likewise, the chromosomal locations of *Taglu1* loci in wheat (homeologous group 2 chromosomes) and *Scglu* in rye (chromosome 2R; Fig. 8; Supplemental Table S3) follow the synteny between wheat and rye.

In maize, genes encoding most of the Bx-pathway enzymes (*ZmBx1*–*ZmBx8*) are clustered on the short arm of chromosome 4 (Frey et al., 2009), and three (*ZmBx9*, *Zmglu1*, and *Zmglu2*) are located outside the cluster (Fig. 8; Supplemental Table S3). *ZmBx9* is located on chromosome 1 (von Rad et al., 2001), and *Zmglu1* and *Zmglu2* are located on chromosome 10 (3.5 Mb apart from each other). Recent high-resolution comparative mapping in grass species revealed microcolinearity among grass chromosomes and demon-

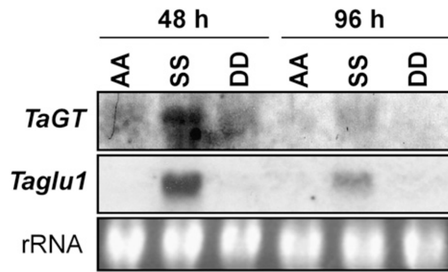

Figure 6. Transcript levels of *TaGT* and *Taglu1* orthologs in diploid progenitors of hexaploid wheat. RNA isolated from 48- and 96-h-old shoots were analyzed by northern hybridization. AA, SS, and DD represent *T. urartu*, *A. speltoides*, and *A. tauschii*, respectively. Ethidium bromide-stained rRNA was used as a loading control.

strated that wheat group 7 chromosomes (where *TaGT* loci are located) are partially orthologous to maize chromosomes 1 (where *ZmBx9* is located) and 4 (where *ZmBx8* is located; Devos, 2005; Salse et al., 2008a, 2009). Therefore, we cannot judge whether the *TaGT* gene corresponds to *ZmBx8* or *ZmBx9* only based on the chromosomal synteny. Sequence identities of the *TaGT*s to *ZmBx8* were slightly higher than those to *ZmBx9* (Supplemental Table S1). In addition, TaGT enzymes showed similar catalytic properties to ZmBX8 rather than to ZmBX9; TaGTs and ZmBX8 showed moderately higher catalytic efficiency to DIMBOA than to DIBOA, while the efficiency of ZmBX9 to DIBOA is extremely lower than that to DIMBOA (von Rad et al., 2001). Therefore, *TaGT* appears to correspond to *ZmBx8*. *ZmBx9* may have originated by duplication of *ZmBx8*, or it may be the trace of paleotetraploidy of the maize genome (Swigonova et al., 2004; Schnable et al., 2011). Localization of the *Taglu1* loci on group 2 chromosomes in hexaploid wheat coincides with the partial synteny between wheat group 2 and maize chromosome 10 (Devos, 2005; Salse et al., 2008a, 2009), on which *Zmglu1* and *Zmglu2* genes are located (Fig. 8). We cannot judge whether *Taglu1* corresponds to *Zmglu1* or *Zmglu2* based on the sequence comparisons (Fig. 2B; Supplemental Table S2). However, considering that *Taglu1* and *Zmglu1* genes are highly expressed in young seedlings while *Zmglu2* starts to express at a later stage (Cicek and Esen, 1999), *Taglu1*s isolated in this study appear to be the counterparts of *Zmglu1*. It should be noted that a BLAST search with the *Taglu1* query against the wheat sequence database did not retrieve sequences other than the *Taglu1*s identified, suggesting that *Zmglu2* also arose only in maize, as mentioned above for *ZmBx9*.

Salse et al. (2008a, 2009) proposed that grass genomes have evolved from a common ancestor with five protochromosomes. According to the model, parts of wheat groups 4 (where *TaBx1* and *TaBx2* are located) and 5 chromosomes (where *TaBx3–TaBx5* are located) are derived from the same protochromosome (designated A11 in the literature), which also is an origin of a part of maize chromosome 4 (where *ZmBx1–ZmBx8* are located), supporting our previous hypothesis that the *Bx1* to *Bx5* genes arose as a cluster and were split into two chromosomes during the evolutionary processes that rearranged the ancient grass genome into seven chromosomes of the tribe Triticeae (Nomura et al., 2003; Fig. 8). However, a part of the wheat group 7 chromosomes (where *TaGT* loci are located) that shows synteny with parts of maize chromosomes 1 (where *ZmBx9* is located) and 4 (where *ZmBx8* is located) is shown to have originated from the other protochromosome, A8, and a part of the wheat group 2 chromosomes (where *Taglu1* loci are located) that shows synteny with a part of maize chromosome 10 (where *Zmglu1* and *Zmglu2* genes are located) is from the protochromosome A4. This implies that the *GT* and *glu* loci had not been included in the ancestral Bx-pathway gene cluster (Fig. 8). However, the fact that one of the maize *GT* genes, *ZmBx8*, is situated only 44 kb apart from *ZmBx1* (Frey et al., 2009) does not allow us to exclude the possibility of the ancestral Bx-pathway gene cluster. Including the *Bx6* and *Bx7* loci in wheat, which remain to be elucidated, in such synteny analysis would help us to know an original form of the Bx-pathway loci.

How Did Barley Lose the Bx-Pathway Loci?

It has been reported that cultivated barley produces no Bxs due to loss of the *Bx1* to *Bx5* loci (Gierl and Frey,

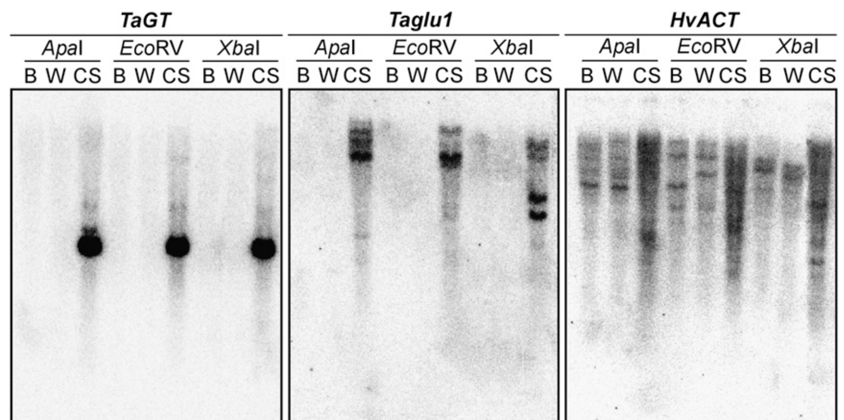

Figure 7. Southern-blot analysis of barley cv Betzes (B) and Wasedori-nijo (W) probed with *TaGTa* and *Taglu1a*. Genomic DNA digests of CS wheat were included as a positive hybridization control. The barley *HvACT* gene whose orthologs are present in wheat (Nomura et al., 2007b) was used as a positive control for hybridization with barley DNA and for cross-hybridization between barley and wheat sequences.

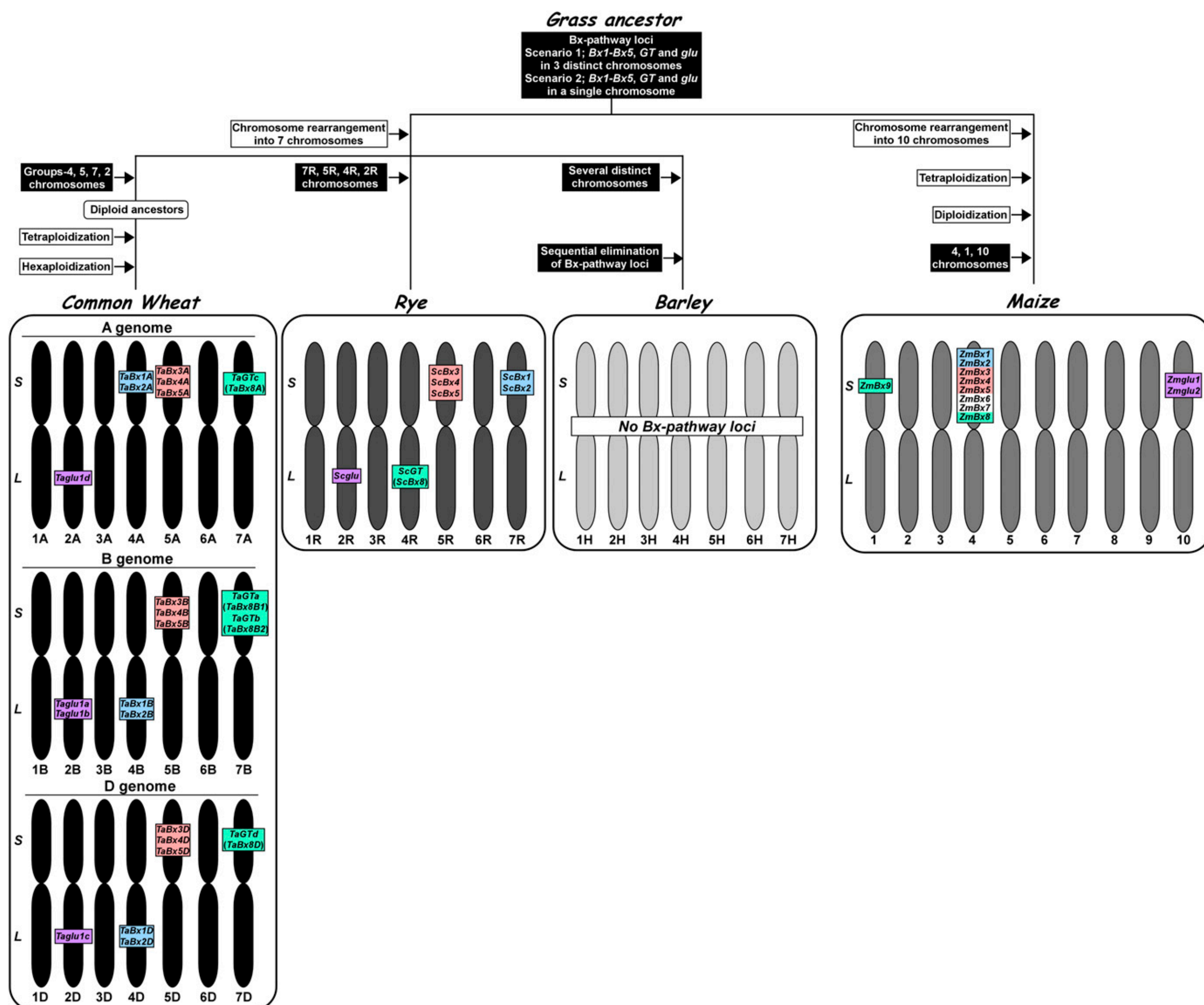

Figure 8. Overview of chromosomal locations of Bx-pathway loci in Triticeae species and maize. Locations of Bx-pathway loci determined so far are shown on each chromosome arm. S and L represent short and long arms, respectively. *TaGT* and *ScGT* genes are also designated as *TaBx8* and *ScBx8*, respectively, in parentheses. Note that orthologs of *ZmBx6* and *ZmBx7* have not yet been identified in wheat and rye. Arm locations of *TaBx1* to *TaBx5* were reported by Nomura et al. (2003, 2005) and those of *Taglu1c* and *Taglu1d* in this study. For the other loci in wheat and rye, chromosomal assignments were performed by Nomura et al. (2003; *ScBx1–ScBx5*) and in this study (*Taglu1a*, *Taglu1b*, *TaGT*s, *Scglu*, and *ScGT*), and their arm locations were estimated based on the chromosomal synteny between wheat and rye (Devos et al., 1993). For the locations of *ZmBx1* to *ZmBx9* in maize, see Frey et al. (1997), Jonczyk et al. (2008), and von Rad et al. (2001). Locations of *Zmglu1* and *Zmglu2* were found by searching the maize sequence database.

2001; Nomura et al., 2003). Grün et al. (2005) demonstrated that one wild barley species (*Hordeum spontaneum*) also lacks those loci, but other wild species (e.g. *H. lechleri*) accumulate Bxs. It has been reported that GT activity is also not detectable in cultivated barley (Leighton et al., 1994). Our study here revealed that this is attributable to loss of the *GT* locus.

Even in maize, where eight *Bx* genes are clustered, *Zmglu1* and *Zmglu2* loci are situated on a chromosome different from that of the *Bx* gene cluster (Fig. 8). Thus, we expected to find that the *glu* locus still exists in cultivated barley even after the loss of all other *Bx* loci. However, Southern analysis revealed that the *glu* locus is also missing in cultivated barley. Nomura et al. (2007a) proposed that degeneration of coding sequence, silencing, or loss of one *Bx* locus triggers the loss of other *Bx* loci, which finally leads to the elimination of all Bx-pathway loci. We previously predicted that Bx-producing wild barley species would have a *Bx* gene cluster on a single chromosome, because it seemed unlikely that *Bx* loci on separate chromosomes would have been eliminated totally in cultivated barley (Nomura et al., 2003).

As mentioned above, however, it now seems reasonable to suggest that the ancient *Bx* loci in barley were already dispersed into distinct chromosomes, as in wheat and rye, as a result of evolutionary processes that rearranged the ancient grass genome into seven chromosomes of the tribe Triticeae (Fig. 8). Presumably, elimination of the *GT* and *glu* loci, as well as the *Bx1* to *Bx5* loci, in barley occurred sequentially according to the same scenario found in the wild A-genome diploid wheat (Nomura et al., 2007a).

Differential Contributions of the Three Hexaploid Wheat Genomes to Reactions Catalyzed by TaGT and TaGlu1 Enzymes

Transcript levels of the four loci differed greatly for both *TaGT* and *Taglu1* genes; B-genome loci were predominantly transcribed, a feature in common with the *TaBx1* to *TaBx5* genes (Nomura et al., 2005). Evidence of differential transcription does not necessarily indicate the actual contribution of each homeolog to its corresponding reaction. Differences in catalytic properties of enzymes encoded by each homeolog must also be considered (Nomura et al., 2005).

TaGT enzymes catalyze the 2-*O*-glucosylation of DIBOA and DIMBOA in vitro, where the reaction efficiencies for DIMBOA are approximately 2- to 3-fold higher than those for DIBOA. In the maize biosynthetic pathway (Jonczyk et al., 2008), however, 2-*O*-glucosylation occurs for DIBOA to form DIBOA-Glc, followed by 7-hydroxylation and 7-*O*-methylation to form DIMBOA-Glc. Therefore, the contribution of individual TaGT enzymes to the reaction in vivo should be estimated based on their reaction efficiencies for DIBOA. Obviously, TaGTa encoded by the B-genome locus plays the major role in the reaction, because its transcript levels are 3- to 37-fold higher in 48-h-old shoots and 4- to 92-fold higher in 96-h-old shoots than are those of the other *TaGT*s, and the reaction efficiency of TaGTa for DIBOA is approximately twice as high as those of the other TaGTs. Even though the reaction efficiencies of TaGTc and TaGTd are comparable to those of TaGTb, their transcript levels are notably lower than those of the B-genome loci *TaGTa* and *TaGTb*, especially in 96-h-old shoots. These results indicated that the B genome contributes most to the TaGT reaction in hexaploid wheat.

TaGlu1 functions as homohexamers and heterohexamers (Sue et al., 2000c, 2006). Judging from transcript levels of individual *Taglu1* loci, the natural hexameric TaGlu1 enzymes are presumed to be composed mainly of TaGlu1a and TaGlu1b, both of which are encoded by B-genome loci. Although the reaction efficiency of the homohexamer of TaGlu1a for DIBOA-Glc is 4- and 9-fold lower than those of TaGlu1d and TaGlu1c, respectively, its transcript level is approximately 31- and 27-fold higher than those of *Taglu1c* and *Taglu1d*, respectively, in 48-h-old shoots and 320- and 8,400-fold higher than those in 96-h-old shoots. These results show that the lower reaction efficiency of TaGlu1a can readily be overcome by its substantially higher transcript level. Similarly, the 2-fold higher reaction efficiency of TaGlu1c over TaGlu1a for DIMBOA-Glc is likely canceled by the low transcript level of *Taglu1c*. Although the reaction efficiency of the TaGlu1b enzyme for DIBOA-Glc was comparable to that of TaGlu1c and 2-fold lower than that of TaGlu1d, its transcript level is remarkably higher than those of *Taglu1c* and *Taglu1d*. Moreover, the reaction efficiency of TaGlu1b for DIMBOA-Glc is obviously higher than those of TaGlu1c and TaGlu1d. Accordingly, we conclude that the main contribution to the deglucosylation reaction is made by the B genome in hexaploid wheat.

In tetraploid wheat, the ratio of transcripts from the A- and B-genome loci of the *TaGT* and *Taglu1* genes was about the same as that observed in hexaploid wheat, in which the B-genome loci were predominantly transcribed. These results suggest that hexaploidization does not influence the transcript profiles of the A- and B-genome loci. In addition, among the three diploid progenitors of hexaploid wheat, transcript levels of the *TaGT* and *Taglu1* orthologs were highest in *A. speltoides* (SS), the B-genome donor to hexaploid wheat. These facts suggest that the transcriptional bias of the *TaGT* and *Taglu1* genes in hexaploid wheat originated at the diploid level and was retained through polyploidization.

The same conclusion has been reported for the *TaBx1* to *TaBx5* genes (Nomura et al., 2005). In allopolyploids, there is no global genomic bias in gene transcription (i.e. genomes in which preferentially transcribed homoeoalleles are present vary from gene to gene; Adams et al., 2003). Our results here, combined with results for the *TaBx1* to *TaBx5* genes, imply a common mechanism allowing preferential transcription of B-genome loci of the Bx-pathway genes. Nomura et al. (2008) analyzed promoter activities of the three homeologs of *TaBx3* and *TaBx4* genes by transient expression of a reporter protein in wheat protoplasts, but no significant differences were detected among the three homeologs. The authors speculated that this might be due to epigenetic gene regulation related to chromatin structure, such as DNA methylation and/or histone modification. In fact, several studies of transcriptional bias in hexaploid wheat showed that epigenetic chromatin modifications were involved (Bottley et al., 2006; Shitsukawa et al., 2007). The preferential transcription of B-genome loci of all Bx-pathway genes may be controlled by such epigenetic alterations.

Is Dispersal of Bx-Pathway Genes Disadvantageous for Wheat and Rye?

Bx-pathway genes are dispersed to homeologous groups 4 (*TaBx1* and *TaBx2*), 5 (*TaBx3*–*TaBx5*), 7 (*TaGT*), and 2 (*Taglu1*) chromosomes in hexaploid wheat and to chromosomes 7R (*ScBx1* and *ScBx2*), 5R (*ScBx3*–*ScBx5*), 4R (*ScGT*), and 2R (*Scglu*) in rye. In contrast, all

maize Bx biosynthetic genes (*ZmBx1–ZmBx8*) are clustered on the short arm of chromosome 4, except for *Zmglu1* and *Zmglu2* on chromosome 10 and *ZmBx9*, a *ZmBx8* homolog, on chromosome 1 (Fig. 8). It is the common feature in prokaryotic actinomycetes that the biosynthetic genes of secondary metabolites are clustered (Dairi, 2005). Also, secondary metabolites are commonly synthesized by groups of genes that form metabolic gene clusters in eukaryotic filamentous fungi, where the clustered genes are not transcribed as a single mRNA, unlike bacterial operons (Osbourn and Field, 2009). In contrast, most plant genes for secondary metabolite pathways characterized so far are not clustered. But now, five examples of such clustering in plants are known: the Bx-pathway genes in maize (Frey et al., 2009), the triterpenoid avenacin pathway genes in oat (Papadopoulou et al., 1999; Qi et al., 2004), the diterpenoids momilactone (Wilderman et al., 2004; Shimura et al., 2007) and phytocassane (Swaminathan et al., 2009) pathway genes in rice, and triterpenoid thalianol pathway genes in Arabidopsis (Field and Osbourn, 2008). Clustering is thought to facilitate the inheritance of beneficial gene combinations and to promote the coordinated transcription of pathway genes by enabling localized changes in chromatin structure (Wegel et al., 2009). In fact, Zhan et al. (2006) demonstrated that neighboring genes are more frequently coexpressed than would be expected by chance. This model would fit the case of *TaBx1* and *TaBx2*, which are 2.2 kb apart (T. Nomura, unpublished data for the A-genome homeologs) and of *TaBx3* and *TaBx4*, which are 7.3 to 11.3 kb apart in the three genomes of hexaploid wheat (Nomura et al., 2008). Nevertheless, all Bx-pathway genes characterized so far in hexaploid wheat, which are dispersed onto four chromosomes, are coordinately transcribed in each genome despite their genome-dependent differential transcript levels, which vary according to juvenile growth stage. The coordinated transcription of Bx-pathway genes has also been observed in maize (Frey et al., 1995; von Rad et al., 2001; Jonczyk et al., 2008) and may be the case in rye, where Bx production peaks in young seedlings (Sue et al., 2000b) as in wheat and maize; transcript levels have not yet been determined for all of the *Bx* genes. Apparently, coordinated transcription of Bx-pathway genes during early growth does not depend on gene clustering in wheat and rye. Perhaps there are cis-elements and transcription factors common to all Bx-pathway genes. A computational survey of promoter sequences of all *TaBx3* and *TaBx4* homeologs in hexaploid wheat and their orthologs in diploid progenitors predicted several cis-elements in common (Nomura et al., 2008). Transcription of some or all genes in a secondary metabolite pathway can be regulated by a small number of transcription factors, such as the OsTGAP1 transcription factor involved in diterpenoid phytoalexin biosynthesis (Okada et al., 2009) and the ORCA3 transcription factor involved in terpenoid-indole alkaloid biosynthesis (van der Fits and Memelink, 2000). The identification of transcription factor(s) and chromatin-related regulatory machinery for Bx-pathway genes should give important clues regarding the metabolic significance of gene clusters in plants.

MATERIALS AND METHODS

Plant Materials

A cultivar of hexaploid wheat (*Triticum aestivum*; $2n = 6x = 42$; genomes AABBDD), Chinese Spring, was used for cDNA cloning, genomic PCR, northern hybridization, qRT-PCR, and Southern hybridization. A tetraploid wheat derived from CS (Tetra-CS; $2n = 4x = 28$; AABB; Yang et al., 1999) was used for northern hybridization and qRT-PCR. For Southern analysis, two cultivars of barley (*Hordeum vulgare*; $2n = 2x = 14$; HH), Betzes and Wasedori-nijo, and three diploid progenitors ($2n = 2x = 14$) of hexaploid wheat, *Triticum urartu* (accession KU199-6; AA), *Aegilops speltoides* (KU5727, SS), and *Aegilops tauschii* (KU20-9, DD) were used. A cultivar of rye (*Secale cereale*; $2n = 2x = 14$; RR), Haru-ichiban, was used for cloning *ScGT* cDNA. To assign chromosomal locations of *GT* and *glu* loci in hexaploid wheat and rye, we used aneuploid lines of CS wheat. Details of each line used are described in Supplemental Materials and Methods S1. Seeds of Wasedori-nijo and Haru-ichiban were purchased from Yukijirushi Shubyo. Other seed stocks were obtained from the National BioResource Project-Wheat in Japan. Seeds were germinated and grown as described by Nomura et al. (2002).

Cloning of *GT* and *Glu* cDNAs

The cDNAs for *TaGTa* to *TaGTd* and *Taglu1d* were isolated from 48-h-old shoots of hexaploid wheat (cv CS) by screening a cDNA library and RT-PCR using primers listed in Supplemental Table S4. *ScGT* cDNA was isolated from a cDNA library of 48-h-old shoots of rye (cv Haru-ichiban). Details are described in Supplemental Materials and Methods S1.

Chromosomal Assignment of *GT* and *Glu* Loci in Hexaploid Wheat and Rye

Chromosomal locations of the *TaGTa* to *TaGTd* loci and the *Taglu1a* to *Taglu1d* loci in hexaploid wheat were assigned using the procedure described by Nomura et al. (2005). Chromosomal locations of the *ScGT* and *Scglu* loci in rye were determined by genomic PCR from wheat-rye chromosome addition lines. Primers used for chromosomal assignment are listed in Supplemental Table S6. Details are described in Supplemental Materials and Methods S1.

Expression and Purification of Recombinant TaGT and TaGlu1 Enzymes

For heterologous expression of N-terminal His-tagged TaGT enzymes, the entire coding region flanked by *Nde*I and *Hin*dIII sites was amplified by PCR using the primers listed in Supplemental Table S5 and was ligated into the *Nde*I and *Hin*dIII sites of a pET28a vector (Novagen). The resulting plasmid was transferred into BL21(DE3)pLysS for protein expression. See Supplemental Materials and Methods S1 for expression and purification of the recombinant TaGT enzymes.

For expression of the N-terminal His-tagged TaGlu1d enzyme, the coding region of mature TaGlu1d enzyme flanked by *Nco*I and *Xho*I sites was prepared by PCR using the primers shown in Supplemental Table S5. Protein expression in *Escherichia coli* and purification were performed as described for TaGlu1a to TaGlu1c (Sue et al., 2006).

Enzyme Assays

Activities of the TaGT enzymes were measured in 50 mM Tris-HCl buffer (pH 7.5) in a total volume of 500 μL. After incubation at 35°C, reactions were terminated by adding 50 μL of 1 N HCl, and reaction products were analyzed by HPLC (eluent, 24% [v/v] methanol containing 0.1% [v/v] acetic acid; column, Wakosil-II 5C18 HG [4.6 × 150 mm]; detection, 280 nm; flow rate, 0.9 mL min^{-1}; temperature, 40°C). To determine the kinetic parameters, UDP-Glc was fixed at 0.5 mM and concentrations of DIBOA or DIMBOA were varied

from 3 to 50 μM. Kinetic parameters for TaGlu1d were determined according to the method described previously (Sue et al., 2006). K_m and V_{max} values were calculated by fitting the data from several experiments to the Michaelis-Menten equation using SigmaPlot 11 (Systat Software).

qRT-PCR Analysis

Total RNA was isolated from 48- and 96-h-old CS and Tetra-CS using an RNeasy Plant Mini Kit (Qiagen), and the first-strand cDNA was synthesized from 2 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) and an oligo(dT) primer. Appropriately diluted RT sample (1 μL per 20 μL of PCR mixture) was subjected to real-time qPCR analysis on a MiniOpticon (Bio-Rad) with SYBR GreenER qPCR Supermix Universal (Invitrogen). Primers specific to each of the four cDNAs of *TaGT* and *Taglu1* genes were used (Supplemental Table S7). All PCR conditions followed the manufacturer's instructions, except the annealing temperature was 60°C. Each sample was quantified with respect to DNA standards (ranging from 10^2 to 10^6 copies per reaction tube). Specificity of the amplification was confirmed by melt-curve analysis and agarose gel electrophoresis.

Northern Analysis

Total RNA was isolated from shoots of CS, Tetra-CS, and diploid progenitors of hexaploid wheat, *T. urartu*, *A. speltoides*, and *A. tauschii*. See Supplemental Materials and Methods S1 for labeling of the probe, hybridization, and signal detection.

Southern Analysis

Total DNA was isolated from 5-d-old shoots of two barley cultivars (Betzes and Wasedori-nijo) and CS wheat using a DNeasy Plant Mini Kit (Qiagen). Aliquots (20 μg) of total DNA were digested individually with *Apa*I, *Eco*RV, or *Xba*I. See Supplemental Materials and Methods S1 for labeling of the probe, hybridization, and signal detection.

The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL/DDBJ databases with accession numbers AB547237 to AB547240, AB548283, and AB548284.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of amino acid sequences of TaGTs with their orthologs in rye (ScGT) and maize (ZmBX8 and ZmBX9).

Supplemental Figure S2. Alignment of amino acid sequences of TaGlu1s with their orthologs in rye (ScGlu) and maize (ZmGlu1 and ZmGlu2).

Supplemental Table S1. Nucleotide and amino acid identities among *GT* genes in hexaploid wheat, rye, and maize.

Supplemental Table S2. Nucleotide and amino acid identities among *glu* genes in hexaploid wheat, rye, and maize.

Supplemental Table S3. *GT* and *glu* cDNAs in hexaploid wheat, rye, and maize.

Supplemental Table S4. Primer sequences used for cloning.

Supplemental Table S5. Primer sequences used for the construction of *E. coli* expression plasmids.

Supplemental Table S6. Primer sequences used for chromosomal assignment.

Supplemental Table S7. Primer sequences used for qRT-PCR.

Supplemental Materials and Methods S1. Detailed experimental procedures.

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