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Cytochrome P450 monooxygenases of DIBOA biosynthesis: Specificity and conservation among grasses

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Abstract

DIBOA and DIMBOA are secondary metabolites of grasses which function as natural pestizides. The four maize genes *BX2* through *BX5* encode cytochrome P450-dependent monooxygenases that catalyse four consecutive reactions in the biosynthesis of these secondary products. Although BX2–BX5 share significant sequence homology, the four enzymes have evolved into specific enzymes each catalysing predominantly only one reaction in the pathway. In addition to these natural reactions, BX3 hydroxylates 1,4-benzoxazin-3-one and BX2 shows pCMA demethylase activity. With respect to DIBOA biosynthesis, identical enzymatic reactions have been found in rye as compared to maize, indicating early evolution of the P450 enzymes in the grasses.

Keywords: Zea mays; Gramineae; Biosynthesis; Cyclic hydroxamic acids; DIBOA; DIMBOA; Cytochrome P450; Yeast expression

1. Introduction

The cyclic hydroxamic acids DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) and DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) play an important role in the chemical defense of cereals against deleterious pests such as insects, pathogenic fungi and bacteria [1]. DIBOA is the main hydroxamic acid in rye, whereas its methoxy derivative DIMBOA is the predominant form in maize and wheat. In maize, a series of five genes that are clustered on the short arm of chromosome four, is sufficient to encode the enzymes to synthesise DIBOA [2]. The first gene in the pathway, *Bx1*, encodes an enzyme resembling a tryptophan synthase α subunit [2, 3] that catalyses the formation of free indole [2] and thereby establishes the branchpoint that leads to the secondary metabolites DIBOA and DIMBOA. The next four genes, *Bx2–Bx5*, encode cytochrome P450-dependent monooxygenases of the CYP71C subfamily [4], that catalyse four consecutive hydroxylations including one ring-expansion to form the defense compound DIBOA (Fig. 1; [2]). The classic forms of cytochrome P450 monooxygenases are membrane-bound heme-containing mixed function oxidases. They utilise NADPH or NADH to reductively cleave molecular oxygen to produce functionalised organic products and a molecule of water. In this generalised reaction, reducing equivalents from NADPH are transferred to the P450 enzyme via a flavin-containing NADPH-P450 reductase (CPR). In plants, P450 enzymes are involved mainly in hydroxylation or oxidative demethylation reactions [5] of a large variety of primary and secondary metabolites including hormones, phytoalexins, xenobiotics and pharmaceutically relevant compounds.

The close genetic linkage and the sequence similarity of the DIBOA-specific P450 genes [4], indicate that these genes most probably arose from gene duplication events. Evolution of these genes generated enzymes of the CYP71C P450 subfamily that catalyse consecutive reactions in the pathway leading to DIBOA. In this report we show that this process has resulted in rather specific P450 enzymes that predominantly catalyse only one reaction in the DIBOA pathway. The question of enzyme specificity was addressed by heter-

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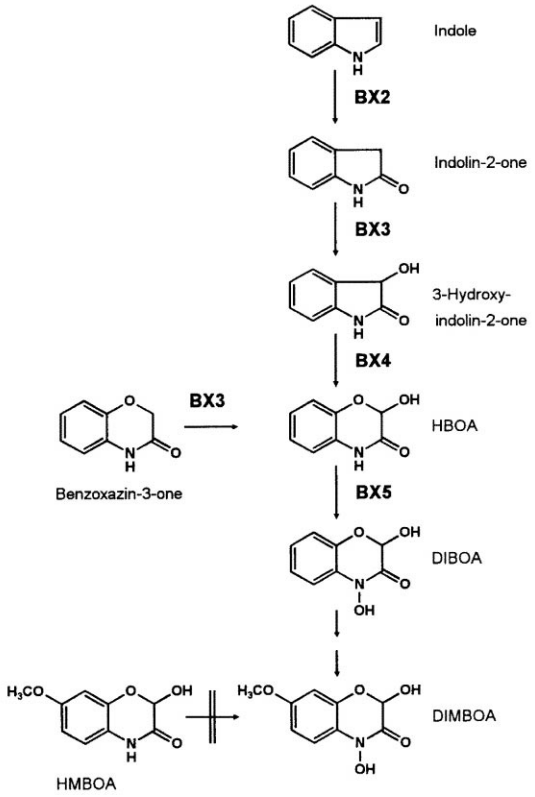

Fig. 1. DIMBOA biosynthetic pathway. The P450 enzymes are indicated by BX2–BX5.

ologous expression of the P450 genes in yeast. In this system we also investigated the interaction of the maize P450 enzymes with one CPR of yeast and two CPRs of Arabidopsis, indicating that the availability of CPR may limit P450 enzyme activity in plants. In order to test whether the DIBOA pathway of maize can be generalised in grasses, microsomes derived from rye and barley seedlings were assayed for the BX2–BX5 specific reactions. In rye (high DIBOA content) the enzyme activities were identical with those in maize, whereas in barley (no detectable DIBOA or DIMBOA) no enzymatic activities were observed.

2. Results

2.1. Characterization of the enzymatic properties of BX2–BX5 by heterologous expression in yeast

The cytochrome P450-dependent monooxygenases BX2–BX5 convert indole to DIBOA by catalysing a series of four hydroxylations (Fig. 1; [2]). These P450 enzymes were expressed in yeast in order to characterise their catalytic properties in more detail. For expression, the *Bx2–Bx5* cDNAs were inserted into the pYeDP60 vector under the control of the galactose inducible GAL10-CYC1 promotor [6]. The P450 genes were expressed in four different *Saccharomyces cerevisiae* strains that were isogenic except for their *CPR* gene locus. Strain W(N) has wild type yeast CPR levels, W(R) overexpresses yeast CPR after induction with galactose [7]. In WAT11 and WAT21 galactose inducible *Arabidopsis thaliana CPRs* (*ATR1* and *ATR2*, respectively) replace the yeast reductase in the genome [6, 8]. Microsomes were isolated from the transgenic yeast strains and tested for the four specific reactions of the DIBOA pathway by HPLC analysis. The concentration of the maize P450 proteins in the microsomes was determined by quantitative Western analysis (Table 1). CPR activities were analysed by cytochrome c reduction [9]. The CPR activity was basically independent of the heterologous P450 gene and similar to the values obtained by Pompon *et al.* [5]. In W(R) overexpression of the yeast CPR results in a 20-fold stimulation of the basal CPR activity. Overexpression of the CPRs from Arabidopsis leads to a 4-5-fold increase over the basal level. The concentration of the maize P450 proteins in the transgenic yeast microsomes was in the range of 0.2–2.6 μg/mg microsomes (Table 1). Except for BX2, these expression levels are lower than in maize microsomes. It is possible that the relative high GC content (65%) of the maize genes [4] does not permit efficient expression in the yeast system. Heterologous expression of the

Table 1
Expression of the cytochrome P450 monooxygenases BX2–BX5 in different yeast strains and in maize. The P450-protein content was quantified by Western analysis and specific activity of microsomes has been determined with the following substrates: BX2: Indole; BX3 (1): Indolin-2-one, (2): Benzoxazin-3-one; BX4: 3-Hydroxy-indoline-2-one; BX5: HBOA; CPR: Cytochrome c; n.d.: no data.

	CPR (nmole × mg^{-1} × min^{-1})	P450 Content (μg/mg microsomal Protein)				specific Activity (nmol product × min^{-1}x μg^{-1})				
		BX2	BX3	BX4	BX5	BX2	$BX3^{(1)}$	$BX3^{(2)}$	BX4	BX5
W(N)	42	1.4	0.3	n.d.	0.2	0.22	n.d.	0.03	n.d	0.02
W(R)	918	2.6	0.4	0.7	0.4	0.37	n.d.	0.16	0.15	0.05
WAT11	174	2.0	0.2	0.8	0.4	0.33	1.94	0.73	0.20	0.16
WAT21	238	1.9	0.4	n.d.	0.4	0.17	n.d.	0.20	n.d.	0.11
Maize	44	0.9	1.0	2.1	4.8	0.9 0.31	0.23	0.09	0.43	0.18

Table 2
Determination of the K_M constant (μM) of BX2–BX5 expressed in yeast WAT11 and in maize microsomes for the metabolised substrates.

		WAT11	Maize
BX2	K_M indole	38	36
BX3	K_M indolin-2-one	10	17
BX3	K_M benzoxazin-3-one	11	18
BX4	K_M 3-hydroxy-ondolin-2-one	36	31
BX5	K_M HBOA	5	7

cinnamate-4-hydroxylase gene from *Helianthus tuberosus* (48% GC) [10] in the same system resulted in protein expression levels that were in the range of 4 to 10 μg/mg microsomal protein [11].

In general, similar P450 activities can be obtained in microsomal preparations derived from the yeast strains as with maize microsomes. The specific activity of the P450 enzymes is dependent on the amount and to a certain extend on the nature of the CPRs present in the microsomes (Table 1). In this respect, some individual features were observed. BX2 shows little preference for one of the CPRs and has a similar specific activity in all yeast strains which is highest in W(R). In contrast, the specific activity of BX3 is about 8 times higher in WAT11 than in maize microsomes. This suggests a productive interaction of BX3 and ATR1 and indicates that in this case the amount of CPR limits the *in vivo* activity of BX3. According to their specific activities, BX4 and BX5 show a preference for the maize CPR(s).

The question of enzyme specificity was addressed with the yeast expression system. The intermediate metabolites of the DIBOA pathway indole, indolin-2-one, 3-hydroxy-indolin-2-on, and HBOA [2] were incubated with microsomal preparations each containing one of the P450 enzymes. Enzymatic conversions occured only in the known productive combinations [2] (Fig. 1), no conversions were detectable (the detection limit is 0.002 $\text{nmol}\times\text{mg}^{-1}\times\text{min}^{-1}$) in the other combinations. Each enzyme is therefore specific for the introduction of only one of the oxygen atoms of the DIBOA molecule.

In addition to the reactions described above, benzoxazin-3-one and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA, Fig. 1) were tested as possible substrates for BX2–BX5. The hydroxylation of benzoxazin-3-one to HBOA (Fig. 1) was previously described for maize microsomes [12]. Of the four P450 enzymes tested, only BX3 catalyses this reaction. Hydroxylation of benzoxazin-3-one is approximately half as efficient as the hydroxylation of indolin-2-one (Table 1). In both cases a C atom at an equivalent position is hydroxylated (Fig. 1). In contrast, for HMBOA, a derivative present in maize seedlings [13], no enzymatic conversions are detectable with BX2–BX5 expressed in yeast or with maize microsomes. In particular, HMBOA is not N-hydroxylated by BX5. This indicates that the 7-methoxy group of HMBOA would interfere with BX5 action.

In a number of species the demethylation of the artificial substrate *p*-chloro-*N*-methylaniline (pCMA) in a P450 specific reaction has been described. This activity is associated with the first plant P450 enzyme that was purified and cloned [14]. This enzyme from avocado (*Persea americana*), for which the natural substrate is still unknown, was designated CYP71A1 and established the CYP71A subfamily [15]. CYP73A1, the cinnamate-4-hydroxylase from *Heliantus tuberosus* shows also pCMA demetylase activity [16]. Since BX2–BX5 have been grouped into the CYP71 family [4], they were tested for pCMA demethylase activity. While BX3–BX5 expressed in WAT11 show low pCMA demethylase activity (0.02–0.04 $\text{nmol}\times\text{min}^{-1}\times\text{mg}^{-1}$ microsomes) WAT11 yeast microsomes containing the BX2 enzyme convert pCMA to *p*-chloro-aniline with a specific activity of 0.74 $\text{nmol}\times\text{min}^{-1}\times\text{mg}^{-1}$ microsomes. This value is about three times the pCMA demethylase activity of CYP71A1 expressed in wild type yeast [17] and one third of the activity of CYP73A1 expressed in W(R) [16].

The K_M values for BX2–BX5 are in the range 5–38 μM. The concentrations determined for the yeast-expressed enzymes are nearly identical to the values measured in maize microsomes (Table 2). This is an indication for the native conformation of the heterologously expressed P450 enzymes. K_M values for other plant P450 enzymes are in the

Table 3
DIBOA-biosynthetic P450 activities in maize, rye and barley

Plant	CPR specific activity (nmole/mg×min)	P450 content (μg/mg)	Specific Activity of microsomes ($\text{nmol}\times\text{mg}^{-1}\times\text{min}^{-1}$) with the substrate				
			Indole	Indolin-2-one	Benzoxazin-3-one	3-Hydroxy-Indoline-2-one	HBOA
Maize	44	5	0.3	0.24	0.09	0.94	0.86
Rye	78	4	0.25	0.19	0.07	0.76	0.45
Barley	29	3	–	–	–	–	–

range of 2 μM for protopin-6-hydroxylase [18] and 350 μM for tyrosin-*N*-hydroxylase [19].

2.2. DIBOA biosynthetic enzymes in other cereals

The hydroxamic acids are widely distributed in grasses [1], suggesting that the aquisation of this pathway occured relatively early in the evolution of the Gramineae. The activity of the DIBOA specific P450 enzymes was assayed in two other cereals, rye (containing hydroxamic acids) and barley (without hydroxamic acids) [1]. These two species are much closer related to each other than either is to maize [20].

The major cyclic hydroxamic acid of rye is DIBOA. As in maize, there are relatively high concentrations (up to 1 mg/g fresh weight) [21] present in the rye seedling. In order to investigate whether identical cytochrome P450-dependent reactions are associated with DIBOA biosynthesis in rye, microsomes were isolated from seedlings. The CPR activity in rye microsomes is about 1.8 times higher than in maize. All five substrates tested (Table 3) were converted to products that were identical to the products obtained with maize microsomes. No additional products were detected. The reactions were strictly dependent on NADPH (data not shown) indicating true cytochrome P450 enzyme reactions. The specific activities determined for the rye microsomes were very similar to the values detected with maize microsomes for the first three reactions in the pathway. The conversion of HBOA to DIBOA was reproducibly lower (a factor of two) in rye microsomes. The identity of the reactions in maize and in rye suggests identical DIBOA biosynthetic pathways for both species.

In microsomes prepared from barley seedlings, no activities of the P450 enzymes of the DIBOA pathway were detectable, although a total P450 content of 3 μg/mg microsomal protein and a CPR activity of 29 $\mathrm{nmol} \times \mathrm{mg}^{-1} \times \mathrm{min}^{-1}$ was determined (Table 3).

3. Discussion

Heterologous expression in yeast strains overexpressing plant CPRs is a powerful tool for the functional analysis of plant cytochrome P450 enzymes [11]. Using this system it was possible to determine the enzymatic properties for BX2–BX5 without using radioactively labelled substrates.

3.1. Enzymatic properties of BX2–BX5

Bx2–Bx5 have probably evolved by gene duplication events as indicated by clustering of the genes on the short arm of chromosome four and the sequence similarity between these genes. They have been grouped into the CYP71C subfamily [4] of cytochrome P450-dependent monooxygenases. The BX3–BX5 proteins share an average amino acid identity and similarity of 58% and 77%, respectively, while the average identity and similarity of these P450 enzymes to BX2 is only 49% and 70% respectively [4]. BX2 is also distinct in that its expression levels in the yeast strains are much higher than that of the other subfamiliy members (Table 1). In addition, the interaction of BX2 with different CPRs seems to be less selective than that of BX3–BX5. The specific activity of BX2 in the microsomes prepared from transgenic W(R) and WAT11 strains are quite similar. In contrast, BX3 shows a clear preference for the CPR of *Arabidopsis thaliana* (ATR1) present in the WAT11 yeast strain. BX4 and BX5 also seem to interact relatively well with ATR1, although their specific activities are lower than in maize microsomes. All heterologous CPRs, however, show a productive interaction with the maize P450 enzymes. This is further proof for the well established structural conservation of the interaction of P450 enzymes and CPRs [22].

The turn-over rate of BX3 expressed in WAT11 is about eight times higher than in maize microsomes. This indicates that CPR is a limiting factor for BX3 activity *in vivo*. Overexpression of CPRs in the plant could therefore result in significantly higher enzymatic activities. This could be important for the engineering of metabolic pathways that involve P450 enzymes.

3.2. Specificity of BX2–BX5

BX2–BX5 show high substrate specificity. Each of the DIMBOA specific enzymatic conversions can be performed only by one enzyme of the subfamily. Such a finding seems to support the idea that plant P450s generally have a much greater substrate specificity than their animal homologues [23]. However, there is emerging evidence that plant P450s in addition to their normal physiological function, can also convert certain xenobiotics with varying efficiencies. The hydroxylation of the herbicide diclofop was shown to occur by a wheat P450 enzyme [24]. Other examples include the demethylation of pCMA by CYP71A1 [17] and by CYP73A1 [16]. The natural substrate for CYP71A1 is not known, although geraniol and nerol have been reported as substrates [25]. CYP73A1 catalyzes the 4-hydroxylation of cinnamic acid, the first oxidative step in the phenylpropan pathway. For CYP73A1 the conversion of extra substrates has been investigated in great detail [23]. Furthermore, plant P450 enzymes can convert different natural substrates. Two P450 enzymes of sorghum that belong to the CYP79 family and the CYP71E subfamily respectively [26] are involved in the

biosynthesis of the cyanogenic glucoside dhurrin. These enzymes are multifunctional and catalyse four distinct steps [27, 28].

Indole appears as the natural substrate for 2-hydroxylation by BX2. In addition, BX2 shows pCMA demethylase activity like CYP71A1 and CYP73A1. Since the other substrates of these P450 enzymes have no structural similarity, it is likely that pCMA is a quite reactive molecule in the context of P450 catalysed reactions, even if binding of pCMA is not optimal. This is underlined by the fact that demetylation of pCMA is also catalysed by several animal P450 enzymes [29].

The structural analogy between 2-naphthoic acid and *trans*-cinnamic acid was shown to be sufficient such that 2-naphthoic acid can serve as an excellent substrate for CYP73A1 [23]. We have probably observed a similar case for BX3 that hydroxylates indolin-2-one and 1,4-benzoxazin-3-one at an equivalent carbon atom. Although indolin-2-one is converted about 2-fold more efficiently, it seems that 1,4-benzoxazin-3-one mimics indolin-2-one in the active site of BX3.

HMBOA was found in maize seedlings [13]. It is neither a substrate for BX5 nor can it be N-hydroxylated in maize microsomes [30]. It is probably the presence of the 7-methoxy group that excludes HMBOA as a substrate for BX5. HMBOA could represent a side product of the DIMBOA pathway, in which the C7-methoxy group was introduced prior to N-hydroxylation by BX5.

3.3. Evolution of the DIBOA pathway

For maize and rye, two distant species among the Gramineae, the same DIBOA biosynthetic pathway exists and therefore a set of proteins homologous to BX2–BX5 can be proposed. If this were the case, the duplications responsible for the evolution of the *Bx2–Bx5* gene cluster must have occurred early in the development of the Gramineae. In maize, the *Bx1* gene is included in this cluster [2]. *Bx1* encodes a protein with homology to the trypthophan sythase α subunit that catalyses the first step in DIMBOA biosynthesis. The isolation of genes homologues to *Bx1–Bx5* from rye and other grasses could give insight into the evolution of the *Bx* gene cluster.

Barley microsomes show no significant DIBOA specific P450 activities. It remains to be shown whether the loss of enzyme activity is due to gene inactivation, or whether the whole *Bx2–Bx5* cluster has been lost. This might have occured during agricultural breeding from wild barley varieties in which DIMBOA is still present [1]. A similar loss of enzyme activity has been observed for the UDP-glucose:DIBOA glycosyltransferase. Glycosylation is required for transport of DIBOA into the vacuole. Glycosyltransferase activity present in wild varieties, was lost during barley cultivation [30].

4. Experimental

4.1. Chemicals

DIBOA and HBOA were isolated from etiolated rye seedlings [31] and further purified by HPLC using a Merck LiChroCART RP-18 column (10×250 mm). The eluent contained 9% HOAc and 10% MeOH in H_2O (v/v).

4.2. Plant materials

The maize hybrid Blizzard (Ciba Geigy), rye seeds Halo (Lochow-Petkus, Germany) and the barley seeds Dissa (original source Seed Union, Germany) were used for preparation of microsomes. Seeds were germinated in the dark on wet filter paper for 3 (maize) or 4 (rye and barley) days. Whole seedlings were utilised for microsomal preparations.

4.3. Expression of BX2–BX5 proteins in E. coli and preparation of antibodies

The coding region of the four P450 genes was obtained by PCR amplification using the cDNA clones [4] as templates. The PCR fragments were inserted into the pET3a expression vector and expressed in *E. coli* strain BL21 (DE3) [32]. 1 l cultures were harvested, lysed and the overexpressed proteins were recovered as inclusion bodies using a modified protocol of Ito *et al.* [33]. Final purification was achieved by preparative SDS/PAGE on a BioRad model 491 Prep Cell using 10% SDS/PAGE according to the recommendations of the supplier. Immunisation of rabbits was carried out by EUROGENTECH (Belgium) using 0.3 mg of the purified proteins per immunisation.

4.4. Expression of P450 proteins in yeast

The yeast strains W(N), W(R) [6], WAT11 and WAT21 [6, 8] and the plasmid pYeDP60 [11] were obtained from P. Urban. The DNA coding sequences for BX2–BX5 were excised from the pET3a constructs by cleavage with NdeI and EcoRI. The NdeI site contains the AUG start codon of the genes. The DNA fragments were inserted into pYeDP60 which was linearized with EcoRI and BamHI by using an adapter (5′-GATCCAGGTGTCCA-3′, 5-TATGGACACCTG-3). Yeast cells were transformed by electroporation [34] and cell culture was performed as described by Urban *et al.* [11].

4.5. Enzyme assays

Plant microsomes were prepared according to Bailey and Larson [35]. Yeast microsomes were prepared as described [11]. The total P450 content of plant microsomes was measured by carbon monoxide differential spectroscopy [36]. pCMA demethylase activity was determined with NADPH as co-substrate as described [37]. Other hydroxylase assays were performed using a 0.2 ml reaction mixture containing 50 mM K-Pi pH 7.5, 0.8 mM NADPH, 5 μM to 1 mM of substrate and 1 mg microsomes (according to the protein content as determined by Bradford). After 10 min incubation at 25°C the assays were terminated by the addition of 0.2 ml MeOH and centrifuged for 5 min. The supernatant was acidified with 0.5 ml 0.1 M HOAc and extracted three times with 0.4 ml EtOAc. The extracts were lyophilised, dissolved in 0.1 ml MeOH and an aliquot was loaded onto a Merck LiChroCART RP-18 HPLC column (4×125 mm) and eluted for 5 min in isocratic conditions with solvent A (H_2O–HOAc, 9:1) followed by a linear gradient from solvent A to B (MeOH–H_2O–HOAc, 70:27:3) for 7 min. Analytes were detected at 254 nm. Microsomal NADPH-cytochrome c reductase (CPR) activity was measured as previously described [38].

4.6. Determination of BX2–BX5 concentration in microsomes

Microsomal proteins were separated by 10% SDS/PAGE and transferred to nitro-cellulose membranes according to the recommendations of the manufacturer (Amersham). For immunostaining, the rabbit polyclonal antibodies specific for BX2–BX5 were used. The blot was stained with anti-rabbit antibodies linked with Cy5 fluorescent dye (Amersham). The fluorescent signal was quantified using a Storm 860 imager (Molecular Dynamics). Purified recombinant P450 proteins were used as a reference for quantification.

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