



Biosynthesis and chemical transformation of benzoxazinoids in rye during seed germination and the identification of a rye *Bx6-like* gene

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DIMBOA (PubChem CID: 2358)

DIMBOA-glc (PubChem CID: 441563)

HBOA (PubChem CID: 322636)

MBOA (PubChem CID: 10772)

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ABSTRACT

Benzoxazinoids are secondary metabolites with plant defense properties and possible health-promoting effects in humans. In this study, the transcriptional activity of *ScBx* genes (*ScBx1-ScBx5*; *ScBx6-like*), involved in benzoxazinoid biosynthesis, was analyzed during germination and early seedling development in rye. Our results showed that *ScBx* genes had highest levels of expression at 24–30 h after germination, followed by a decrease at later stages. For *ScBx1-ScBx5* genes expression was higher in shoots compared with root tissues and vice versa for *ScBx6-like* gene transcripts. Moreover, methylated forms of benzoxazinoids accumulated in roots rather than in shoots during seedling development, in particular reaching high levels of HMBOA-glc in roots. Chemical profiles of benzoxazinoid accumulation in the developing seedling reflected the combined effects of *de novo* biosynthesis of the compounds as well as the turnover of compounds either pre-stored in the embryo or *de novo* biosynthesized. Bioinformatic analysis, together with the differential distribution of *ScBx6-like* transcripts in root and shoot tissues, suggested the presence of a *ZmBx6* homolog encoding a 2-oxoglutarate dependent dehydrogenase in rye. The *ScBx6-like* cDNA was expressed in *E. coli* for functional characterization *in vitro*. LC-MS/MS analysis showed that the purified enzyme was responsible for the oxidation of DIBOA-glc into TRIBOA-glc, strongly suggesting the *ScBX6-like* enzyme in rye to be a functional ortholog of maize *ZmBX6*.

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Abbreviations: 2-ODD, 2-oxoglutarate dependent dehydrogenase; BOA, 2-benzoxazinone; Ct, Cycle threshold; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIBOA-glc, 2-β-D glucopyranosyl-4-hydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIMBOA-glc, 2-β-D glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; ES⁻, Electro spray negative mode; FeSO₄, Iron sulphate; G6PDH, Glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HBOA-glc, 2-β-D-glucopyranosyl-1,4-benzoxazin-3-one; HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HMBOA-glc, 2-β-D glucopyranosyl-7-methoxy-1,4-benzoxazin-3-one; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LC-MS/MS, Liquid chromatography-mass spectrometry; MBOA, 6-methoxy-2-benzoxazinone; MRM, Multiple reaction monitoring; PCR, Polymerase chain reaction; qRT-PCR, Quantitative real-time polymerase chain reaction; SPF, Putative splicing factor; TRIBOA-glc, 2,4,7-trihydroxy-1,4-benzoxazin-3-one-glucoside; TRIBOA, 2,4,7-trihydroxy-2H-1,4-benzoxazin-3(4H)-one.

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1. Introduction

Benzoxazinoids, a specialized group of defense and allelopathic compounds in plants, have been studied in a few dicots and several monocots such as maize (*Zea mays*), wheat (*Triticum aestivum*), rye (*Secale cereale*), and wild barley (*Hordeum* spp.) (Etzerodt et al., 2008; Schullehner et al., 2008; Søltøft et al., 2008; Niemeyer, 2009). Benzoxazinoids are characterized as hetero-aromatic metabolites that are categorized into three groups: 1) benzoxazinones (benzoxazin-2-one, BOA; and its 6-methoxy derivative, MBOA), 2) lactams (2-hydroxy-1,4-benzoxazin-3-one, HBOA; its 7-methoxy derivative, HMBOA and their glycosides), and 3) hydroxamic acids (2,4-dihydroxy-1,4-benzoxazin-3-one, DIBOA; its 7-methoxy derivative, DIMBOA, and their glycosides). Table 1

Table 1
Classification of benzoxazinoids based on their chemical structures. 2-Benzoxazolinone (BOA); 6-methoxy-2-benzoxazolinone (MBOA); 2-hydroxy-1, 4-benzoxazin-3-one (HBOA); 2-hydroxy-7-methoxy-1, 4-benzoxazin-3-one (HMBOA); 2-β-D-glucopyranosyloxy-1,4- benzoxazin 3-one (HBOA-glc) 2-β-D glucopyranosyloxy-7-methoxy-1, 4-benzoxazin-3-one (HMBOA-glc); 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA); 2-β-D glucopyranosyloxy-4-hydroxy-1,4 benzoxazin-3-one (DIBOA-glc); 2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA); 2-β-D glucopyranosyloxy-4-hydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA-glc). Double hexose derivatives of HBOA and DIBOA (HBOA-glc-hex and DIBOA-glc-hex).

Benzoxazolinones		Lactams		Hydroxamic acids		
R ₁		R ₁	R ₂	R ₁	R ₂	
H	BOA	H	H	H	H	DIBOA
OCH ₃	MBOA	H	glc	H	glc	DIBOA-glc
		OCH ₃	H	OCH ₃	H	DIMBOA
		OCH ₃	glc	OCH ₃	glc	DIMBOA-glc
		H	glc-hex ^a	H	glc-hex ^a	DIBOA-glc-hex

^a The nature of the second sugar molecule (hexose) is not yet identified.

describes the most common benzoxazinoids found in plants and shows their chemical structures. In vegetative plant tissue, DIMBOA and its glycosides are the predominant benzoxazinoids in maize and wheat, whereas DIBOA and its glycosides are the predominant form found in rye (Jonczyk et al., 2008). Recent studies revealed that the not yet fully identified benzoxazinoids DIBOA-glc-hexose and HBOA-glc-hexose are two other major forms of benzoxazinoids, present in the mature wheat and rye seeds and products thereof (Adhikari et al., 2012a; Jensen et al., 2015; Tanwir et al., 2013).

Benzoxazinoid biosynthesis, with respect to gene isolation and functional characterization, has been extensively elucidated in monocots (Gierl and Frey, 2001; Nomura et al., 2002) compared with dicots. To date, genes involved in benzoxazinoid biosynthesis in monocots have been isolated from maize (Frey et al., 1995, 1997), wild barley (Grün et al., 2005), wild diploid wheat (Nomura et al., 2007) and hexaploid wheat (Nomura et al., 2005, 2003, 2002). In maize, nine genes (*ZmBx1-ZmBx9*) are involved in DIMBOA-glc biosynthesis. Eight *ZmBx* genes are specifically clustered together on the short arm of chromosome 4 (Jonczyk et al., 2008; Von Rad et al., 2001), whereas *ZmBx9*, which is a duplicate of *ZmBx8*, is found outside the cluster on chromosome 1 (Von Rad et al., 2001). In contrast to maize, only five *Bx* genes have so far been described for DIBOA biosynthesis in hexaploid wheat and rye. The wheat *TaBx* genes are localized on chromosome 4 (*TaBx1-TaBx2*) and chromosome 5 (*TaBx3-TaBx5*), whereas the rye *ScBx* genes are localized on chromosome 7 (*ScBx1-ScBx2*) and chromosome 5 (*ScBx3-ScBx5*) (Nomura et al., 2003). All members of the *Bx* gene clusters exhibit different enzymatic properties and perform distinct functions, shown for the genes studied in this work in Table 2. Chromosome clustering of the *Bx* genes has been proposed to facilitate the transfer of benzoxazinoid pathway genes among distant relatives (Nomura et al., 2003) whereas it appears not to be essential for the coordinated transcription in wheat and rye (Sue et al., 2011).

Earlier studies reported that benzoxazinoids are absent from

mature seeds and only appear in plants during germination at early developmental stages (Frey et al., 2003). The highest concentrations of total benzoxazinoids have been found in young leaves, e.g. in four-day-old seedlings in wheat (Zuniga et al., 1983), and big differences in the contents have been observed among cultivars and under different growth conditions. The tissue-specific localization of benzoxazinoids in vegetative tissues has also been addressed in maize, wheat and rye (Cambier et al., 2000; Carlsen et al., 2009; Copaja et al., 2006; Villagrasa et al., 2006). However, recent studies showed that considerable amounts of these compounds are indeed present in the mature seeds of wheat and rye (Pedersen et al., 2011; Tanwir et al., 2013), and these novel findings emphasize the use of more accurate analytical tools to gain a better understanding of their biosynthesis. Thus far, the effects on benzoxazinoid levels at various stages of plant growth have mostly been described in terms of chemical contents under stress conditions, i.e., insect attack in maize and wheat or during tissue degradation in rye when used as green manure. In addition, the effects of cooking processes on benzoxazinoids were recently analyzed in food products (Tanwir et al., 2013). The biosynthesis of benzoxazinoids at the molecular level in rye has not been elucidated in details, which is pertinent since rye plants in general show the highest concentrations of benzoxazinoids among the cereals.

Rye, an important crop in the northeastern regions of Europe and southern United States, is used as a green manure crop during the early stages of growth, and the mature grains are used for bread and breakfast products. High biomass production, tolerance to low temperature, and allelopathic effects have increased its importance as a cover crop (Barnes et al., 1987). Potential health effects of benzoxazinoids (Adhikari et al., 2013) and their bioavailability in animals (Adhikari et al., 2012a, 2012b, 2013) have generated interest in knowledge about the biosynthesis of these compounds and their bio-transformations during food processing, in particular in rye products with high contents of benzoxazinoids (Tanwir et al., 2013).

Table 2
Bx genes studied in this work which are involved in benzoxazinoid biosynthesis, with their corresponding enzymes, function and cellular localization.

Gene	Enzyme	Function	Cellular Localization
<i>Bx1</i>	indole-3-glycerol phosphate lyase	Converts indole-3 glycerol phosphate into indole	Chloroplast
<i>Bx2-Bx5</i>	cytochrome P450 oxygenases	Converts indole into DIBOA	Endoplasmic reticulum
<i>Bx6</i>	2-oxoglutarate dependent dioxygenase (2-ODD)	Converts DIBOA-glc into TRIBOA-glc	Cytoplasm (?)
<i>Bx7</i>	O-methyltransferase	Methylates TRIBOA-glc turning it into DIMBOA-glc	Cytoplasm

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