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REVIEW

The genetic background of benzoxazinoid biosynthesis in cereals

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Abstract Benzoxazinoids (BXs) are important compounds in plant defense. Their allelopathic, nematode suppressive and antimicrobial properties are well known. BXs are found in monocot plants and in a few species of dicots. Over 50 years of study have led to the characterization of the chromosomal locations and coding sequences of almost all the genes involved in BX biosynthesis in a number of cereal species: *ZmBx1–ZmBx10a÷c* in maize, *TaBx1–TaBx5*, *TaGT* and *Taglu* in wheat, *ScBx1÷ScBx5*, *ScBx6-like*, *ScGT* and *Scglu* in rye. So far, the ortholog of the maize *Bx7* gene has not been identified in the other investigated species. This review aims to summarize the available data on the genetic basis of BXs biosynthesis in cereals.

Keywords Hydroxamic acid · Monocots · Allelochemicals · Secondary metabolites · *Bx* genes

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Occurrence, characterization and biological role of BXs

Benzoxazinoids (BXs) are protective and allelophatic secondary metabolites found in numerous species belonging to the *Poaceae* family, including maize, rye, wheat (Niemeyer 1988a; Grün et al. 2005; Nomura et al. 2007; Frey et al. 2009; Chu et al. 2011; Sue et al. 2011), the wheat progenitors *Triticum urartu*, *Aegilops speltoides*, *Aegilops squarrosa* (Niemeyer 1988b), and wild barleys: *Hordeum roshevitzii*, *Hordeum flexuosum*, *Hordeum brachyantherum*, *Hordeum lechleri* (Grün et al. 2005) and in genera—*Chusquea*, *Elymus*, *Arudo* (Zùñiga et al. 1983), *Coix* (Nagao et al. 1985), but not in *Avena* (Hamilton 1964), *Hordeum vulgare* or its progenitor *Hordeum spontaneum* (Grün et al. 2005). These compounds are also present in single species within a few dicot families: *Acathaceae—Acanthus mollis* (Wolf et al. 1985), *Aphelandra tetragona*, *Blepharis edulis* and *Aphelandra squarrosa* (Baumeler et al. 2000); *Rannuncuaceae* (Gierl and Frey 2001)—*Consolida orientalis* (Sicker et al. 2000), *Plantaginaceae* (Gierl and Frey 2001)—*Scoparia dulcis* (Chen and Chen 1976) and *Lamiaceae—Lamium galeobdolon* (Sicker et al. 2000).

BXs were first discovered and characterized in rye (Virtanen and Hietala 1955a, b), wheat and maize (Wahlroos and Virtanen 1959), in the 1950s. According to Hanhineva et al. (2011), three classes of compounds comprise the BXs: hydroxamic acids (HAs), lactams and benzoxazolinones, whereas Niemeyer (2009) divided BXs into HAs, lactams and methyl derivatives (Table 1).

According to Niemeyer (2009), HAs are the most active class of BXs by virtue of a hydroxyl group bound to the heterocyclic nitrogen atom, although studies on pests of maize done by Cambier et al. (2001) and Glauser et al. (2011) showed that the methylated forms of HAs are far

Table 1 Classification of benzoxazinoid compounds (according to Niemeyer 2009; Hanhineva et al. 2011)

BXs class	Compounds belonging to the given class
HAs	Indolin-2-one-3-hydroxy-indolin-2-one
	DIBOA-2,4-dihydroxy-1,4-benzoxazin-3-one
	DIBOA-Glc-2-*O*-*β*-D-glucopyranosyloxy-4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one
	DIMBOA-2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
	DIMBOA-Glc-2-*O*-*β*-D-glucopyranosyloxy-4-hydroxy-7-(2H)-methoxy-1,4-benzoxazin-3(4H)-one
	DIM_2BOA-7,8-dimethoxy-2,4-dihydroxy-1,4(2H)-benzoxazin-3-one
	DIM_2BOA-Glc-2-*O*-*β*-D-glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-(2H)-1,4-benzoxazin-3(4H)-one
	TRIBOA-2,4,7-trihydroxy-1,4-benzoxazin-3-one
	TRIBOA-Glc-2,4,7-trihydroxy-2H-1,4-benzoxazin-3(4H)-one
Lactams	HBOA-2-hydroxy-1,4-benzoxazin-3-one
	HBOA-Glc-2-*O*-*β*-D-glucopyranosyloxy-1,4-benzoxazin-3(4H)-one HMBOA-2-hydroxy-7-methoxy-1,4-benzoxazin-3-one
	HMBOA-Glc-2-*O*-*β*-D-glucopyranosyloxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one
	HM2BOA-2-Hydroxy-6,7-dimethoxy-(2H)-1,4-benzoxazin-3(4H)-one
	HM2BOA-Glc-2-*O*-*β*-D-glucopyranosyloxy-7,8-dimethoxy-(2H)-1,4- benzoxazin-3(4H)-one
	DHBOA-2,7-dihydroxy-1,4-benzoxazin-3-one
	DHBOA-Glc-2-*O*-*β*-D-glucopyranosyl-7-hydroxy-2H-1,4-benzoxazin-3(4H)-one
Benzoxazolinones	BOA-2-benzoxazolin-2(3H)-one
	MBOA-6-methoxy-benzoxazolin-2-one
Methyl derivatives	HDMBOA-2-hydroxy-4,7-dimethoxy-(2H)-1,4-benzoxazin-3(4H)-one
	HDMBOA-Glc-2-*O*-*β*-D-glucopyranosyloxy-4,7-dimethoxy-(2H)-1,4-benzoxazin-3(4H)-one

more toxic than de-methylated ones. For example, HDMBOA-Glc was proved to have higher toxic impact on aphid *Metopolophium dirhodum* than DIMBOA-Glc (Cambier et al. 2001).

The biosynthesis of BXs is usually at its highest during the juvenile stage of plant growth, then it subsequently declines and becomes stabilized at a lower level (Ebisui et al. 1998; Nomura et al. 2005, 2008). This pattern of biosynthesis is reflected by the transcript levels of the relevant enzymes (Rad et al. 2001; Nomura et al. 2005; Sue et al. 2006).

It should be pointed out that BXs biosynthesis is influenced by the plant genotype and cultivar (Niemeyer 2009) environmental conditions such as photoperiod (Epstein et al. 1986), light intensity (Manuwoto and Scrrber 1985) and the application of fertilizer (Manuwoto and Scrrber 1985). Moreover, introduction of foreign genes may also have an impact on BX production: in transgenic *Bt* corn, the level of BXs was found to be significantly lower than in the control non-transgenic plants (Nie et al. 2005). The authors suggested that the *Bt* gene has adverse effects on the biosynthesis and accumulation of DIMBOA and some phenolic acids, such as ferulic acid. However, under conditions of either water or nitrogen stress, the accumulation of DIMBOA in the leaves of the *Bt* corns could be enhanced.

In dicot plants, the BX content is comparable with that of monocot seedlings (Frey et al. 2009). However, in monocots, both shoots and roots produce BXs, while dicot species (e.g. *A. squarrosa*, *L. galeobdolon* and *C. orientalis*) synthesize these compounds in the above-ground parts of plants and in roots but at low levels. High levels of BXs have been detected in several adult parts of dicots: flower buds and flowers of *C. orientalis*, and leaves of *L. galeobdolon* and monocots: crown roots of maize (Schullehner et al. 2008). In some cases, the elevated biosynthesis of those compounds was observed in older plants; for instance in older whole plants (including the roots) of rye wounded mechanically (Kruidhof et al. 2014).

BXs are secondary metabolites that play roles in allelopathy and defense

The most effective allelopathic compounds are DIBOA, DIMBOA and their breakdown products BOA and MBOA (Barnes and Putnam 1987; Tabaglio et al. 2008). Their toxicity is due to the oxidation of cell wall peroxidases with associated production of H_2O_2, accumulation of lignins, disruption of lipid metabolism and protein synthesis, reduction of transport and/or secretory capabilities, and decreased H^+-ATPase activity (Barnes and Putnam 1987; Tabaglio et al. 2008).

Among cereals, rye shows the strongest allelopathic potential with the ability to reduce the germination, growth and development of many weeds (e.g. *Lepidium sativum*, *Amaranthus retroflexus*, *Echinoehloa crusgalli*, *Portulaca*

oleracea) and crop plants (e.g. cucumber, melon, tomato, lettuce, maize, tobacco) by up to 98 % (Barnes and Putnam 1987; Tabaglio et al. 2008). The allellopathic activity of rye depends on the season of the year: in autumn it is far higher than in early spring and, additionally, may increase after mechanical damage (Kruidhof et al. 2014). It seems to have a particular application effect: by wounding-increased allelopathic activity of rye applied as cover crop plant it would be possible to reduce the amount of around-growing weeds more efficiently, although, as underlined by the above mentioned authors, these effects might not be sufficient to compensate for the loss in biomass resulting from wounding.

In many species belonging to the *Poaceae* family, BXs are a crucial element in their defense mechanisms against pests, e.g. European Corn Borer (ECB, *Ostrinia nubilalis*) in maize (Klun et al. 1967; Barry et al. 1994), aphids (*Sitobion avenae*) in wheat (Bohldar et al. 1986) and nematodes in rye (Zasada et al. 2005). BX-based plant defenses against aphids and whorl feeding larvae seem to be connected with the anti-feeding properties of these compounds, namely their inhibition of digestive proteases responsible for detoxification and pest salivation (Feng et al. 1992). In addition, BXs appear to increase plant resistance to viruses transmitted by aphids, e.g. BYDV in wheat transmitted by *Rhopalosiphum padi* (Givovich and Niemeyer 1991).

Several studies have shown a relationship between BXs and disease resistance. However, the correlation between BXs content and disease resistance is not always positive, and is dependent on their site of synthesis and the character of the pathogen (Long et al. 1978; Søltoft et al. 2008).

Enhanced BXs synthesis can be induced by pathogenic organisms and also by tissue wounding (Basse 2005; Kruidhof et al. 2014). Wounding of maize plants caused increased DIMBOA synthesis, producing levels similar to those after infection by *Ustilago maydis* (Basse 2005). Persans et al. (2001) showed that the *CYP71C1* and *CYP71C3* of maize, encoding cytochrome P450s, involved in DIMBOA biosynthesis, were induced in response to wounding and treatment with naphthalic anhydride. After successive defoliations, the BX content in rye shoots was found to decline, but simultaneously it increased in the roots (Collantes et al. 1999). Glauser et al. (2011) showed also that BX derivatives such as HDMBOA-Glc and HDM2BOA-Glc have a toxic impact both on *S. littoralis* and *S. frugiperda* despite that *S. frugiperda* have a strong detoxification capacity in relation to DIMBOA.

BXs have also been shown to play a role in (1) improving plant tolerance to soil salinity (Makleit 2005), (2) the detoxification of triazine derivatives (Marcacci et al. 2005) and aluminum (Poschenrieder et al. 2005), (3) preventing chlorotic symptoms by forming chelates with iron (Pethô 2002), and (4) the inhibition of gibberellin-induced α-amylase activity in barley seeds (Kato-Noguchi 2008).

BXs importance is not only limited to the plant defense strategies, but also, like in case of majority plant secondary metabolites, they have strong, positive influence on human health. They are considered to be capable of lowering cancer risk (Zhang et al. 1995; Roberts et al. 1998) and insulin secretion (Landberg et al. 2010). Moreover, they have anti-allergic properties (Otsuka et al.1988; Poupaert et al. 2005), appetite suppression and weight reduction effects (Rosenfeld and Forsberg 2009). However, the content of BXs in final food products strongly depends on the type of grain processing (Tanwir et al. 2013; Hanhineva et al. 2014).

The biosynthesis of BXs

The BXs biosynthetic pathway has been studied in maize (Frey et al. 1997; Rad et al. 2001; Jonczyk et al. 2008), diploid, tetraploid, hexaploid *Triticales* (Nomura et al. 2002, 2005, 2008) and wild barley (Grün et al. 2005), but so far not in dicots (Schullehner et al. 2008). The most detailed study was performed in maize.

The first step, a branchpoint in BXs biosynthesis that occurs in chloroplasts, is the conversion of indole-3-glycerolphosphate to indole. The products of the next four reactions that take place in endoplasmic reticulum are indolin-2-one, 3-hydroxy-indolin-2-one, HBOA and DIBOA (Frey et al. 1997, 2009; Gierl and Frey 2001; Grün et al. 2005; Chu et al. 2011). Subsequently, the glucosylation of DIBOA to 2-*O*-β-glucoside (which is stored in vacuole) occurs prior to hydroxylation and O-methylation reactions in the cytoplasm that produce TRIBOA-Glc and DIMBOA-Glc, respectively (Gierl and Frey 2001; Grün et al. 2005; Jonczyk et al. 2008). Then DIMBOA-Glc and DIBOA-Glc can be transported to the vacuole. After hydroxylation DIBOA-Glc and DIMBOA-Glc are converted into DIBOA and DIMBOA, respectively, and released from vacuole to cytosol. Recently, Meihls et al. (2013) discovered other step: O-methylation reaction catalyzed by *O*-methyl transferases ZMBX10a ÷ ZMBX10c resulting in converse of DIMBOA-Glc into HDMBOA-Glc (Fig. 1). In several dicot plant species, DIBOA-Glc is the final product of BXs biosynthesis (Sicker et al. 2000).

Upon disintegration of the cell due to pathogen or pest attack and mobilization of jasmonic acid and/or its methyl ester, glucosidases stored in the chloroplast are activated to produce toxic aglucons (Oikawa et al. 2002; Niemeyer 2009). DIMBOA is the main aglucon in maize and wheat (Niemeyer 1988a; Frey et al. 1997), whereas in rye (Niemeyer 1988a; Gierl and Frey 2001; Zasada et al. 2005) and wild barley (Niemeyer et al. 1992; Grün et al. 2005) it is DIBOA.

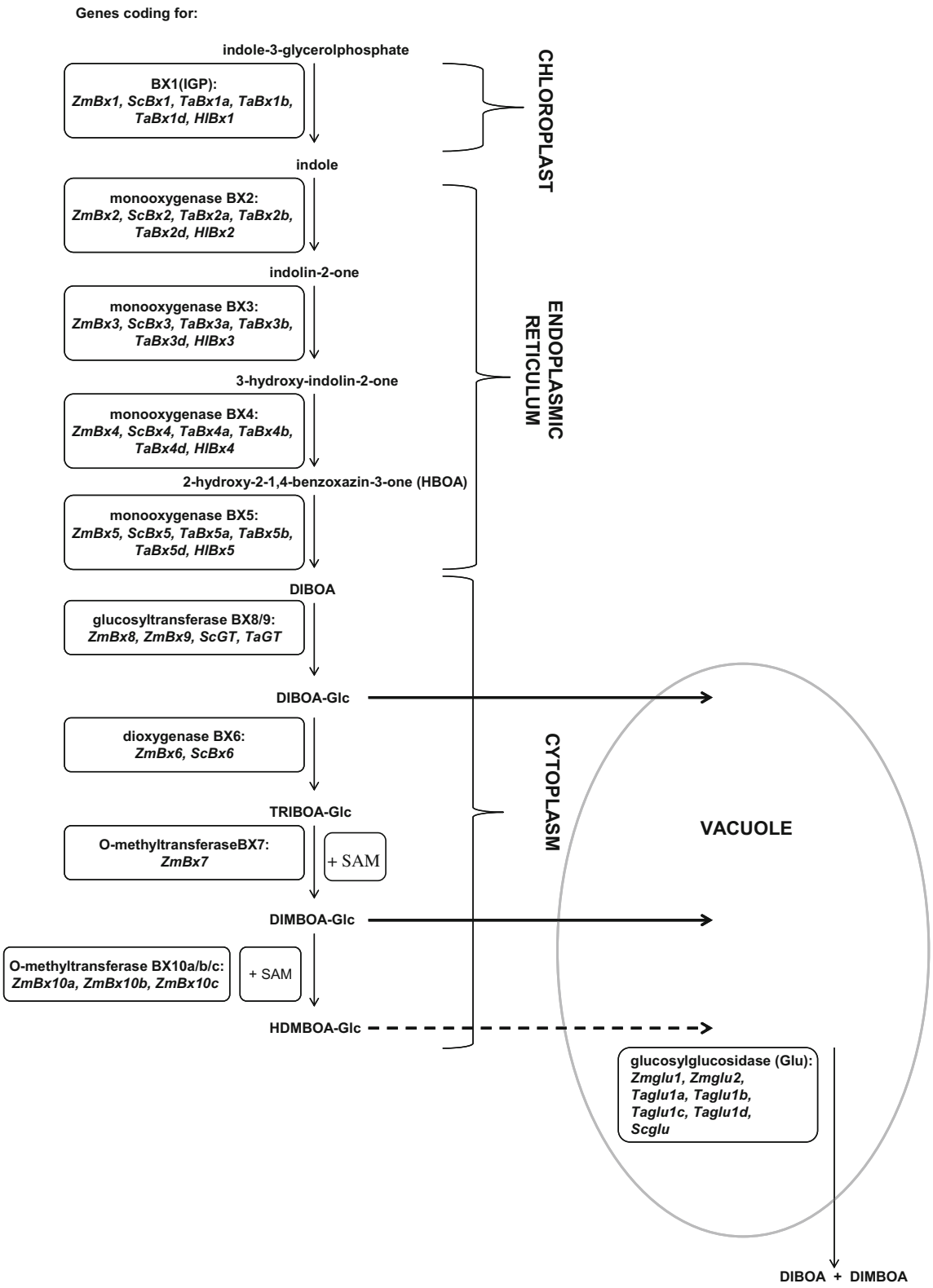

Fig. 1 BX biosynthesis pathway. Based on Sue et al. (2011), Dutartre et al. (2012) and Meihls et al. (2013)

Aglucons with an open ring structure generate the highly reactive α-oxo-aldehyde, which reacts with numerous nucleophiles occurring in amino acid residues (particularly thiols and amines) of proteins including catalytic enzymes, and this may account for the inhibition of various metabolic processes, such as electron transport in mitochondria

and chloroplasts or NADH oxidation by the cell wall (Niemeyer 2009). DIBOA and DIMBOA are unstable compounds, and are spontaneously degraded in the soil to BOA and MBOA, respectively (Zasada et al. 2005; Meyer et al. 2009).

Genetic basis of BX biosynthesis

Several genes controlling the biosynthesis of BXs have been isolated and characterized. The BX biosynthetic genes of maize are *ZmBx1–ZmBx10a÷c*, *Zmglu1* and *Zmglu2*. These encode the following enzymes: *ZmBx1*–indole-3-glycerol phosphate lyase; *ZmBx2–ZmBx5*—members of the CYP71C subfamily of cytochrome P450 monooxygenases; *ZmBx6*–2-oxoglutarate dependent dioxygenase, *ZmBx7–O*-methyltransferase; *ZmBx8*, *ZmBx9*—glucosyltransferases; *ZmBx10a÷ZmBx10c–O*-methyltransferase; *Zmglu1*, *Zmglu2*–glucosylglucosidases (Frey et al. 1997; Gierl and Frey 2001; Rad et al. 2001; Jonczyk et al. 2008; Meihls et al. 2013).

Orthologs of the maize genes *Bx1–Bx5* have also been identified in hexaploid *Triticum aestivum* (Nomura et al. 2002, 2003), diploid wheat *Triticum boeoticum* (Nomura et al. 2007), *Secale cereale* (Rakoczy-Trojanowska et al. 2013; Bakera et al. 2015) and *Hordeum lechleri* (Grün et al. 2005). Genes encoding glucosylglucosidases (*Taglu1a*, *Taglu1b*, *Taglu1c*, *Taglu1d*, *Scglu*) and glucosyltransferases (*TaGTa*, *TaGTb*, *TaGTc*, *TaGTd*, *ScGT*) have been identified in hexaploid wheat (Sue et al. 2006) and rye (Nikus et al. 2003; Sue et al. 2011).

For the majority of *Bx* genes, only the sequences of their mRNAs are known (Table 2). The exceptions are: maize *ZmBx1÷ZmBx5* and wheat *TaBx3* and *TaBx4*, for which cds, introns, 3′ UTRs, 5′ UTRs and putative promoters sequences have been described (Kramer and Koziel 1995; Frey et al.1997; Nomura et al. 2005, 2008) and in rye: *ScBx1÷ScBx5* sequences, including cds, introns, 3′ UTRs (http://www.ncbi.nlm.nih.gov/nuccore: KF636828, KF620524, KF636827, KF636826, KF636825, Table 2). La Hovary 2012 published the complete cDNA with 3′ UTR of *ScBx1* gene. Promoter sequences and 5′ UTRs have also been reported for the genes *TuBx3*, *TuBx4* (*Triticum urartu*), *AtBx3*, *AtBx4* (*Aegilops tauschii*), *AsBx3* and *AsBx4* (*Aegilops speltoides*), (Nomura et al. 2008). However, the availability of genomic sequence data means that full sequences of the other *Bx* genes of maize and wheat may readily be predicted. For example, the *ZmBx1* gene sequence was used as the query sequence in a BLAST screen of the B73 maize genome and a homologous gene was identified (http://www.ncbi.nlm.nih.gov/nuccore/AC200309.3:82,911–85,155,GRMZM2G085381), (Butrón et al. 2010). These authors then used an identical approach

Table 2 GenBank accession numbers of *Bx* genes of selected *Poaceae* species

Species	Gene	Sequence available	Accession number
Zea mays	*ZmBx1*	WG	X76713
	ZmBx2	WG	Y11368
	ZmBx3	WG	Y11404
	ZmBx4	WG	X81828
	ZmBx5	WG	Y11403
	ZmBx6	mRNA	AF540907
	ZmBx7	mRNA	EU192149
	ZmBx8	Complete cds + intron	AF331854
	ZmBx9	Complete cds + intron	AF331855
	Zmglu1	Protein	AAA65946
	Zmglu2	Protein	AAD09850
	ZmBx10a	Complete cds	KC754962
	ZmBx10b	Complete cds	KC754963
	ZmBx10c	Complete cds	KC754964
Triticum aestivum	*TaBx1A*	mRNA	AB094060
	TaBx1B	mRNA	AB124849
	TaBx1D	mRNA	AB124850
	TaBx2A	mRNA	AB042630
	TaBx2B	mRNA	AB042631
	TaBx2D	mRNA	AB124851
	TaBx3A	WG	AB298184
	TaBx3B	WG	AB298185
	TaBx3D	WG	AB298186
	TaBx4A	WG	AB298184
	TaBx4B	WG	AB298185
	TaBx4D	WG	AB298186
	TaBx5A	mRNA	AB042629
	TaBx5B	mRNA	AB124856
	TaBx5D	mRNA	AB124857
	Taglu1a	mRNA	AB100035
	Taglu1b	mRNA	AB236422
	Taglu1c	mRNA	AB236423
	Taglu1d	mRNA	AB548284
	TaGTa	mRNA	AB547237
	TaGTb	mRNA	AB547238
	TaGTc	mRNA	AB547239
	TaGTd	mRNA	AB547240
Triticum monococcum subsp. *aegilopoides*	*TbBx1-1P*	mRNA	AB255437
	TbBx1-2P	mRNA	AB255438
	TbBx1-3P	mRNA	AB255439
	TbBx1-4P	mRNA	AB255440
	TbBx1-5P	mRNA	AB255447
	TbBx2-1	mRNA	AB255441
	TbBx3-1	mRNA	AB255442
	TbBx4-1P	mRNA	AB255443
	TbBx5-1P	mRNA	AB255444
	TbBx5-2P	mRNA	AB255445
	TbBx5-3P	mRNA	AB255446
	TbBx5-4	mRNA	AB255448
	TbBx5-5	mRNA	AB255449

Table 2 continued

Species	Gene	Sequence available	Accession number
Triticum urartu	*TaBx3* ortholog	Promoter and 5′ UTR	AB297462
	TaBx4 ortholog	Promoter and 5′ UTR	AB297465
Aegilops tauschii	*TaBx3* ortholog	Promoter and 5′ UTR	AB297464
	TaBx4 ortholog	Promoter and 5′ UTR	AB297467
Aegilops speltoides	*TaBx3* ortholog	Promoter and 5′ UTR	AB297463
	TaBx4 ortholog	Promoter and 5′ UTR	AB297466
Secale cereale	*ScBx1*	Complete cds	HG380515
		complete cds + 3′ UTR	JQ716987
		complete cds + introns + 3′ UTR	KF636828
	ScBx2	Complete cds	HG380516
		mRNA	JX442061
		Complete cds + intron + 3′UTR	KF620524
	ScBx3	Complete cds	HG380517
		Complete cds + introns + 3′UTR	KF636827
	ScBx4	Complete cds	HG380518
		Complete cds + introns + 3′UTR	KF636826
	ScBx5	Complete cds	HG380519
		Complete cds + introns + 3′ UTR	KF636825
	ScBx6	Complete cds	HG380520
	Scglu	Protein	AAG00614
	ScGT	mRNA	AB548283
Hordeum lechleri	*HlBx1*	Partial cds + 3′ UTR	AY462226
	HlBx2	mRNA	AY462227
	HlBx3	mRNA	AY462228
	HlBx4	mRNA	AY462229
	HlBx5	mRNA	AY462230

WG whole gene including complete cds, 3′ UTR, predicted 5′ UTR and introns

to obtain maize gene orthologs of *Bx2–Bx5* and *Bx8*. By the same method, TAC clones of *T. aestivum* the full-length sequences of the *TaBx3* and *TaBx4* genes (http://www.ncbi.nlm.nih.gov/nuccore/AB298184.1-AB298186.1) were identified (Nomura et al. 2008).

Interestingly, the genes *Bx6* and *Bx7*, which encode enzymes catalyzing sequential 7-hydroxylation and 7-O-methylation of DIBOA-Glc to DIMBOA-Glc, have only been identified in maize, and appear to be absent from wheat, *Hordeum lechleri* and rye (Sue et al. 2011). Therefore, the identity of the genes controlling the transformation of DIBOA-Glc to DIMBOA-Glc in these cereal species is unknown. Recently, however, Tanwir et al. isolated *Bx6-like* gene from rye cv. Picasso (http://www.ncbi.nlm.nih.gov/nuccore/HG380520.1) which showed, on the nucleotide level, more than 78 % identity to *Zea* mays 2-oxoglutarate dependent dioxygenase (*Bx6*) (http://www.ncbi.nlm.nih.gov/nuccore/AF540907).

The regulation of *Bx* gene expression has been studied in *Triticum*. In hexaploid wheat, the expression of the *TaBx1–TaBx5* genes is co-regulated, but despite this, their level of transcription depends on the genome (A or B or D) on which the particular genes are located (Nomura et al. 2005). All genes located on chromosomes in genome B (*TaBx1B–TaBx5B*, *TaGTa–TaGTb*, *Taglu1a* and *Taglu1b*) are transcribed at a higher level than those from genomes A and D (Nomura et al. 2005; Sue et al. 2011). The same phenomenon has been observed in diploid progenitors of hexaploid wheat: *T. urartu* (genome A) and *A. tauschii* (genome D) display lower level *Bx* gene transcription than *A. speltoides*, the donor of genome B (Sue et al. 2011).

Phylogenetic relationships between *Bx* genes

As mentioned above, the biosynthesis of BXs is catalyzed by enzymes encoded by *Bx* genes: *Bx1–Bx9*. *Bx1* is considered a gene at the cluster branch point (whose evolution can be traced back to the duplication and functionalization of an ancestor encoding the alpha-subunit of tryptophan synthase—TSA) because the BX biosynthesis pathway is initiated by the indole-3-glycerol phosphate lyase encoded by this gene, which mediates the transformation of indole-3-glycerol-phosphate into indole (Frey et al. 2009). In *Hordeum spontaneum* and most *Hordeum vulgare* varieties, in which the defensive system is based on the indole alkaloid gramine [3-(dimethylaminomethyl)-indole], a *Bx1* ortholog is absent. Analysis of a phylogenetic tree of *Bx1* sequences from maize, wheat, rye and wild barley drawn according to Saitou and Nei (1987) indicates that they share a monophyletic origin (Fig. 2). However, there is relatively high structural dissimilarity between *HlBx1* and *ZmBx1*. To explain this difference, Grün et al. (2005) proposed that *HlBx1* should be evaluated separately. Phylogenetic analysis of the *Bx1*-encoded enzymes in three BX-producing dicots, *C. orientalis*, *L. galeobdolon* and *A. squarrosa*, led to the conclusion that BX biosynthesis evolved independently in dicot and monocot plants. Surprisingly, despite little similarity between their amino acid sequences, the BX1 enzymes of maize and wheat, and CoBX1, their ortholog from *C. orientalis*, have comparable catalytic properties (Schullehner et al. 2008).

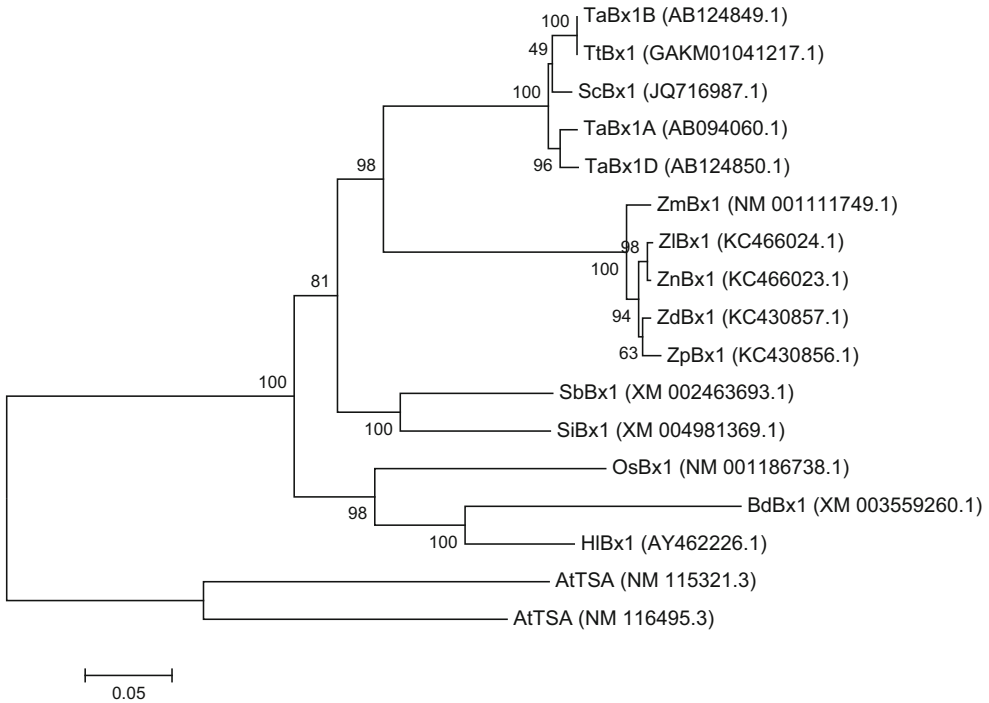

Fig. 2 Phylogenetic tree of *Bx1* sequences in chosen *Poaceae* species. The phylogenetic tree is generated by Mega 6 software (Tamura et al. 2013) based on the Neighbor Joining (NJ) algorithm (Saitou and Nei 1987) and Maximum Composite Likelihood substitution method with pairwise deletion and bootstrap analysis of 500. cDNA sequences from GenBank were translated into protein sequences followed by alignment done by means of ClustalW (attached to Mega6). After that, the protein sequences were transformed again into cDNA sequences and used for further analysis. The bootstrap values are indicated at the branch points. Scale bar indicates number of substitutions per site. Sequences from *Arabidopsis thaliana TSA* genes were used as outgroup. Individual labels were used for each *Bx1 cds* from organisms: Sc: *Secale cereale*, Ta: *Triticum aestivum*, Zm: *Zea mays*, Zl: *Zea luxurians*, Zn: *Zea nicaraguensis*, Zd: *Zea diploperennis*, Zp: *Zea perennis*, Sb: *Sorghum bicolor*, Si: *Setaria italica*, Os: *Oryza sativa*, Bd: *Brachypodium distachyon*, Hl: *Hordeum lechleri*, Tt: *Triticum turgidum*, At: *Arabidopsis thaliana*

A second group of genes controlling BX biosynthesis is composed of *Bx2–Bx5*, encoding four CYP71 monooxidases that are responsible for the sequential introduction of four oxygen atoms into the indole moiety, yielding DIBOA. Structurally, the *Bx2–Bx5* genes of wheat, maize, rye and wild barley share high homology and form four clades on the phylogenetic tree (Fig. 3). Their conserved structure suggests that their progenitor evolved before the divergence of the *Triticeae* and the *Panicoideae* (Grün et al. 2005; Frey et al. 2009). The CYP71 family proteins encoded by the *Bx2–Bx5* genes are highly substrate specific, which most probably is a result of duplication of a common ancestor gene followed by neofunctionalization (Frey et al. 2009; Chu et al. 2011).

Orthologs of *Bx2–Bx5* have so far not been identified in rice or sorghum. The rice genes *Cyp71c16* and *Cyp71c17* appear to be related to *Bx2* of other *Poaceae*, but their function is unclear. The remaining BX enzymes: BX3 ÷ BX5 have also not been detected in these species (Frey et al. 2009).

A third group of *Bx* genes comprises those encoding glucosyl transferases and glucoside glucosidases. From the studies of von Rad et al. (2001) and Sue et al. (2011), and our own unpublished analysis, it may be concluded that the wheat and rye *GT* and *glu* genes correspond to maize *ZmBx8* and *Zmglu*, respectively. The *ZmBx9* gene seems to have arisen by the duplication of *ZmBx8*, or it may be a remnant of paleotetraploidy of the maize genome (Swigoňová et al. 2004).

The structural polymorphism of *Bx* genes has so far been investigated in only two species: maize and rye. Using a study population consisting of 281 maize diverse inbred lines, Butrón et al. (2010) identified 45 INDELs and 44 SNPs in four amplicons for *ZmBx1*, 6 INDELs and 11 SNPs in one amplicon for *ZmBx2*, 3 INDELs and 19 SNPs in one amplicon for *ZmBx3*, 7 INDELs and 2 SNPs in two amplicons for *ZmBx4*, 10 INDELs and 35 SNPs in three amplicons in *ZmBx5*, and no polymorphisms of greater than 5 % frequency for *ZmBx8*. Twenty-eight polymorphisms of *ZmBx1* and one polymorphism of *ZmBx2* were significantly associated with the leaf content of DIMBOA and DIMBOA-Glc. Numerous SNPs and INDELs were found within fragments (675–832 bp) of the *ScBx5* gene (comprising part of first and second exon, and first intron) in winter rye (inbred line L318) and its wild relatives: *Secale cereale* ssp. *africanum*, *S. cereale* ssp. *ancestrale*, *S.*

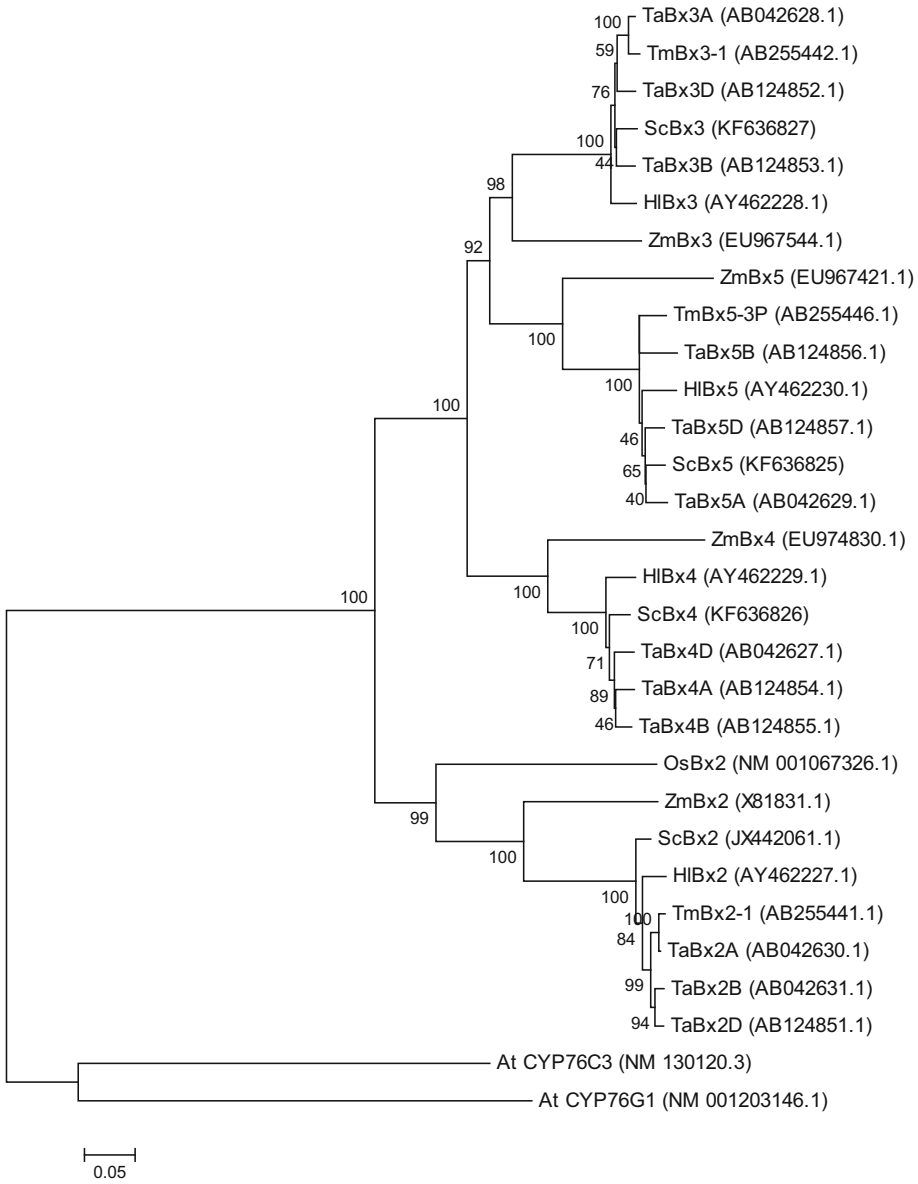

Fig. 3 Phylogenetic tree of *Bx2÷5* sequences. Mega 6 software (Tamura et al. 2013) based on the Neighbor Joining (NJ) algorithm (Saitou and Nei 1987) and Maximum Composite Likelihood substitution method with pairwise deletion and bootstrap analysis of 500. cDNA sequences from GenBank were translated into protein sequences followed by alignment done by means of ClustalW (attached to Mega6). After that, the protein sequences were transformed again into cDNA sequences and used for further analysis. The bootstrap values are indicated at the branch points. Scale bar indicates number of substitutions per site. Sequences from *Arabidopsis thaliana CYP76* genes were used as outgroup. Individual labels were used for each *Bx2÷5 cds* from organisms: Sc: *Secale cereale*, Ta: *Triticum aestivum*, Zm: *Zea mays*, Os: *Oryza sativa*, Hl: *Hordeum lechleri*, Tm: *Triticum monococcum* subsp. *aegilopoides*, At: *Arabidopsis thaliana*

cereale ssp. *dighoricum*, *S. cereale* ssp. *segetale*, *S. strictum*, *S. strictum* ssp. *africanum*, *S. strictum* ssp. *anatolicum*, *S. strictum* ssp. *ciliatoglume*, *S. strictum* ssp. *kuprianovii S. strictum* ssp. *strictum*, *S. sylvestre* and *S. vavilovii* (Rakoczy-Trojanowska et al. 2013). The analyzed sequence is partially collinear with the cytochrome P450 domain, including CYP cysteine heme–iron ligand signature. The total number of SNPs was 201, on average 2.7 SNP occurred per 10 nt and the total number of INDELs-50, averagely 0.7 INDELs per 10 nt. It should be emphasized that the majority of SNPs (5 times more abundant in intron than in two analyzed exons) and all INDELs were present in the intron. The most repeated SNP type in exons was C/G when in intron A/T and the most rare A/T and C/G, respectively. The amount of SNPs and INDELs differed between the examined species and ranged from 1 (*S. cereale* ssp. *dighoricum* and *S. strictum* ssp. *ciliatoglume*) to 99 (*S. strictum*) and from 1 (*S. cereale ssp. africanum*) to 13 (*S. strictum*), respectively. The longest insertion (106 bp) was identified in *S. strictum* and the longest deletion (51 bp)—in *S. strictum* ssp. *kuprijanovii*. No INDELs were detected in amplicons of *S. cereale* ssp. *segetale*, *S. vavilovii*, *S. cereale* ssp. *dighoricum* and *S. strictum* ssp. *ciliatoglume*.

Mapping

All identified genes controlling the biosynthesis of BXs in maize, wheat and rye have been mapped. Most of them are organized in clusters, especially the maize *Bx* genes. Gene clustering is a common feature in bacteria (Zheng et al.

2002), but less so in plant genomes, particularly in the case of genes encoding enzymes participating in secondary metabolism, which are mostly unlinked (Frey et al. 2009). However, recent studies have shown several examples of occurrence of genes clusters in plants and all of them are involved in secondary metabolite biosynthesis; besides BXs, to this group belong linamarin and lotaustralin in *Lotus japonicus*, diterpenes in *Oryza sativa*, avenacin in *Avena* spp., thianol and marneral in *Arabidopsis thaliana*, noscapine in *Papaver somniferum*, steroidal glycoalkaloid in *Solanum lycopersicum* and *Solanum tuberosum* (reviewed in Boycheva et al. 2014).

It is generally agreed that the maize genes encoding the oxidative enzymes of BX biosynthesis (*ZmBx1–ZmBx6*) as well as the *O*-methyl transferase (*ZmBx7*) and glucosyl transferase (*ZmBx8*) are localized on the short arm of chromosome 4, while *ZmBx9*, a close homolog of *ZmBx8*, is situated on chromosome 1, and two genes encoding glucosyl glucosidases (*Zmglu1*, *Zmglu2*) have been mapped to the short arm of chromosome 10 (Frey et al. 1997; Rad et al. 2001; Jonczyk et al. 2008). The chromosomal location of *Bx6* gene in maize genome is controversial. Although Dutartre et al. (2012) failed to locate the *ZmBx6* gene in the maize genomic sequence and they found only a close paralog on the long arm of chromosome 2, our recent analysis applying B73 genome revealed that the sequence deposited in NCBI data base (http://www.ncbi.nlm.nih.gov/nuccore/AF540907.1) is identical with 1,252,700–1254047 nt fragment of chromosome 4 (http://www.ncbi.nlm.nih.gov/nuccore/NC_024462.1) and similar to the 231,954,236–231,953,295 nt fragment of chromosome 2 (http://www.ncbi.nlm.nih.gov/nuccore/NC_024460.1) at 84 %. The newest discovered genes in maize-*ZmBx10a÷ZmBx10c* are located on chromosome 1, mapped to bin 1.04, approximately 66,304,872–66,500,692 bp (Meihls et al. 2013).

In hexaploid wheat, the *Bx* gene cluster is divided between group 4 (*TaBx1* and *TaBx2*) and group 5 (*TaBx3÷TaBx5*), with an additional copy of *TaBx3* on genome B (Nomura et al. 2003). The genes *Taglus* and *TaGTs* have been mapped to chromosomes belonging to groups 2 and 7, respectively (Sue et al. 2011). The order of genes *TaBx3÷TaBx5* is still not agreed. According to Nomura et al. (2008) these genes are ordered in the following manner (in relation to centromere): *Bx4–Bx3–Bx5* whereas from the figure published by Nomura et al. (2003) and Sue et al. (2011) there might be concluded that these genes are located on the short arms of chromosomes from group 5 in another way: *Bx3–Bx4–Bx5*.

In rye, genes *ScBx1* and *ScBx2* are located on chromosome 7R, *ScBx3÷ScBx5*—on chromosome 5R (Nomura et al. 2003) and *ScGT* (an ortholog of *ZmBx8/ZmBx9* coding for BX glucosyltransferase) and *Scglu* (an ortholog of *Zmglu1/Zmglu2* coding for BX glucosylglucosidase) are located separately on chromosomes 4R and 2R, respectively (Sue et al. 2011). Similarly as in wheat, the chromosomal arrangement of *ScBx3÷ScBx5* has not been determined yet.

The differences between the locations of the *Bx* genes in the genomes of maize, wheat and rye can be explained by numerous rearrangements of monocotelydoneous protochromosomes during the course of evolution (Salse et al. 2009). This hypothesis might explain the ancestry of individual chromosomes of modern cereals and the *Bx* genes they carry: (1) wheat chromosome group 4 (with *TaBx1*, *TaBx2*) and 5 (with *TaBx3÷TaBx5*), rye chromosomes 5 (with *ScBx3÷ScBx5*) and 7 (with *ScBx1*, *ScBx2*), and maize chromosome 4 (with *ZmBx1÷ZmBx8*) originated from protochromosome A11; (2) wheat chromosome group 2 (with *Taglua*, *Taglub*, *Tagluc*, *Taglud*), rye chromosome 2 (with *Scglu*) and maize chromosome 10 (with *Zmglu1*, *Zmglu2*) originated from protochromosome A4, and (3) wheat chromosome group 7 (with *TaGTa*, *TaGTb TaGTc TaGTd*), rye chromosome 4 (with *ScGT*) and maize chromosomes 1 (with *ZmBx9*) and 4 (*ZmBx8*) originated from protochromosome A8 (Sue et al. 2011; Dutartre et al. 2012). Rye chromosomes 7R and 5R show synteny with the long arms of hexaploid wheat group-4 chromosomes where *TaBx1* and *TaBx2* are located, and the short arms of group 5 chromosomes (with *Tabx3*, *TaBx4*), respectively (La Hovary 2012 upon Devos et al. 1993).

Although 5 *Bx* genes (*HlBx1–HlBx5*) have been isolated from *Hordeum lechleri* (Grün et al. 2005), their genomic location is currently unknown (Sue et al. 2011).

Bx genes have not so far been identified in cultivated barley (Grün et al. 2005). It is possible that *Hordeum vulgare* has "lost" these genes during the domestication process (Gierl and Frey 2001) or through chromosomal rearrangement (Grün et al. 2005).

Several QTLs controlling resistance to leaf feeding by a native American lepidopteran species (Bohn et al. 2001), European corn borer (ECB), (Cardinal et al. 2006; Butrón et al. 2010) and the Asian corn borer (ACB), (Li et al. 2010) have been identified in maize on chromosome 4, close to the *Bx* gene cluster (Cardinal et al. 2006; Li et al. 2010). Furthermore, two of these QTLs (*umc123* and *php200713*) have been mapped to the region of the *Bx* cluster (Cardinal et al. 2006). Further studies are required to prove the proposed correlation between the level of DIMBOA biosynthesis and resistance to leaf feeding damage by ACB and ECB (Li et al. 2010).

Outstanding questions

Despite the growing body of data on the genetic mechanisms controlling the biosynthesis of BXs, there are still many unanswered or poorly resolved questions: (1) which

enzymes mediate the transformation of DIBOA-Glc into DIMBOA-Glc in wheat, rye and other cereals besides maize, and which genes encode these enzymes? (2) Is the *ScBx6-like* gene of rye a functional ortholog of maize *ZmBx6*? (3) On which chromosome(s) are located genes *Bx6* and *Bx7* of rye, wheat and other cereals accumulating BX? (4) How are *Bx3*, *Bx4* and *Bx5* loci ordered in wheat and rye? (5) On which arm of chromosome 1 are mapped genes *ZmBx10a÷ZmBx10c*? (6) What is the location of *ZmBx6* gene in maize genome and if there is only one or two copies of this gene? (7) How has cultivated barley lost the ability to synthesize BXs and has the proposed elimination of all *Bx* loci due to degeneration of the coding sequences, silencing, or loss of one *Bx* locus activated the loss of all other *Bx* loci, as proposed by Nomura et al. (2007)? (8) How do different biotic and abiotic stresses regulate the expression of *Bx* genes? (9) Which polymorphisms are functionally relevant to BX biosynthesis?

Author contribution statement Contribution to this publication inserted through the co-authors was equal.

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