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REVIEW

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Evolution of benzoxazinone biosynthesis and indole production in maize

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Abstract The synthesis of a diverse spectrum of secondary metabolites has allowed plants to develop sophisticated chemical defense mechanisms. Maize (*Zea mays* L.), for example, releases a cocktail of volatile compounds when attacked by a caterpillar. These compounds attract a parasitic wasp, which deposits its eggs in the larvae, thereby controlling the population size of the herbivore. Indole, which is part of the cocktail, is produced by an enzyme recruited from primary metabolism. Indole can either function as a volatile signal or be converted by specific cytochrome P450 enzymes into benzoxazinoids, which function as important defense chemicals.

Keywords Chemical defense mechanisms · Cytochrome P450 enzymes · 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) biosynthesis · Evolution (secondary metabolic pathways) · Secondary metabolism · *Zea* (defense mechanisms)

Abbreviations DIBOA: 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one · DIMBOA: 2,4-dihydroxy-7-methoxy-2*H*-1, 4-benzoxazin-3(4*H*)-one · HBOA: 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one · IGL: indole-3-glycerol phosphate lyase · IGP: indole-3-glycerol phosphate · TS: tryptophan synthase

Introduction

The hundreds of thousands of unique plant secondary metabolites constitute a large field of chemical biodiversity that is not only important for the survival strategies of plants but also represents an immense source for the discovery of new drugs and lead compounds. The distribution of certain metabolites in plants sometimes reflects their phylogenetic origin. On the other hand, closely related plant taxa often differ in their spectra of secondary products. Therefore, the evolution of the synthetic capacity for these substances has constantly accompanied plants from their origin onwards. In *Arabidopsis thaliana* (L.) Heynh., it is estimated that about 5,000 genes, i.e. about a quarter of all genes, are involved in secondary metabolism (The *Arabidopsis* genome initiative 2000). Many of these genes that encode enzymes or regulatory proteins are probably recruited from "primary" functions. In order to understand the evolution of these secondary pathways, we have to identify the genes specific for secondary metabolic pathways, determine their function and try to reconstruct their origins from primary metabolism by sequence and functional comparisons with putative ancestral genes. The ongoing genome projects will be indispensable in this respect.

A secondary metabolic pathway can be defined by the branch point from primary metabolism and the consecutive downstream reactions that lead to specific end products. Obviously, catalysis of the branch reaction is crucial for the establishment of a secondary metabolic pathway. This reaction produces the first intermediate, which can be processed further into "useful" products that may be favored by natural selection. In this review, indole production and formation of the benzoxazinoid 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) are used as examples to discuss the evolution of secondary metabolic pathways.

Many plant species respond to herbivore damage by the release of volatile compounds. Herbivore predators and parasitic wasps exploit these chemical signals to locate their prey or hosts. Several such chemically mediated tritrophic interactions have been documented for agrarian systems including lima bean (Dicke et al. 1990), cotton and maize (Turlings et al. 1990). Maize seedlings damaged by beet armyworm caterpillars release a specific cocktail of volatile terpenoids and indole (Turlings et al. 1991) that is recognized by parasitic wasps.

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Volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] present in the saliva of beet armyworm caterpillars has been identified (Alborn et al. 1997) as the major active elicitor for the formation of volatiles in maize. Recently, two genes, *Igl* and *stc1*, that are specifically elicited by volicitin have been isolated from maize. *Igl* encodes an indole-3-glycerol phosphate lyase (IGL; Frey et al. 2000) and *stc1* encodes a sesquiterpene cyclase (Shen et al. 2000). IGL cleaves indole-3-glycerol phosphate (IGP) to form indole and glyceraldehyde-3-phosphate.

The benzoxazinoids 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA) and its methoxy derivative DIMBOA are found predominantly in the Gramineae. Benzoxazinoids are natural pesticides and serve as important factors in host-plant resistance to microbial diseases and insects, and as allelochemicals (reviewed in Niemeyer 1988; Sicker et al. 2000). In maize, a series of five genes is sufficient to encode the enzymes to synthesize DIBOA (Frey et al. 1997). The first gene in this pathway, *Bx1*, encodes an enzyme function identical to that of IGL which catalyses the formation of free indole (Frey et al. 1997). Conversion of IGP to indole is the branch reaction that leads to the production of both secondary metabolites, indole and benzoxazinoids (Fig. 1).

IGL and BX1 have evolved from a tryptophan synthase α-subunit

Tryptophan synthase (TS) catalyzes the conversion of IGP and serine to tryptophan. The well-characterized bacterial TS enzyme is an $\alpha_2\beta_2$ heterotetramer linked via the β-subunits (Creighton and Yanofsky 1966). The individual subunits catalyze two independent reactions: IGP is converted by the α-subunit to indole and glyceraldehyde-3-phosphate, and indole and serine are converted by the β-subunit to tryptophan and H_2O. It is important to note that the enzyme activity of the subunits is increased dramatically in the intact TS complex (Table 1). Indole is not released from the TS complex but rather travels through a tunnel connecting the active sites of the α- and β-subunits (Fig. 2). There is evidence that plant TS, like the bacterial complex, functions as an α β heteromer (Radwanski et al. 1995). The α- and β-subunits are encoded by independent genes (*TSA* and *TSB*) and the interaction of α and β was inferred from complementation experiments.

The BX1 and IGL proteins from maize share an amino acid sequence identity of more than 60% to plant TSAs. Unlike the α-subunit of TS, BX1 and IGL can efficiently cleave IGP to form free indole without being activated by a β-subunit (Fig. 2). Kinetic analysis of purified BX1 (Frey et al. 1997) and IGL (Frey et al. 2000) proteins expressed in *Escherichia coli* demonstrated that homomeric BX1 and IGL proteins are about 30-fold and 3-fold, respectively, more efficient in catalyzing IGP cleavage than the *E. coli* TS $\alpha_2\beta_2$ heterotetramer (Table 1).

The genes *Bx1* and *Igl* are evolutionarily related to *TSA* genes. The exon/intron structure of *Bx1* and *Igl* and the *A. thaliana TSA* gene is almost conserved (Fig. 3). However, the sequences of BX1 and IGL

Table 1 Comparison of catalytic properties of indole-3-phosphate lyase-type enzymes

Parameter	*Escherichia coli*		*Zea mays*	
	α	$\alpha_2\beta_2$	BX1	IGL
$K^{IGP}{}_m$	0.5 mM	0.03 mM	0.013 mM	0.1 mM
k^{cat}	0.002 s^{-1}	0.2 s^{-1}	2.8 s^{-1}	2.3 s^{-1}
$k^{cat}/K^{IGP}{}_m$	0.004 $mM^{-1}s^{-1}$	7.4 $mM^{-1}s^{-1}$	215 $mM^{-1}s^{-1}$	23 $mM^{-1}s^{-1}$

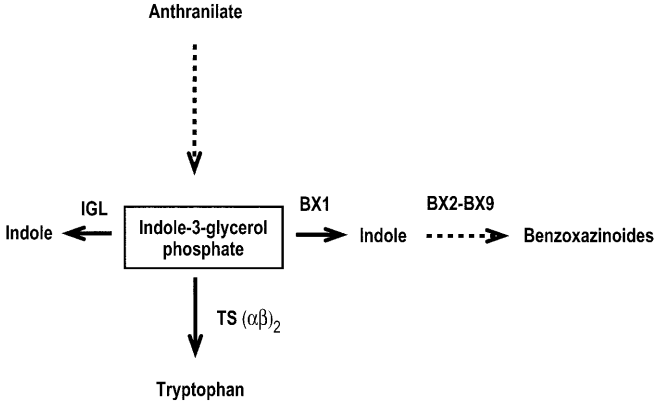

Fig. 1 Indole and benzoxazinoid secondary metabolite formation branches from tryptophan biosynthesis. The two lyases IGL and BX1 cleave IGP into indole and glyceraldehyde-3-phosphate and serve as committing enzymes for indole-derived secondary metabolites. Indole can directly function as a volatile signal or is converted by other enzymes (BX2–BX9) to benzoxazinoids that have an important function in the chemical defense of grasses

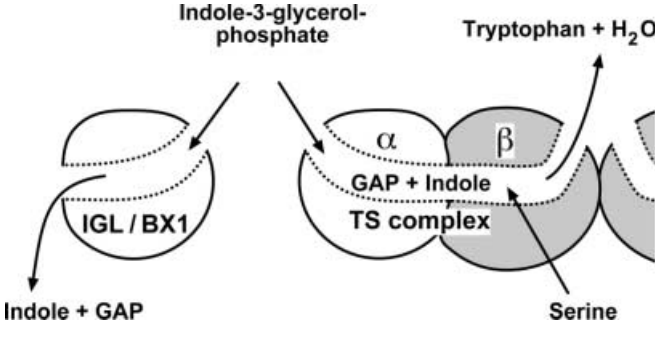

Fig. 2 Comparison of the indole-3-glycerol phosphate lyases IGL and BX1 with the tryptophan synthase complex. BX1 and IGL cleave IGP to form free indole and glyceraldehyde-3-phosphate (*GAP*). The tryptophan synthase (*TS*) complex catalyses the conversion of IGP and serine to tryptophan. This complex is an $\alpha_2\beta_2$ heterotetramer linked via the β-subunits. BX1 and IGL have homology to α-subunits and catalyze the same reaction. The difference is, however, that BX1 and IGL are highly active in a homomeric form, while α-subunits have substantial activity only in the intact TS complex (Table 1). Indole is not released from the TS complex but rather travels through a tunnel connecting the active sites of the α- and β-subunits

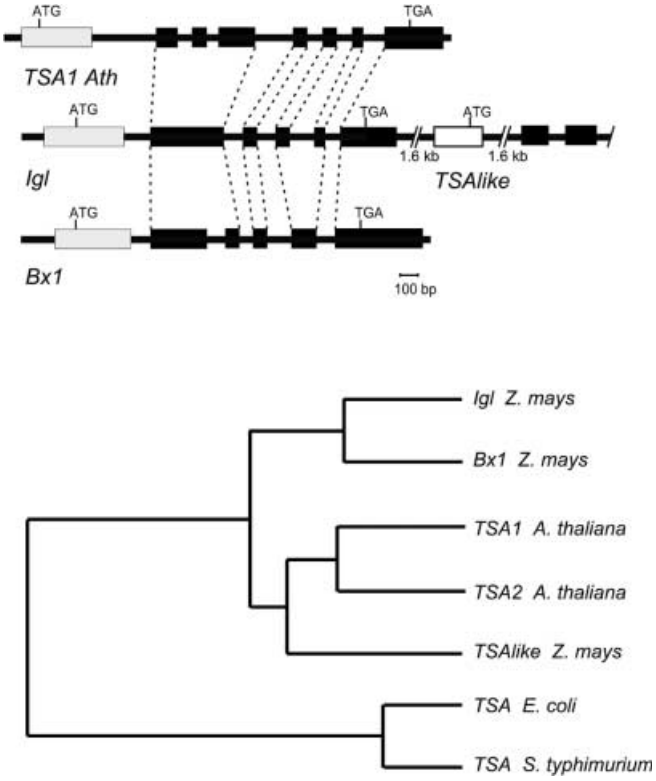

Fig. 3 Genes related to *Bx1* and *Igl*. *Upper diagram* The gene structures of *Bx1* and *Igl* are compared with the *TSA* gene from *Arabidopsis thaliana* (*TSA1 Ath*). Exons are represented by *filled boxes*, and corresponding exons are indicated by *dashed lines*. In maize, a putative *TSA* gene (TSA_{like}) is located 1.6 kb downstream of *Igl*. The first exons share little homology and encode the signal peptides required for plastid import. The mature BX1 and IGL proteins are 82% identical to each other and share about 65% homology with TSA_{like} and TSA from *A. thaliana*. *Lower diagram* Phylogenetic tree indicating that TSA_{like} is more homologous to *TSA* genes from *A. thaliana* than to the maize paralogues *Bx1* and *Igl*

deviate at several positions from the TSA consensus, including the domain required for interaction with TSB (Frey et al. 2000). These amino acid changes might reflect the different enzymatic properties of these proteins. *Bx1* and *Igl* are more closely related to each other (Fig. 3) than to the maize candidate gene for *TSA* (TSA_{like}), which is in turn more closely related to the *TSA* genes of *A. thaliana*. The fact that *Igl* and TSA_{like} are separated by only 1.6 kb on chromosome 1 of maize indicates an ancient gene-duplication event.

In conclusion, a gene involved in primary metabolism (*TSA*) was duplicated and subsequently recruited for secondary metabolism. In this process, *Bx1* and *Igl* were modified during evolution to obtain their specific functions. Not only the enzymatic properties had to be adjusted such that free indole is produced, but the expression pattern also had to be altered in order for the genes to function in secondary metabolism. *Bx1* is under developmental control in the young seedling and *Igl* is induced later in development in leaves in response to herbivore damage (Frey et al. 1997, 2000). The synthesis of several other plant metabolites, such as auxin, indole glucosinates, anthranilate-derived alkaloids and tryptamine derivatives (Kutchan 1995; Radwanski and Last 1995; Bartel 1997), could depend on indole as an intermediate. IGP has been proposed as a branch point from the tryptophan pathway for the synthesis of the indolic phytoalexin camalexin (3-thiazol-2′yl-indole) in *A. thaliana* (Tsuji et al. 1993; Zook 1998). Indole is also found in the scent of flowers such as lilac and robinia. Hence, the recruitment of an indole-3-glycerol phosphate lyase from TSA genes might have occurred several times independently in plant evolution.

There are two other examples of the recruitment of genes from primary metabolism. The homospermidine synthase from *Senecio vernalis* is derived from deoxyhypusine synthase, an enzyme required for activation of translation factor 5A (Ober and Hartmann 1999), and a serine carboxypeptidase-like protein that functions as an acyltransferase in secondary metabolism is found in *A. thaliana* (Lehfeldt et al. 2000).

Duplicated cytochrome P450 genes are required for conversion of indole to benzoxazinoids

The biosynthesis of benzoxazinones commences by conversion of indole to DIBOA. In certain grasses like rye (*Secale cereale* L.), DIBOA is glycosylated and stored in the vacuole. In other species like maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.), DIBOA is first converted to its 7-methoxy derivative DIMBOA and then glycosylated for vacuolar storage, (Sicker et al. 2000; Fig. 4). The introduction of four oxygen atoms into the indole moiety that yields DIBOA is catalyzed by four cytochrome P450-dependent monooxygenases. These enzymes are membrane-bound heme-containing mixed-function oxidases. They utilize NADPH or NADH to reductively cleave molecular oxygen to produce functionalized organic products and a molecule of water. In this generalized reaction, reducing equivalents from NADPH are transferred to the P450 enzyme via a flavin-containing NADPH-P450 reductase. In plants, P450 enzymes are involved mainly in hydroxylation or oxidative demethylation reactions of a large variety of primary and secondary metabolites, including hormones, phytoalexins, xenobiotics and pharmaceutically relevant compounds. The plant P450 genes represent a fairly large gene family. In *A. thaliana*, 286 P450 genes have been annotated (The *Arabidopsis* genome initiative 2000). An even greater number of P450 genes can be expected in plants containing more secondary metabolites.

The four P450 genes involved in DIBOA biosynthesis have been termed *Bx2–Bx5* (Frey et al. 1997). They are members of the *CYP71C* subfamily of plant cytochrome P450 genes and share an overall amino acid identity of 45–65%. The stepwise conversion of indole to DIBOA occurs as follows (Fig. 4): BX2 catalyses the formation of indolin-2(1*H*)-one, which is converted to 3-hydroxyindolin-2(1*H*)-one by BX3. Then, BX4 catalyses the

conversion of 3-hydroxy-indolin-2(1*H*)-one to 2-hydroxy-2*H*-1,4-benzoxazin-3(*4H*)-one (HBOA). This unusual ring expansion has been investigated by labelling experiments, and a mechanism for this transformation has been proposed (Spiteller et al. in press). The N-hydroxylation of HBOA to DIBOA is catalyzed by BX5.

The sequence homology, the similar exon/intron structures, and the gene clustering of *Bx2–Bx5* (see below) indicate that these genes have been derived by gene duplications from one precursor (Frey et al. 1997). However, each of the four P450 enzymes has evolved a high degree of distinct substrate specificity. Only one intermediate in the pathway is converted by each respective P450 enzyme to a specific product. Each enzyme is specific for the introduction of one specific oxygen atom into the DIBOA molecule. The relatively high specificity of the enzymes seems to support the idea that plant P450 enzymes generally have a much greater substrate specificity than their animal homologues. However, there is emerging evidence that plant P450 enzymes, in addition to their normal physiological function, can also convert certain xenobiotics with varying efficiencies. For example, the artificial substrate *p*-chloro-*N*-methylaniline (pCMA) is efficiently demethylated by BX2 and by several other plant P450 enzymes (Glawischnig et al. 1999).

Recently the two genes encoding specific benzoxazinoid glucosyltransferases of maize have been characterized in our group (U. von Rad, personal communication). These genes have been termed *Bx8* and *Bx9*. Their gene products convert DIBOA and DIMBOA to the respective D-glucosides. The two enzymes for conversion of DIBOA to DIMBOA, presumably another oxygenase and a methyltransferase, encoded by the genes *Bx6* and *Bx7* remain to be isolated.

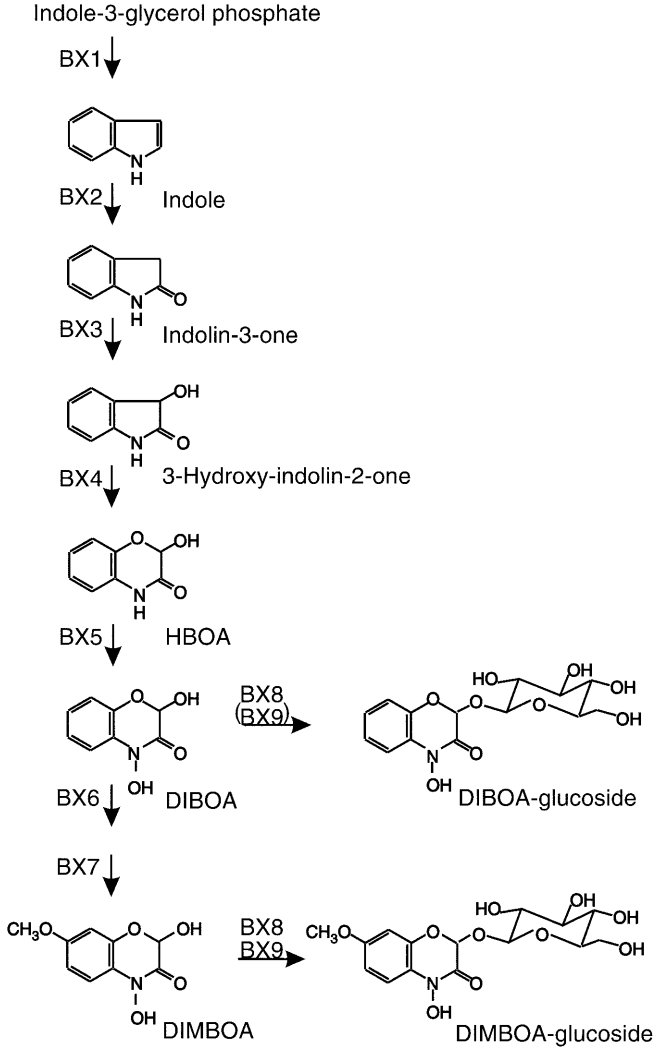

Fig. 4 Benzoxazinoid biosynthetic pathway. Indole is synthesized in the chloroplast by BX1. The P450 enzymes BX2 through BX5 convert indole to DIBOA. The enzymes for transformation of DIBOA to DIMBOA have not yet been identified. DIBOA and DIMBOA are converted to the respective D-glucosides by the glucosyltransferases BX8 and BX9

The *Bx* genes are clustered on one chromosome

Genetic mapping of the *Bx* genes in maize had the surprising result that these genes are clustered on the short arm of chromosome 4 (Frey et al. 1997; Fig. 5). Gene clustering is often associated with gene duplication. Therefore the relatively close arrangement of the P450 genes *Bx2–Bx5* within 6 cM is not unexpected. The cluster of P450 genes is tightly linked to the *Bx1* gene and to *Bx8* encoding the DIBOA/DIMBOA-specific glucosyltransferase. *Bx1* and *Bx2* are separated by only 2.5 kb; the exact position of *Bx8* relative to these two genes remains to be determined. The gene cluster

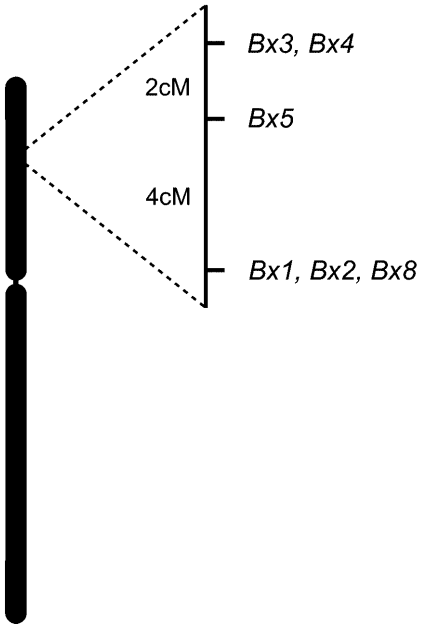

Fig. 5 The maize *Bx* gene cluster. The *Bx* genes on the short arm of chromosome 4 are shown. The distances are given in centimorgans (cM)

comprises three different enzymatic functions and a complete set of genes for the biosynthesis of DIBOA glucoside. The pathway starts with the branch reaction that leads to indole formation (*Bx1*), is followed by the subsequent hydroxylation reactions that convert indole into DIBOA (*Bx2–Bx5*), and terminates with the formation of DIBOA glucoside (*Bx8*). At present, there is no other example of plant genes integrated in one biosynthetic pathway that are all located in one gene cluster. The chromosomal positions of *Bx6* and *Bx7* have not yet been determined by molecular mapping. *Bx9*, the homologue of *Bx8*, is located on chromosome 1.

It is unclear how gene clustering is related to the evolution of benzoxazinoid biosynthesis and whether for example, gene clustering has any influence on the expression of the *Bx* genes. Since the *Bx* genes are located within 6 cM, these genes will frequently be transferred to the next generation as one functional unit, encoding all enzymes required for the biosynthesis of DIBOA. Whether or not this genetic co-segregation is of any advantage to maize is presently unclear. One could speculate that the loss of one enzyme would interrupt the pathway, which could lead to the formation of a potentially deleterious intermediate. In any case, it remains to be determined whether the *Bx* genes are also clustered in other species.

Evolution of benzoxazinoid biosynthesis

Benzoxazinoids are widely distributed in grasses and are also found in several dicotyledonous species in the families Acanthaceae, Ranunculaceae and Scrophulariaceae (Sicker et al. 2000), suggesting that the acquisition of this pathway occurred relatively early in the evolution of the Gramineae and probably even before monocots and dicots diverged. The activity of the DIBOA-specific P450 enzymes has been assayed in two other cereals, rye (*Secale cereale* L.) and barley (*Hordeum vulgare* L.) (Glawischnig et al. 1999). These two species are much more closely related to each other than either is to maize (Devos and Gale 1997).

The predominant benzoxazinoid of rye is DIBOA. As in maize, there are relatively high concentrations (up to 1 mg/g fresh weight) present in the rye seedling. The cytochrome P450-dependent reactions are very similar to those detected in maize. Indole, indolin-2(1*H*)-one, 3-hydroxy-indolin-2(1*H*)-one, and HBOA were converted to the same products that were obtained with maize microsomes (Glawischnig et al. 1999). No additional products were detected and the reactions were strictly dependent on NADPH, indicating true cytochrome P450 enzyme reactions. The similarity of the reactions in maize and in rye suggests identical DIBOA biosynthetic pathways for both species.

In microsomes prepared from barley (*H. vulgare*) seedlings, no activities of the P450 enzymes of the DIBOA pathway were detectable, although the total P450 content in microsomes and the NADPH-P450 reductase activity were similar to those in maize (Glawischnig et al. 1999). In contrast, DIBOA was detected in several wild barley species, e.g. *Hordeum lechleri*. Cloning and functional analysis of the P450 homologues to *Bx2–Bx5*, indicated the presence of fully orthologous genes (S. Grün, personal communication). In *H. vulgare*, however, the *Bx* genes are lost and cannot be detected in genomic Southern experiments. A similar loss of enzyme activity has also been observed for the UDP-glucose:DIBOA glycosyltransferase. Glycosyltransferase activity is present in some wild varieties but is absent in varieties that do not contain benzoxazinoids. It has been proposed (Leighton et al. 1994) that this loss occurred in the process of cultivating modern barley (*H. vulgare*).

Although maize and rye are distantly related, the DIBOA biosynthetic pathway seems to be identical in both species. Therefore, a set of *Bx* orthologous genes exists in all benzoxazinoid-containing grasses. If this were the case, the gene duplications responsible for the evolution of the *Bx2–Bx5* gene cluster must have occurred early in the development of the Gramineae. If the occasional presence of benzoxazinones in dicots is considered, then the evolution of the pathway might have occurred even before monocots and dicots diverged. The isolation of dicot genes homologues to *Bx* genes from maize could give more insight into the evolution of this pathway.

Concluding remarks

Gene duplications seem to play an important role in the evolution of secondary metabolic pathways. In the examples presented, duplicated *TSA* genes from primary metabolism are recruited for the production of free indole. This compound is either used directly for signaling or converted to a defense chemical. For the latter steps, gene duplications have generated the P450 genes required for DIBOA biosynthesis. The redundancy potentially created by gene duplication does not necessarily result in functional or genetic redundancy, because the gene products have evolved towards a defined substrate specificity or their specific expression patterns generate non-overlapping functions. In the analysis of the *A. thaliana* genome sequence, a fairly high degree of gene duplication was detected (The *Arabidopsis* genome initiative 2000). Detailed analysis indicated that these duplications are not due to a single event leading to polyploidy (Vision et al. 2000). Rather, they have accompanied the evolution of *A. thaliana* for the last 200 million years.

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