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Evolution of the indole alkaloid biosynthesis in the genus *Hordeum*: Distribution of gramine and DIBOA and isolation of the benzoxazinoid biosynthesis genes from *Hordeum lechleri* ☆

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Abstract

Two indole alkaloids with defense related functions are synthesized in the genus *Hordeum* of the Triticeae. Gramine (3(dimethyl-amino-methyl)-indole) is found in *H. spontaneum* and in some varieties of *H. vulgare*, the benzoxazinoid 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) is detected in *H. roshevitzii*, *H. brachyantherum*, *H. flexuosum*, *H. lechleri*. Biosynthesis of DIBOA and of gramine was found to be mutually exclusive in wild *Hordeum* species, indicating that there was selection against simultaneous expression of both pathways during evolution. The full set of genes required for DIBOA biosynthesis in *H.lechleri* was isolated and the respective enzyme functions were analyzed by heterologous expression. The cytochrome P450 genes *Bx2–Bx5* demonstrate a monophyletic origin for *H. lechleri*, *Triticum aestivum* and *Zea mays*. *HlBx2–HlBx5* share highest homology to the orthologous genes of *T. aestivum.* In contrast, the branch point enzyme of the DIBOA pathway, the indole-3-glycerol phosphate lyase BX1, might have evolved independently in *H. lechleri*. In all *Hordeum* species that synthesize DIBOA, DNA sequences homologous to *Bx* genes are found. In contrast, these sequences are not detectable in the genomes of *H. vulgare* and *H. spontaneum* that do not synthesize benzoxazinoids.

Keywords: Gramineae; *Hordeum* species; DIBOA; Gramine; Cytochrome P450 enzymes; Indole biosynthesis; Evolution

1. Introduction

Plant secondary metabolites have committed functions in communication, and serve often as weapons in chemical defense. Benzoxazinoids represent protective metabolites that are abundant in the Gramineae, including the major agricultural crops maize, wheat and rye. Outside the Gramineae these secondary metabolites are found dispersed in isolated dicotyledonous species (Sicker et al., 2000). Benzoxazinoids are important factors of plant resistance against microbial diseases and insects, and serve allelochemical functions. A unique situation is observed in the Triticeae genus *Hordeum* L. The benzoxazinoid 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) is synthesized in some *Hordeum* species but is missing in others, including *H. vulgare* (Niemeyer, 1988). A second defense related indole alkaloid, 3(dimethyl-amino-methyl)-indole (gramine),

Abbreviations: PCR, polymerase chain reaction.

☆ Accession numbers: *HlBx1*, AY462226; *HlBx2*, AY462227; *HlBx3*, AY462228; *HlBx4*, AY462229; *HlBx5*, AY462230.

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is detected in *Hordeum*. Benzoxazinoids and gramine have common features: they control a broad spectrum of pests and pathogens, and the *in planta* concentrations are highest in seedlings and young plants. Both secondary metabolites are derived from tryptophan biosynthesis. Gramine is synthesized via tryptophan (Gross et al., 1974), and indole-3-glycerol phosphate (IGP) is the branch point for DIBOA biosynthesis. While no genes have been identified for gramine biosynthesis, the biosynthetic pathway of benzoxazinoids has been elucidated in maize (Frey et al., 1997, 2003; v. Rad et al., 2001). The committing enzyme of benzoxazinoid biosynthesis, BX1, evolved via gene duplication and modification from the alpha subunit of the tryptophane synthase. Indole-3-glycerol phosphate (IGP) is cleaved by BX1 into indole and glyceraldehyde-3-phosphate. The introduction of four oxygen atoms into the indole moiety that yields DIBOA is catalyzed by the cytochrome P450 enzymes BX2–BX5 (Fig. 1). Orthologues to *Bx1–Bx5* have been identified for wheat (*TaBx1–TaBx5*, Nomura et al., 2002, 2003).

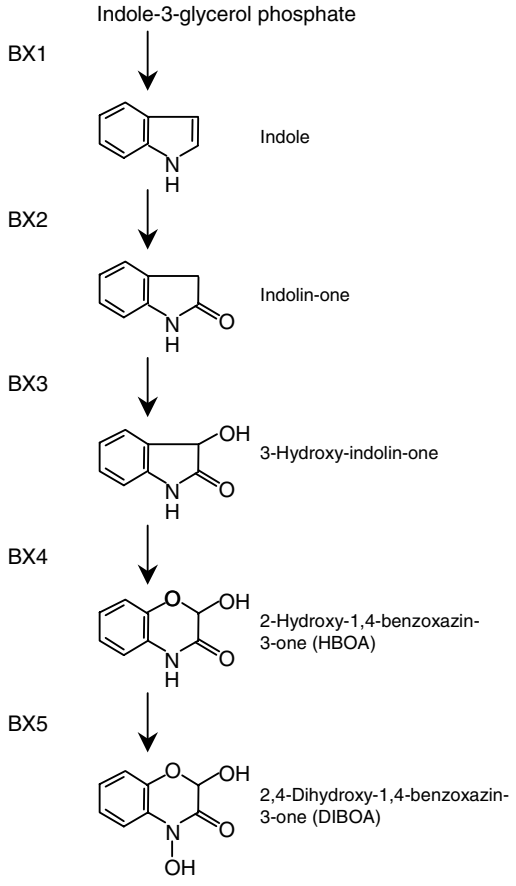

Fig. 1. Schematic presentation of the DIBOA biosynthetic pathway showing enzyme reactions catalyzed by the enzymes BX1–BX5. The pathway was first elucidated in maize (Frey et al., 1997) and later in wheat (Nomura et al., 2002, 2003).

Hordeum provides the possibility to study the evolution of pathways with functional equivalent products that compete for substrates from tryptophan biosynthesis. Due to the missing data on genes involved in gramine biosynthesis, the investigations on co-evolution of DIBOA and gramine biosynthesis were restricted to the evaluation of the product pattern. We show that the two pathways are mutually exclusive. The knowledge about benzoxazinoid biosynthesis in *Zea mays* allowed the isolation of the orthologous genes from *H. lechleri*. The data demonstrate a monophyletic origin of the P450 genes of benzoxazinoid biosynthesis in the Gramineae and a collective loss of these genes in the non-DIBOA producing species. The *Bx1* gene function may have evolved by independent duplications of the *TSA* gene.

2. Results

2.1. Biosynthesis of defense related indole alkaloids is restricted to one structural class, DIBOA or gramine, in Hordeum species

Five different wild barley species (*H. brachyantherum*), karyotype H, diploid; *H. flexuosum*, karytope H, diploid; *H. lechleri*, karyotype H, hexaploid; *H. roshevitzii*, karyotype H, diploid, and three different lines of *H. spontaneum*, karyotype I, diploid (Jacob et al., 2004) and four lines of cultivar barley *H. vulgare* (Alexis, Baccara, Nuernberg and Tellus, karyotype I, diploid,) were analyzed for their indole alkaloid content at the seedling stage (first fully expanded leaf) via HPLC. No concurrent production of both types of indole alkaloids was detected in any line (Table 1). The four cultivar lines contain neither gramine nor benzoxazinoids (Table 1). Seedlings of the wild barley species *H. brachyantherum*, *H. flexuosum*, *H. lechleri*, *H. roshevitzii* contain DIBOA in concentrations of 300–1000 nmol g^{-1} fresh weight, gramine concentrations remained below the detection limit (100 nmol g^{-1} fresh weight) in these species. In the three tested *H. spontaneum* lines gramine concentrations of 300–1600 nmol g^{-1} fresh weight are found whereas benzoxazinoid concentrations remained below the detection limit.

2.2. Characterization of the indole-3-glycerolphosphate lyase HlBX1

The branch point gene *Bx1* of DIBOA biosynthesis is derived from the α-subunit of tryptophan synthase, *TSA*, via gene duplication and modification. TSA and BX1 catalyze the same reaction, the conversion of indole-3-glycerol phosphate (IGP) to indole and glyceraldehyde-3-phosphate. There are two fundamental differences between BX1 and TSA: monomeric BX1

Table 1
DIBOA and gramine content in seedlings of *Hordeum* species

Species	Line	DIBOA [nmol/g fresh weight]	Gramine [nmol/g fresh weight]
H. vulgare	Alexis	0	0
	Baccara	0	0
	Nürnberg	0	0
	tellus	0	0
H. spontaneum	42–48	0	364 (±52)
	150–31	0	829 (±105)
	160–53	0	1401 (±216)
H. brachyantherum	(H 2012)	647 (±121)	0
H. flexuosum	(H 1110)	404 (±124)	0
H. lechleri	(H 1550)	977 (±163)	0
H. roshevitzii	(H 179)	327 (±16)	n.d.

n.d., not determined.

catalyzes the reaction and the formed indole is released. In contrast, efficient catalysis by TSA requires the formation of a complex with the β-subunit (TSB) of the tryptophan synthase (TS), the synthesized indole is not released but travels through a tunnel connecting the active sites of the α- and β-subunits and is used for the formation of tryptophan by TSB within the complex. A second indole-3-glycerol phosphate lyase with homology to *TSA* and *Bx1* and the characteristic enzymatic properties of BX1, has been identified in maize. This BX1-type enzyme, termed IGL, is induced by herbivore attack and delivers indole in the so-called tritrophic interaction (Alborn et al., 1997; Frey et al., 2000).

In order to isolate the gene that catalyzes the formation of indole for DIBOA biosynthesis in *H. lechleri*, a cDNA library derived from seedling tissue was screened with the maize *Bx1* gene cDNA (*ZmBx1*) as a probe at reduced stringency. One hybridization signal was detected and the recombinant phage isolated. The isolated sequence was named *HlBx1*. Sequencing revealed a strong homology with *ZmBx1* (72% identity). The gene was expressed in *Escherichia coli* and the kinetic data of the purified recombinant enzyme were determined (Table 2). HlBX1 catalyzes efficiently the biosynthesis of indole, the K_m and k^{cat} values for the substrate IGP are close to the constants determined for the indole-3-glycerol phosphate lyases BX1 and IGL from *Z. mays*. The homomeric HlBX1 (k^{cat}/K_m^{IGP} 31 mM^{-1} s^{-1}) is about 4-fold more efficient in catalyzing IGP cleavage than the *E. coli* TS $\alpha_2\beta_2$ (k^{cat}/K_m^{IGP} 7.4 mM^{-1} s^{-1}). Since maximum DIBOA concentrations are found in the *H. lechleri* seedling and no other *Bx1*-homologous clone was detected in the seedling-specific cDNA library, HlBX1 is very likely the BX1 function of *H. lechleri*.

Table 2
Comparison of catalytic properties

	Z. mays		*H. lechleri*
	BX1	IGL	HlBX1
K_m^{IGP} (mM)	0.013	0.1	0.080
k^{cat} (s^{-1})	2.8	2.3	2.5
k^{cat}/K_m^{IGP} (mM^{-1} s^{-1})	215	23	31

2.3. Isolation of HlBx2–HlBx5 from H. lechleri

Indole is converted to DIBOA by the action of four cytochrome P450 enzymes, BX2–BX5, that introduce four oxygen atoms. The genes *Bx2–Bx5* are members of the *CYP71C* subfamily of plant cytochrome P450 genes and share overall amino acid identities of 45–65%. Poly(A)$^+$ RNA was extracted from *H. lechleri* seedlings and used in an RT-PCR approach to isolate partial sequences with homology to the maize genes *Bx2–Bx5*. Two fragments were isolated and used for screening of a cDNA library. Six recombinant phages were isolated and the sequence of the cloned cDNAs determined. Four different cDNAs, named *HlBx2*, *HlBx3*, *HlBx4* and *HlBx5* according to their respective closest maize homologue, were identified. Two clones represented partial sequences of the *HlBx2* sequence.

Identities of the deduced amino acid sequences constitute 80% for HlBX2 and ZmBX2, 76% for HlBX3 and ZmBX3, 81% for HlBX4 and ZmBX4 and 78% for HlBX5 and ZmBX5 (Table 3). This is significantly higher than the respective identities of HlBX sequences among themselves (47–67%, Table 3) and of the four maize BX sequences among themselves (43–60%, Frey et al., 1995).

2.4. Substrate specificity of the enzymes HlBX2–HlBX5

The stepwise conversion of indole to DIBOA in maize starts with the formation of indolin-2(1*H*)-one catalyzed by BX2. Indolin-2(1*H*)-one is converted to 3-hydroxy-indoline-2(1*H*)-one by BX3. Then BX4 catalyzes the conversion of 3-hydroxy-indoline-2(1*H*)-one to 2-hydroxy-2*H*-1,4-benzoxazin3(4*H*)-one (HBOA). The *N*-hydroxylation of HBOA to DIBOA is catalyzed by BX5 (Fig. 1). Substrate specificity of the ZmBX2–ZmBX5 enzymes was tested by heterologous expression

Table 3
Comparison of the amino acid sequences of *H. lechleri* and *Z. mays*

	HlBX2	HlBX3	HlBX4	HlBX5
HlBX3	51/70	–	–	–
HlBX4	52/71	67/83	–	–
HlBX5	47/66	67/80	58/76	–
ZmBX2	**80/90**	51/71	50/68	49/69
ZmBX3	50/72	**76/88**	64/80	65/81
ZmBX4	52/72	62/79	**81/90**	57/77
ZmBX5	46/65	61/76	54/74	**78/86**

Percentage of identities/percentage of similarities are given. Combinations with the highest degree of identity and similarity are highlighted by bold letters.

in *S. cerevisiae* WAT11 (Frey et al., 1997; Glawischnig et al., 1999). This yeast system expresses an *A. thaliana* P450 reductase gene (*ATR1*) (Pompon et al., 1996) and the same system was used for expression of HlBX2–HlBX5. Intermediates of the DIBOA biosynthesis pathway were incubated with microsomal fractions from yeast cells expressing one of the *H. lechleri* P450 enzymes. As their maize homologues, HlBX2 converts specifically indole to indolin-2(1*H*)-one and HlBX4 specifically 3-hydroxy-indolin-2(1*H*)-one to HBOA. HlBX5 had to be modified to meet the requirements for efficient translation in yeast cells to get detectable enzyme activity (see Section 4). The modified HlBX5 specifically converts HBOA to DIBOA. None of the other substrates was converted by one of these enzymes. All efforts to get a functional expression of HlBX3 failed, most likely due to limitations of the heterologous yeast system. However the wheat enzyme TaBX3 (Nomura et al., 2002) and HlBX3 share 95% identity and 98% similarity, this extremely high degree of sequence conservation makes it most likely that the *H. lechleri* sequence indeed represents the BX3 function although in this one case a functional verification of the HlBX enzyme function in the recombinant yeast system was not possible.

2.5. Distribution of HlBx-homologous sequences in Hordeum species

We examined whether the genes orthologous to *HlBx1–HlBx5* are present in different wild barley species and *H. vulgare* cultivars. A clear correlation between DIBOA biosynthesis and presence of *HlBx* gene sequences was detected. Strong hybridizing bands were observed for all *Hordeum* species producing DIBOA for all five genes assayed (Fig. 2, representative data for *HlBx1* and *HlBx2* hybridization probes are shown). Under the same hybridization conditions no hybridization bands were detected for *H. vulgare* cultivars and *H. spontaneum* ssp. with the probes of the P450 genes *HlBx2–HlBx5*, although clear signals were displayed in parallel with wheat DNA. All four modifying genes of the pathway seem to be absent from the genomes of these non-DIBOA producing species. *HlBx1* homologous sequences were detected in all *Hordeum* species independent of the status in DIBOA biosynthesis. Interestingly, the hybridization pattern with the *HlBx1* probe is identical for *H. vulgare* and *H. spontaneum* ssp., this result is in accord with the assumption that *H. spontaneum* is the progenitor of cultivated barley. However, it is not clear whether the bands lighting up in *H. vulgare* and *H. spontaneum* represent an orthologue of *HlBx1* ore simply the *TSA* gene. The maize *ZmBx1*-probe cross-hybridizes with *Igl* and maize *TSA* under the hybridization conditions used.

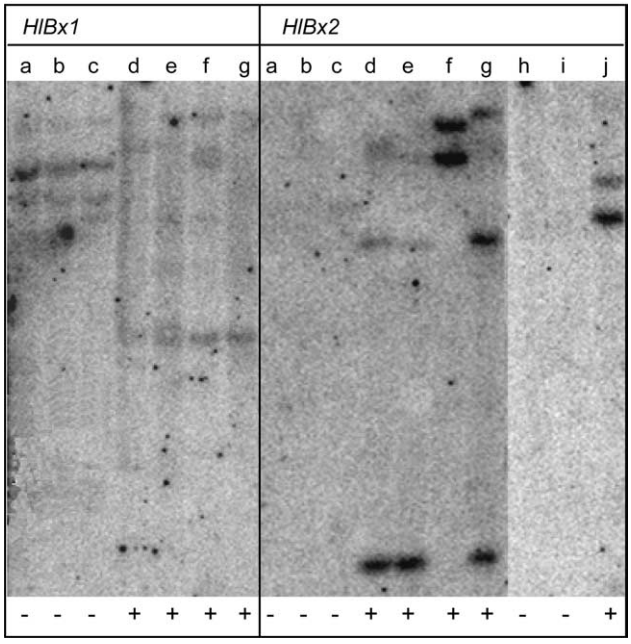

Fig. 2. Southern analysis of genomic DNAs (*Hin*dIII digest) from various *Hordeum* species and *T. aestivum* with *H. lechleri Bx gene* cDNA. Representative data for hybridization probes (*HlBx1* and *HlBx2*) are shown. Hybridization with *HlBx2* revealed no signal for *H. spontaneum* ssp. and *H. vulgare* cultivars, bands light up for all plants that synthesize DIBOA. The same distribution of hybridization signals was displayed for *HlBx3*, *HlBx4*, *HlBx5* (data not shown). The membrane was probed consecutively with the probes. + and − indicate the presence and absence of DIBOA in the investigated species. (a,h,i) *H. vulgare* cultivars Alexis, Nade and B87; (b) *H. spontaneum* ssp. 42–48; (c) *H. spontaneum* ssp. 160–130; (d) *H. lechleri*; (e) *H. brachyantherum*; (f) *H. roshevitzii*; (g) *H. flexuosum*; (j) *T. aestivum* cultivar Trakos.

3. Discussion

3.1. Biosynthesis of DIBOA and of gramine is mutually exclusive in wild Hordeum species

The genus *Hordeum* L. comprises 31 species which occur under a wide variety of climates in Eurasia and the New World. The species can be grouped into four clades (Xu, Xa, H and I). Clade H includes Asian (H1) and New World members (H2). Representatives of subclades H1 (*H. roshevitzii*) and H2 (*H. brachyantherum*, *H. flexuosum*, *H. lechleri*) contain DIBOA, *H. spontaneum ssp*, grouped in clade I, have substantial levels of gramine while DIBOA is not detectable (Table 1). Nothing is published about indole alkaloid content in *Hordeum* species from clade Xu and Xa.

Benzoxazinoids are found in the subfamilies Panicoideae (e.g., *Z. mays*), and Pooideae (e.g., *Triticum aestivum*), suggesting that the acquisition of the pathway occurred relatively early in the evolution of the Gramineae. Gramine is synthesized in *Hordeum* and *Phalaris*, both belonging to the subfamily Pooideae but grouped into the tribes Triticeae and Avenae, respectively. It

can be assumed that gramine biosynthesis evolved prior to the division into the two tribes. Hence, it can be hypothesized that the pathways for the biosynthesis of gramine and DIBOA were coexisting in the precursors of *Hordeum*. The overlap in biological function of the two secondary metabolites may have allowed the loss of one pathway without unfavorable consequences. Loss may even be beneficial, since biologically relevant concentrations of DIBOA and gramine in defence reactions are relatively high (0.1–1.0 mg per g fresh weight, Sicker et al., 2000; Hanson et al., 1981), and a substantial channeling of intermediates from the tryptophan biosynthetic pathway into the secondary metabolic pathways is required. The loss of one secondary biosynthetic pathway might help to balance primary and secondary metabolism of the plant. Another example of two plant secondary metabolites that share the biosynthetic origin and are mutually exclusive is given by glucosinolates and cyanogenic glucosides. Both derive from amino acids that are converted into aldoximes by cytochrome P450 enzymes belonging to the CYP79 family (Glawischnig et al., 2003). Cyanogenic glucosides occur widely throughout the plant kingdom, glucosinolates are found among the order Capparales and in the genus *Drypetes* in the order Euphorbiales. *Carica papaya* is the only known example that contains both groups of secondary metabolites (Tang, 1971; Spencer and Seigler, 1984; Bennett et al., 1997). When the sorghum cyanogenic glucoside-specific pathway is expressed in *Arabidopsis thaliana* a competition for substrates shared by the two pathways is observed (Bak et al., 2000). Such a competition may eventually lead to the elimination of one pathway.

DIBOA biosynthesis was lost already in *H. spontaneum*, the proposed wild progenitor of barley, and likewise, DIBOA is not present in *H. vulgare* cultivars. The picture is not consistent for gramine. All cultivars in our analysis do not have gramine at detectable levels, but other cultivars have 0.2–0.4 mg/g fresh weight of gramine in young leaves (e.g., Argandoña et al., 1987). The amount of gramine might increase after fungal attack and elicitation (Matsuo et al., 2001). The dispersed occurrence of gramine biosynthesis in cultivated barley shows that breeding processes did not select for gramine biosynthesis.

3.2. Evolution of DIBOA biosynthesis in the Gramineae

Benzoxazinoid biosynthesis first has been elucidated in maize (Frey et al., 1997). Conversion of IGP to indole by BX1 is the branch reaction that leads to the production of the benzoxazinoids. The biosynthesis continues with the conversion of indole to DIBOA catalyzed by the P450 enzymes BX2–BX5. In certain grasses like rye and wild species of *Hordeum* DIBOA is glucosylated and stored in the vacuole. In maize and wheat, DIBOA is converted to its 7-methoxy derivative DIMBOA and glucosylated (Niemeyer, 1988). The biosynthesis of DIBOA-glucoside can be considered as the basic biosynthetic pathway of defence related benzoxazinoids in the Gramineae. Recently, orthologous genes, *TaBx1–TaBx5*, have been identified in wheat (Nomura et al., 2002, 2003) and the respective enzymatic functions have been described in rye (Glawischnig et al., 1999). A phylogenetic tree (Fig. 3) was constructed using the deduced amino acid sequence data from maize, wheat and *H. lechleri*. The genes can be divided into two functional sets, the branch point gene *Bx1* (Fig. 3A) and the cytochrome P450 genes *Bx2–Bx5* (Fig. 3B) that are required for modification of the branch point reaction product. In the phylogenetic analysis of the cytochrome P450 enzymes, proteins with identical functions group together. The closest relationship exists between the genes from wheat and those from *H. lechleri* (between 95% and 97% identity on amino acid level), hence the phylogeny of these *Bx* genes reflects the phylogenetic relationship of the plant species. The data demonstrate a monophyletic origin of the P450 genes involved in DIBOA biosynthesis.

The picture may be different for the BX1 enzyme function. In the phylogenetic tree, the TSA proteins and the BX1-type enzymes group into two branches. In the case of a monophyletic origin of BX1-function, a closer relationship of HlBX1 and TaBX1 compared to ZmBX1 would be expected, but almost identical levels of identity were revealed in the analysis (identities on amino acid level: HlBX1and ZmBX1, 72%; HlBX1 and TaBX1, 73%). Hence the phylogeny of maize, wheat and *H. lechleri* is not reflected, and independent *TSA* gene duplication events very likely have created *Bx1*-function in maize and wheat on the one hand, and in barley on the other hand. Likewise *Igl* might also represent an independent gene duplication event in maize.

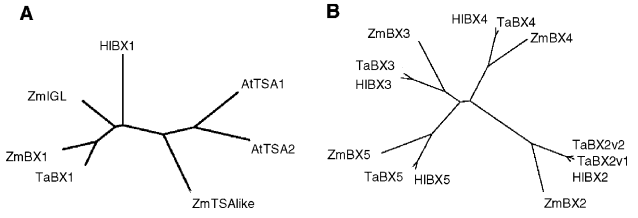

Fig. 3. Phylogenetic tree of indole-3-glycerole phosphate lyases and cytochrome P450 enzymes involved in DIBOA biosynthesis. Neighbor joining trees (Saitou and Nei, 1987) were constructed using the ClustalW program. (A) The amino acid sequences of *Bx1* orthologues from *Z. mays*, *T. aestivum* and *H. lechleri* were compared with *Igl* and *TSA* genes from *Z. mays* and *Arabidopsis thaliana*. (B) The comparison of the amino acid sequences of the P450 genes involved in benzoxazinoid biosynthesis comprised the genes from *Z. mays*, *T. aestivum*, and *H. lechleri*. There are two functional genes for *Bx2* in *T. aestivum*. Data were taken from Frey et al. (1997, 2000) and Nomura et al. (2002, 2003).

In maize eight genes of benzoxazinoid biosynthesis, *Bx1–Bx8*, are found within 23 cM on the short arm of chromosome 4 (Frey et al., 2003). This gene cluster is split in wheat. The genes *TaBx1* and *TaBx2* are located at chromosome 4, while *TaBx3–TaBx5* map on chromosome 5 (Nomura et al., 2002). In rye these two gene groups are found at syntenic positions (chromosomes 5 and 7, Nomura et al., 2003). The chromosomal location for *Hordeum* has not yet been determined. It can be assumed that the genes were present in the progenitor of *Hordeum*. The age of the branching point between the *Hordeum* I-clade that includes *H. spontaneum*, *H. vulgare*, and the H-clade, comprising the DIBOA-producing species, was estimated with 12 Myr (Jacob et al., 2004). During this time of independent evolution *Bx-gene* homologous sequences were lost in *H. vulgare* and *H. spontaneum*. A detailed analysis of the genome size in *Hordeum* revealed considerable differences between diploid species that evolved during this time, e.g., *H. spontaneum ssp.* have about 10.7 pg DNA per haploid genome, *Hordeum flexuosum* about 8.5 pg per haploid genome (Jacob et al., 2004). Hence processes like gene duplications, deletions, and inversions were active. Nomura et al. (2003) hypothesize that the original arrangement of *Bx* genes in the Gramineae is a cluster. This might explain the concomitant loss of *Bx2–Bx5* by rearrangement of that chromosomal region.

4. Experimental

4.1. Chemicals

Indole and indolin-2-one were purchased from Fluka Chemie, Buchs, CH, 1,4-benzoxazin-3-one from Aldrich. 2-Hydroxy-1,4-benzoxazin-3-one (HBOA) was synthesized chemically according to Honkanen and Virtanen (1960) and Glawischnig et al. (1999). DIBOA was purified from rye seedlings according to Bailey and Larson (1991) and Glawischnig et al. (1999). DIMBOA was purified from maize seedlings according to Hartenstein et al. (1992). 3-Hydroxyindolin-2-one was obtained by reducing isatin (indole-2,3-dione, Sigma) according to Frey et al. (1997). Gramine was purchased from Sigma–Aldrich. Oligonucleotides were purchased from MWG Biotech, GIBCO BRL and Sigma Genosys.

4.2. Plant material and growth conditions

H. vulgare cultivars Alexis, Baccara, and Nürnberg, were obtained from the Bavarian State Research Center for Agriculture, Germany; *H. vulgare* cultivars B87 and Nade and *T. aestivum* cv. Trakos, respectively, from the Department of Plant Sciences, Center for Life and Food Sciences, Weihenstephan, Technical University Munich, Germany. The three *H. spontaneum* lines 42–48, 150–31 and 160–53 were from Risø National Laboratory, Roskilde, Denmark. *H. brachyantherum* Nevski (H2012), *H. flexuosum* Steud. (H1110), *H. lechleri* (Steud.) Schenck (H1550), *H. roshevitzii* Bowden (H179) and *H. vulgare* cultivar Tellus were obtained from Institute of Plant Genetics and Crop Plant Research, Gatersleben Germany, *Z. mays* "LG11" from Lima Grain, France and *Secale cereale* "Halo" from Lochow-Petkus, Bergen, Germany.

Seeds were germinated after stratification on wet filter paper under following conditions: 16 h daylight 16 °C, 8 h dark, 12 °C, light intensity: 400 μ Einstein/m^2 s.

4.3. Isolation of indole alkaloids from seedlings

Gramine was isolated from seedlings as described by Hoult and Lovett (1993). 0.2–3.0 g of plant material ground in liquid nitrogen were homogenized and incubated for 24 h in 30 ml of 0.01% acetic acid. After filtration the pH of the homogenate was adjusted to 9.15 with 0.2 M KOH, followed by centrifugation (5 min, 3000 rpm). Sep-Pak C_{18} Cartridges (Waters Associates) were washed with 2 ml acetonitrile and equilibrated with 2 ml of 1 mM KH_2PO_4, pH 7.0. 10 ml of the plant extract were applied to the cartridge. After washing with 2 ml of 0.05 M KH_2PO_4, pH 9.5/isopropanol (70/30) and 2 ml of 0.05 M KH_2PO_4, pH 9.5/isopropanol (95/5), gramine was eluted with 2 ml of 0.05 M KH_2PO_4, pH 2.3/isopropanol (70/30). After lyophilisation the residue was dissolved in 120 μl of 0.05 M KH_2PO_4 + 0.1% triethylamine, pH 7.65/acetonitrile (60/40) and analyzed by HPLC.

DIBOA was purified from seedlings according to Bailey and Larson (1991) and Glawischnig et al. (1999) with 2.0 g of plant material. The ground material was incubated for 1 h in 5 volumes of H_2O to generate the aglucon of the benzoxazinoid. After adjusting the pH to 2.0 with HCl the homogenate was incubated at 65 °C for 5 min. and centrifuged (10 min, 5000 rpm). The supernatant was extracted two times with each 0.7 volumes ethyl acetate. The organic phases were evaporated by centrifugation in vacuum at room temperature and the DIBOA containing residue was dissolved in 60 μl methanol for HPLC-analysis.

Two replicates of every plant sample were analyzed in duplicate.

4.4. HPLC-based quantification of indole alkaloids and their biosythesis intermediates from plant extracts and P450 enzyme assays

Plant extracts and enzyme assays were analyzed by reverse phase high performance liquid chromatography (HPLC) using LiChroCart RP18 columns (125 × 4, flow rate 1 ml/min) and the system "Gold V810" or "Gold Nouveau" Beckman. Gramine was eluted for 12 min

under isocratic conditions (0.05 M KH_2PO_4 + 0.1% triethylamine, pH 7.65/acetonitrile (60/40)). Benzoxazinoids and intermediates of the DIBOA-biosynthesis were eluted for 5 min in isocratic conditions with solvent A (H_2O/acetic acid, 9:1) followed by a linear gradient from solvent A to B (methanol/H_2O/acetic acid, 70:27:3) for 7 min. Analytes were detected at 254 nm (benzoxazinoids) and 221 nm (gramin) and analyzed as described (Glawischnig et al., 1999).

4.5. Construction and screening of the H. lechleri cDNA library

RNA was isolated from 10 days old *H. lechleri* seedlings according to Logemann et al. (1987). Poly(A)$^+$ RNA was prepared using Oligotex Direct mRNA Midi Kit (Qiagen). cDNA was synthesized from 1 μg poly(A)$^+$ RNA with the TagMan Kit (Perkin–Elmer). The cDNA library was constructed using the Lambda-ZAP system (Stratagene) according to the manufacturer's specifications. The primary library (5×10^5 pfu) was amplified and 5×10^5 pfu of the amplified library were screened.

For the identification of sequences homologous to *Bx1* the maize cDNA was used for hybridization (Frey et al., 1997). The fragments for screening the cDNA library for sequences homologous to *Bx2–Bx5* were isolated by an RT-PCR approach that combines enrichment for cytochrome P450 genes (Fischer et al., 2001, primer perf-1, -5) and specific amplification using sequence information of isolated *Bx* genes (primer 2df, c2wr). A first PCR was performed as described by Fischer et al. (2001). The combination of primers perf-1 and perf-5 with the T-anchor primer was productive. The generated DNA fragments were subjected to a second PCR with specific primer pairs. Two fragments with homology to *Bx2* (435 bp, primer 2df) and *Bx3* (179 bp, primer c2wr) were amplified. Fragments were labeled with ^{32}P and used for hybridization of the cDNA library under low stringency conditions (2× SSPE, 55 °C).

4.6. Heterologous expression of HlBx1 in E. coli

HlBx1 was expressed in the pET3a *E. coli* expression system and assayed as described (Frey et al., 1997, 2000). Transit peptide sequences at the amino terminus were not included in the expression clone.

4.7. Heterologous expression of the cytochrome P450 genes HlBx2 to HlBx5

The alignment gave strong evidence that the isolated sequences were full-size cDNAs of *HlBx2*, *HlBx3*, *HlBx5* since the ATG codon is present at analogous position to the translational start of each respective maize *Bx* gene. In the *HlBx4* cDNA a start codon is missing but the complete amino terminal region of *ZmBx4* is encoded. Hence *HlBx4* seems to have an extended amino terminal anchor domain. For heterologous expression the ACC codon located at homeologous position to the start codon in *ZmBx4* was replaced by the codon ATG using an adaptor sequence.

Adaptor HlBx4:

fw: 5′CTAGAGAATTCGGCACCATGGCTCTTG
AAGCAGCGTACCACTACCTGCA3′;
rev: 5′CCATCACCATGCGACGAAGTTCTCGGT
ACCACGGCTTAAGA3′.

The yeast expression vector pYeDP60 (Urban et al., 1990) was modified by ligation of an adaptor between the *Bam*HI and *Eco*RI restriction sites. cDNAs cloned in the lambda-ZAP system are integrated in the yeast vector in the correct orientation for expression after digestion with *Eco*RI and *Xho*I.

Adapter pYad:

fw: 5′GATCCGAATTCATACGTTAGCATGGCT
ACGGTCGACC3′;
rev: 5′TTAACCAGCTGGCATCGGTACGATTGC
ATACTTAAGC3′.

Grasses and yeast differ in their codon usage. Genes of grasses are biased to G/C-codons. This can result in reduced gene expression. For expression of *HlBx3* and *HlBx5* an A/T-rich region was introduced and the 5′-untranslated leader was reduced in size. The site directed mutagenesis altered the DNA sequence without changing the encoded amino acid sequence, 129 bp of the *HlBx3* and 123 bp of the *HlBx5* at the respective 5′-ends were exchanged. For each sequence the 5′-end was replaced by a combination of mutagenesis-adaptor and site directed mutagenesis PCR derived fragment in a three point ligation. The resulting clones were sequenced to exclude primer- and PCR-based errors of the modified sequences. The following adaptor and primer pairs were employed:

HlBx3:

adaptor1fw: 5′GATCCATGGCTCTTGAAGCAGC
ATACCACTACCTGCA3′;
adaptor1rev: 5′CCATCACCATACGACGAAGTT
CTCGGTAC3′;
adaptor2fw: 5′GATCCATATAAATGGCTTTGGA
AGCTGCTTACCACTACTTGCAAATCGCTGTC
GGCCATGGTACTTCTACGCCAGCTGCTTTGT
T3′;
adaptor2rev: TTGTTTCGTCGACCGCTACTTCA
TGGAACCGGCTGTCGCTAAACGTTCATCACC
ATTCGTCGAAGGTTTCGG TAAATATAC3′;
mismatch primer fw: 5′TCTACGCCAGCTGCTTT
GTTGACTGTTTTGTTGTTGTTGATTATTAGA

TTGGCTTGGGTTAGAACTACTACTGCTTCTA CTAGATTGAGCAAGCAGCAGCAGCTC3′;
mismatch primer rev: 5′GAATGATGGCCGGCGG CCATGGATG3′.

HlBx5:

adaptor1fw: 5′ AATTCATATAAATGGCTCTTGA AGCTGCTCATCACTACTTGAGACACGCTTGG TCATGGTACTTCTGCTCCAG3′;
adaptor1rev: 5′GACCTCGTCTTCATGGTACTGG TTCGCACAGGAGTTCATCACTACTCGTCGAA GTTCTCGGTAAATATAC3′;
mismatch primer1fw: AGTTGTGGACGGAATTC ATGGCTCTTGAAG3′;
mismatch primer2fw: 5′ GGTACTTCTGCTCCAG CTGCTTTGTTGTTGGTTTGTGTTCCATTGTTG TTGTTGTTGTTGTTGTTCGCTTCTTTGAGAA CCTCAGCGTCGACAAGA3′;
mismatch primer1/2rev: 5′AAGGGCGAGAAGG CGACGTCGGTGG3′.

4.8. DNA sequencing and computer analysis

Sequence analysis was done with an ABI PRISM 377 DNA Sequencer (Perkin–Elmer). Sequence data were analyzed using the program SeqMan (DNA-Star, Lasergene) and homology searches were done with BLAST analysis (Altschul et al., 1997). Phylogenetic trees were generated using CLUSTALW (http://clustalw.genome.jp/), based on the Neighbor Joining (NJ) algorithm.

4.9. Transformation of yeast cells and preparation of microsomal fractions

Yeast cells of the Arabidopsis P450 reductase *ATR1* expressing *S. cerevisiae* WAT11 were grown according to Pompon et al. (1996) and transformed by heat shock according to Gietz et al. (1992) or electroporation according to Becker and Guarente (1991). Yeast microsomal proteins containing heterologously expressed barley P450 enzymes were prepared according to Urban et al. (1990).

The cytochrome P450 enzyme mediated conversions of different substrates were analyzed according to Glawischnig et al. (1999).

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