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Benzoxazinoid biosynthesis in dicot plants

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ABSTRACT

Benzoxazinoids are common defence compounds of the grasses and are sporadically found in single species of two unrelated orders of the dicots. In the three dicotyledonous species *Aphelandra squarrosa, Consolida orientalis* and *Lamium galeobdolon* the main benzoxazinoid aglucon is 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA). While benzoxazinoids in *Aphelandra squarrosa* are restricted to the root, in *Consolida orientalis* and *Lamium galeobdolon* DIBOA is found in all above ground organs of the adult plant in concentrations as high as in the seedling of maize. The initial biosynthetic steps in dicots and monocots seem to be identical. Indole is most probably the first specific intermediate that is oxygenated to indolin-2-one by a cytochrome P450 enzyme. *C. orientalis* has an active indole-3-glycerolphosphate lyase for indole formation that evolved independently from its orthologous function in maize. The properties and evolution of plant indole-3-glycerolphosphate lyases are discussed.

1. Introduction

Plant secondary metabolites have essential functions in protection against herbivory and microbial infection, as attractants for pollinators and seed-dispersing animals and as allelopathic agents. Secondary plant products often are differentially distributed among limited taxonomic groups within the plant kingdom. Benzoxazinoids represent protective and allelophatic metabolites that are found in a multitude of species of the family Poaceae (Gramineae), of the monocot plants. Interestingly, these secondary metabolites are synthesised by the major crops maize, wheat and rye. Outside the Poaceae, benzoxazinoids are found in two distant orders of the eudicots, the Ranunculales and the Lamiales (Fig. 1). In contrast to the situation in the Poales, benzoxazinoid biosynthesis in these orders is restricted to single isolated species within the families Ranunculaceae (i.e. *Consolida orientalis*), Lamiaceae (*Lamium galeobdolon*) and Plantaginaceae (*Scoparia dulcis*) (Fig. 1; Sicker et al., 2000; Alipieva et al., 2003). Several species are reported to synthesise benzoxazinoids in the Acanthaceae (*Acanthus mollis*, *Aphelandra tetragona*, *A. squarrosa*, *Blepharis edulis;* Sicker et al., 2000). DIBOA [2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one] (Fig. 1) and its C7-methoxy derivative DIMBOA are the predominant representatives of benzoxazinoids in plants (Niemeyer, 1988).

In the grasses, a series of five genes is sufficient to encode the enzymes to synthesise DIBOA (Frey et al., 1997; Nomura et al., 2002, 2003; Grün et al., 2005; Fig. 6). The committed enzyme of benzoxazinoid biosynthesis, BX1, links primary to secondary metabolism. Most likely it evolved *via* gene duplication and modification from the alpha-subunit of the tryptophan synthase (TSA). Both enzymes are functionally indole-3-glycerol phosphate lyases (IGLs) that cleave indole-3-glycerol phosphate (IGP) into indole and glyceraldehyde-3-phosphate (alpha-reaction of tryptophan biosynthesis). TSAs are subunits of the tryptophan synthase complex as shown in plants for *Arabidopsis thaliana* (Radwanski et al., 1995) and *Zea mays* (Kriechbaumer et al., 2008). TSA mutant plants exhibit a deficiency in tryptophan biosynthesis, leading to a distinguished phenotype (Radwanski et al., 1996). While TSA-activity is to a large extent dependent on interaction with the beta-subunit of the tryptophan synthase, IGLs can also exist as functionally active monomers. This has been shown for BX1 (Frey et al., 1997; Nomura et al., 2005; Grün et al., 2005) and the maize enzyme ZmIGL (Frey et al., 2000). These enzymes catalyse efficiently the formation of indole that can serve as first intermediate in benzoxazinoid biosynthesis or as a volatile signal. *ZmBx1* and *ZmIgl* mutants have normal tryptophan levels.

Four cytochrome P450 enzymes (P450s), BX2–BX5, and one 2-oxoglutarate dependent dioxygenase (BX6) introduce the oxygen atoms in DIBOA/DIMBOA biosynthesis in maize. Orthologs of *Bx1* to *Bx5* have been identified for wheat (Nomura et al., 2002, 2003)

Abbreviations: PCR, polymerase chain reaction.

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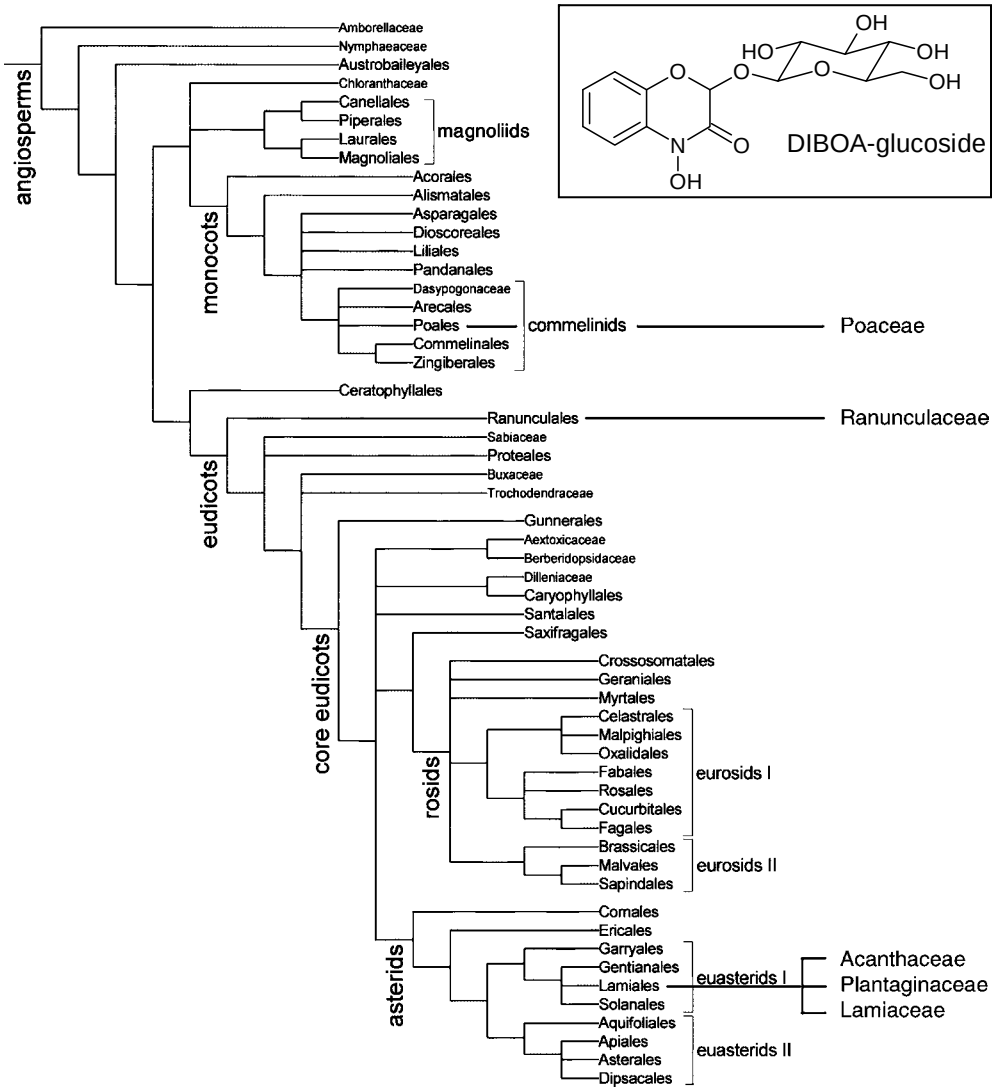

Fig. 1. Phylogeny of angiosperms. Benzoxazinoids are detected in the Poaceae, Ranunculaceae, Acanthaceae, Plantaginaceae and Lamiaceae. The chemical structure of DIBOA-glucoside is given in the insert. Tree according to Angiosperm Phylogeny Group (APG II, 2003).

and *Hordeum lechleri* (Grün et al., 2005). In the grasses, the genes of DIBOA-biosynthesis have a monophyletic origin.

The investigation of the benzoxazinoid biosynthetic pathway in three dicot plants, *Aphelandra squarrosa* (zebra plant; Acanthaceae), *Consolida orientalis* (larkspur; Ranunculaceae) and *Lamium galeobdolon* (yellow archangel; Lamiaceae) indicate that the first biosynthetic steps are biochemically identical to the benzoxazinoid biosynthesis in the grasses. A comprehensive survey of *Igl* genes in *C. orientalis*, *L. galeobdolon* and *A. squarrosa* revealed a wide spectrum of catalytic properties for IGLs in plants and identifies candidate genes for the branchpoint reaction in benzoxazinoid biosynthesis. However, the gene duplication and evolution of the putative *C. orientalis Bx1* gene must have occurred independently of the events that specified BX1 in the grasses.

2. Results

2.1. Benzoxazinoid distribution in different plant organs is non-uniform in dicot plants

The biological significance of benzoxazinoids as natural pesticides and insecticides is well documented. Additionally, exudation by the root has allelopathic function (Barnes and Putnam, 1987). In maize, the presence of benzoxazinoids is a characteristic feature of the seedling; the only adult organ with significant benzoxazinoid levels is the crown root (Table 1). Two of the investigated dicot plants, *C. orientalis* and *L. galeobdolon*, exhibit benzoxazinoid concentrations of the same level as maize (up to 40 mM). In contrast to the grasses, high benzoxazinoid concentrations are present in organs of the adult dicot plant: in flower bud and flower of *C. orientalis*, and in adult leaves of vegetatively propagated *L. galeobdolon* plants (Table 1). In *C. orientalis* no benzoxazinoids are detectable in the root, and in *L. galeobdolon* the concentrations in the root are low. Hence, benzoxazinoids have no allelopathic function *via* root exudation in these plant species. In contrast, in *Aphelandra squarrosa*, traceable levels of benzoxazinoids are exclusively present in the root. In the family Acanthaceae DIMBOA occurs in addition to DIBOA. DIMBOA is present in *A. squarrosa* roots in minor amounts (Table 1). Benzoxazinoids are not evenly distributed along the root of *A. squarrosa*, highest levels are present in the root tip; DIBOA and DIMBOA are present in *in vitro* cultured roots. In essence, the three dicot plants exhibit distinct patterns of benzoxazinoid distribution.

Table 1
Benzoxazinoid contents (mmol/kg fr. wt) in dicot plants

Species	Tissue	DIBOA	DIMBOA
Aphelandra squarrosa[a]	Root <1 cm	0.042	0.0004
	Root 1–2 cm	0.032	0.0002
	Root 2–5 cm	0.0034	0,0001
	Root 10 cm, tip	0.150	n.d.
	Root 10 cm, lateral root	0.058	n.d.
	Root 10 cm, root body	0.059	>0.0001
	Root culture 10 cm	0.064	0.0001
Consolida orientalis	Root, 2 weeks	n. d.	n.d.
	Cotelydon, 2 weeks	0.2	n.d.
	Young leaf, 8 weeks	24.0	n.d.
	Old leaf, 8 weeks	17.0	n.d.
	Flower bud, 8 weeks	34.0	n.d.
	Flower, 8 weeks	36.0	n.d.
Lamium galeobdolon[b]	Adventitious root	1.1	n.d.
	Young leaf	19.0	n.d.
	Old leaf	17.0	n.d.
	Flower bud	7.7	n.d.
	Flower	5.0	n.d.
Zea mays (inbred B73)	Root, 3 days	<0.1	19.0
	Root, 4 days	<0.1	13.0
	Crown root, 8 weeks	<0.1	2.8
	Shoot, 3 days	<0.1	19.0
	Shoot, 4 days	<0.1	13.0
	Scutellum, 3 days	<0.1	5.7
	Leaf, 10 weeks	n.d.	n.d.

fr. wt fresh weight.
n.d. not detectable.
Mean values of 3 replicates are given.
[a] No benzoxazinones are detectable in above ground tissue.
[b] Plants were propagated by planting of adventitious shoots and cuttings.

2.2. Characterization of benzoxazinoid biosynthesis of dicot plants by labelling studies

In the grasses, benzoxazinoid biosynthesis branches off from the tryptophan biosynthetic pathway, the first specific intermediate is indole. Radioactively labelled indole and tryptophan were used in feeding studies to get a hint whether a connection between benzoxazinone and tryptophan biosynthetic pathways exists in the dicot species. Roots (*A. squarrosa*) and shoots (*C. orientalis*, *L. galeobdolon*) were incubated in aqueous solution of the radioactively labelled precursors. Subsequently benzoxazinoids, free and protein-bound tryptophan were isolated and analysed for radioactive label. Both precursors resulted in incorporation of radioactive label into proteins (data not shown). In contrast, radioactive benzoxazinoids were only detectable when labelled indole was applied (Supplemental Figure S1). This result implies that indole can enter the biosynthetic pathway and may be an intermediate in benzoxazinoid biosynthesis in all three dicot species.

The finding that radioactively labelled indole is incorporated into benzoxazinoids allows the investigation of *de novo* DIBOA-biosynthesis in different plant parts (Fig. 2). Detached organs were used to demonstrate the biosynthetic capacity of various tissues. Consistent with the low amount of DIBOA present in *L. galeobdolon* roots, significantly lower biosynthetic rates are determined in the roots compared to above ground tissues. In *C. orientalis,* lowest levels of ^{14}C-labelling are detected in young leaves of mature plants and fully open flowers; roots were not assayed. Benzoxazinoid levels in *A. squarrosa* roots were too low to allow a reliable analysis.

2.3. A P450 enzyme generates indolin-2-one in C. orientalis and L. galeobdolon

De novo biosynthesis in *C. orientalis* seedlings and young *A. squarrosa* plantlets is sufficiently high to monitor reliably the incorporation of ^{14}C-indole. These systems appear suitable to characterise enzymatic steps of the pathway by applying specific inhibitors. In *C. orientalis* and *L. galeobdolon* incubation of shoots with the P450-enzyme inhibitor 1-aminobenzotriazol (Mugford et al., 1992; Kim et al., 2004) significantly reduced the *de novo* synthesis of DIBOA (Supplemental Figure S2) indicating that at least one P450 enzyme is integrated in the pathway.

In grasses indole is converted to indolin-2-one by the P450 enzyme BX2 (Frey et al., 1997; Nomura et al., 2002; Grün et al., 2005). Knowing that indole is also a potential intermediate of the pathway in dicots and that P450s might contribute to DIBOA biosynthesis, microsomal preparations from *C. orientalis* and *L. galeobdolon* shoots were assayed for the conversion of indole. In both cases a product was generated that was identified as indolin-2-one by HPLC and LC–MS analysis. The transformation of U-$^{13}C_8$-^{15}N-indole into labelled indolin-2-one was further verified by LC–MS (Supplemental Figure S3). The biosynthesis is strictly dependent on the addition of NADPH to the assay. This is an indication that the second reaction of DIBOA biosynthesis in grasses can, in principle, take place in these dicot tissues and it suggests that indole and indolin-2-one are intermediates of the pathway in *C. orientalis* and *L. galeobdolon*.

2.4. Benzoxazinoid synthesizing dicot plants differ with respect to the arsenal of indole-3-glycerolphophate lyases

In the labelling experiments, label from indole and not from tryptophan is incorporated in DIBOA. Therefore, it is unlikely that the substrate indole is delivered *via* tryptophan by a tryptophanase-type enzyme, rather in the dicots indole could be provided by an indole-3-glycerol phosphate lyase, like in the grasses. In the grasses gene duplication of TSA and 'partial' neo-functionalization resulted in indole-3-glycerol phosphate lyases that are active as monomers and optimized for the production of free indole (Frey et al., 2000). The alignment of plant TSA and BX1 enzymes reveals highly conserved regions (Supplemental Figure S4) that are even preserved in cyanobacteria (*Synechocystis* sp.). These regions were used to design primer pairs for the amplification of *Igl*-sequences of *A. squarrosa*, *C. orientalis* and *L. galeobdolon* (Supplemental Table S1) using cDNA isolated from benzoxazinoid containing tissue. Cloning and sequencing of the PCR-fragments verified the amplification of *Igl*-sequences. The isolated partial sequences were used for the screening of cDNA libraries under low stringent conditions and full-size cDNA-clones were isolated. In the following, we name indole-3-glycerol phosphate lyases '*Igl*' as a neutral designation for enzyme function, '*Bx1*' when the catalytic parameters are in the range of ZmBX1 and we restrict the name '*TSA*' to subunits of the tryptophan synthase.

Two *Igl*-sequences isolated from *A. squarrosa* are 98% identical and typify most probably alleles of one gene (*AsIgl version 1*(*AsIglv1,* EU747710) and *AsIgl version 2(AsIglv2*, EU747711). Sequences representing different *Igl*-genes were isolated for *C. orientalis* (*CoIglv1*, EU747713, *CoIglv2*, EU747714, and *CoBx1*, EU747712) and *L. galeobdolon* (*LgIgl1*, EU747715, *LgIgl2*, EU747716). *CoIglv1* and *CoIglv2* are 99.2% identical and are likely allelic (*CoIgl*), but *CoBx1* is definitely a different *Igl* gene; only about 60% identity is found on protein level when compared with *CoIglv1* or *CoIgvl2*. The two *L. galeobdolon* genes have 72.5% identity on protein level. CoBX1 is the most divergent enzyme; a similarity of about 70% is displayed in comparisons with the other genes, whereas all other pair wise comparisons reveal an about 10% higher similarity (Supplemental Table S2). Southern analysis under low stringency conditions was applied to reveal the presence of *Igl*-sequences in the three plant genomes (Supplemental Figure S5). In the case of *A. squarrosa*, 5′-, middle- and 3′-probes of *AsIgl1* identify two bands each, a finding consistent with the presence of the homologous

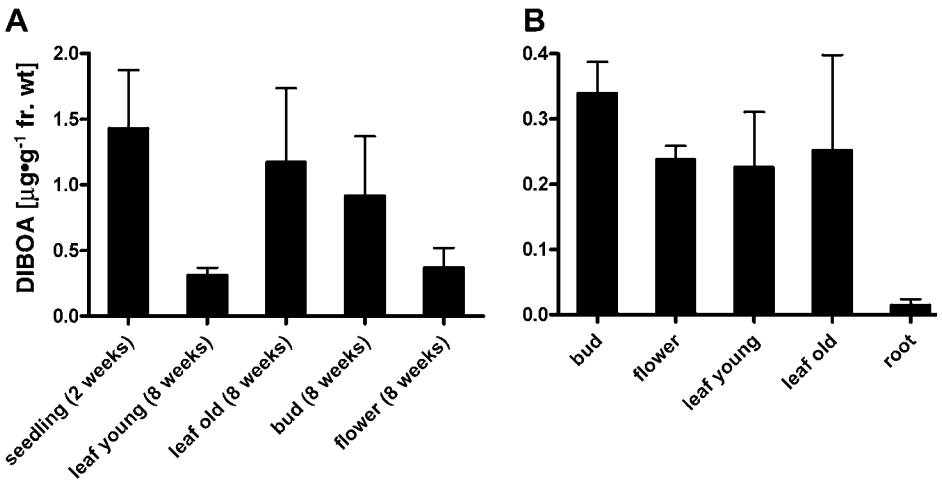

Fig. 2. *De novo* DIBOA-biosynthesis in different plant organs. (A) *C. orientalis* and (B) *L. galeobdolon*. The explants were incubated in ^{14}C-indole solution for 24 h before extraction of benzoxazinoids. The radioactively labeled DIBOA was quantified after thin layer chromatography. Two independent experiments with two repetitions of each incubation were performed. The standard deviation of the means for each experiment are indicated by the bars.

sequences found in the cDNA library. A complex hybridization pattern is revealed for *C. orientalis*, up to four bands are detected in different restriction digets with the *CoBx1* probe, additional hybridization signals are unveiled with the *CoIgl* cDNA. Hence, it is not clear whether the isolated genes represent all *Igl*-genes present in *C. orientalis*. In the Southern analysis of *L. galeobdolon* individual hybridisation signals can be associated with *LgIgl1* and *LgIgl2* probes. Most bands are specific for the one or the other gene, however, it cannot be excluded that minor bands are signals of additional *Igl*-genes not yet isolated as a cDNA clone.

The transcript levels of the genes were determined by qRT–PCR. The levels of glyceraldehydphosphate dehydrogenase (*GAP C*) were determined in parallel and used for normalisation of the data (Supplemental Table S3). *AsIgl* transcript is present in roots at a low level, a similar expression level is detected for *LgIgl1* in flower buds, flower and leaf. *LgIgl2* has an about 10-times higher expression level in these organs. In the root both *L. galeobdolon* genes display the same moderate expression. No correlation between benzoxazinoid content and *Igl*-gene expression was detected. The *C. orientalis* genes *CoIgl* and *CoBx1* differ in absolute levels and expression pattern (Fig. 3). *CoIgl* is expressed at a low level in flower, leaf and root. *CoBx1* transcript level is highest in flowers, even the levels of *GAP C* transcript are exceeded, and lowest in root. *CoBx1* expression corresponds to the distribution of DIBOA in *C. orientalis*.

The kinetic parameters of the IGL enzymes in the alpha-reaction were assayed using recombinant protein expressed in *E. coli*. In addition, analogous N-terminal fusions of the his-tag to the *Igl*-sequence were analysed for ZmBX1, AtTSA1 and AtTSA2. The enzymes can be divided into three groups according to the k_{cat}-value (Table 2): the value is beyond 0.5 s^{-1} for ZmBX1 and CoBX1, between 0.02 and 0.016 s^{-1} for the two AsIGLs, LgIGL1 and AtTSA2, and below 0.004 s^{-1} in the last group. Interestingly, AtTSA1, the alpha-subunit of the *Arabidopsis* tryptophan synthase complex, is in the group with lowest k_{cat}-values, together with the two CoIGLs and LgIGL2. Every plant in the analysis seems to have one IGL with the catalytic properties that allow the deliverance of indole for biosynthetic processes. In the case of *A. squarrosa*, *C. orientalis* and *L. galeobdolon*, the released indole may be used in benzoxazinoid biosynthesis. Highly efficient enzymes are expressed in the grasses and in *Consolida orientalis*.

A phylogenetic analysis (Fig. 4) of the isolated *Igl* genes was performed comprising also the functionally-characterized genes *ZmBx1*, *ZmIgl*, *ZmTSA*, *ZmTSAlike* from maize, the *Arabidopsis thaliana* genes *AtTSA1* and *AtTSA2* and the homologous genes *PpTSA*, *SsTSA*, *StTSA* from *Physcomitrella patens*, *Synechococcus* sp. and *Salmonella typhimurium*. The tree shows a clear separation between the dicot and the maize genes, especially, CoBX1 evolved independently of ZmBX1.

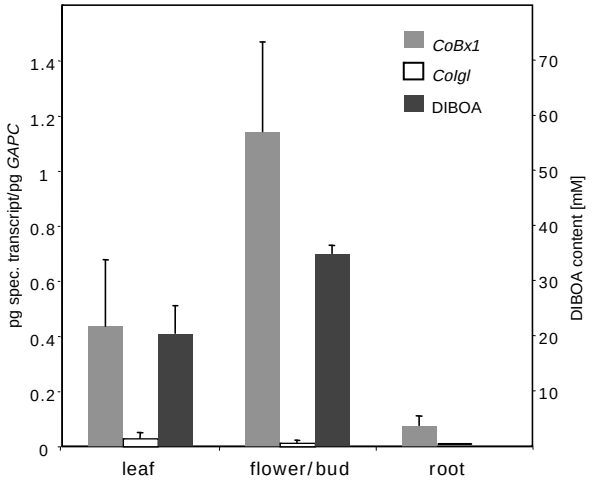

Fig. 3. Expression of *CoBx1* and *CoIgl* in different parts of the plant. RNA was isolated from leaf, flower and root of *Consolida orientalis* and the transcript levels were determined by qRT–PCR. The transcript levels are given in relation to the *GAP C*-mRNA. The DIBOA levels of the respective tissues are indicated. The mean values of three (leaves and root) and two (flower/bud) experiments are given and the standard deviation indicated by the bars.

2.5. Structure-function predictions deduced from modelling of Igl-sequences

The X-ray structures of bacterial TSA (StTSA, Hyde et al., 1988; TtTSA, Yamagata et al., 2001; EcTSA, Nishio et al., 2005) and of BX1 (Kulik et al., 2005) have been determined. The enzymatic activity of the *Salmonella* enzyme is highly dependent on complex formation with the beta-subunit of the tryptophan synthase (Kawasaki et al., 1987). The allosteric interaction of the two subunits is a prerequisite for the switch from inactive to active conformation. In ZmBX1 the monomer is in the active conformation. According to the X-ray structure of the *Salmonella* TSA and BX1 two structurally

Table 2
Comparison of catalytic properties

	$K_{0.5}^{IGP}$ mM	k_{cat} s^{-1}	$k_{cat}/K_{0.5}^{IGP}$ mM^{-1} s^{-1}
CoBX1	0.038	1.900	50.00
ZmBX1	0.031	0.510	16.45
LgIGL1	0.610	0.020	0.033
AsIGLv1[a]	0.214	0.019	0.089
AsIGLv2[a]	0.241	0.018	0.075
AtTSA2	0.399	0.016	0.040
CoIGLv1	0.390	0.0042	0.011
CoIGLv2	0.279	0.0012	0.004
AtTSA1[a]	0.530	0.0014	0.003
LgIGL2	0.024	0.0003	0.013

[a] Enzyme displays no Michaelis–Menten-kinetics. In all other cases $K_{0.5}^{IGP} = K_m^{IGP}$.

equivalent positions are supposed to contribute to the formation of the active monomer conformation. These positions are StTSA Tyr173 *versus* ZmBX1 Phe253, and StTSA Leu127 *versus* ZmBX1 Ile207 (Supplemental Figure S6). However, the sequence alignment of all IGL proteins assayed in this publication does not suggest a correlation between this particular sequence pattern and the activity of the enzyme monomers, e.g. LgIGL2, having poor catalytic values, has the ZmBX1 pattern. Modelling of IGLs, including active monomers (CoBX1, ZmIGL) and AtTSA1 that is part of the tryptophan synthase complex (Radwanski et al., 1995) revealed another amino acid position that might influence the enzymatic properties of the gene. According to the modelled structure the amino acid residue in the position structurally equivalent to the *S. typhimurium* site 98 influences the orientation of the glutamate (position StTSA 49, Fig. 5, Supplemental Figure S6) that is directly involved in catalysis. Valin or leucin present in ZmIGL, ZmBX1 and CoBX1 push the glutamate into the substrate-binding pocket (Fig. 5B, Supplemental Figure S5). Two conformations are possible in the case of an alanine in the respective site (AsIGL, AtTSA2, CoIGL, LgIGL1, LgIGL2, Fig. 5C and D, Supplemental Figure S6) and further interactions may influence the actual conformation of the enzyme. In AtTSA1 serine pulls the glutamate out of the substrate cavern (Fig. 5A, Supplemental Figure S6) and this would explain the low catalytic activity of the *Arabidopsis* TSA monomer.

3. Discussion

3.1. The expression of the benzoxazinoid biosynthesis pathway is different in monocots and dicots

It has been shown previously (Baumeler et al., 2000) that benzoxazinoids in *A. squarrosa* are exclusively found in the root. Consistent with this investigation, DIBOA and minor amounts of DIMBOA were detected in young roots and in root organ culture. The concentration is significantly increased in the root-tip region. However, the concentration of benzoxazinoids in *A. squarrosa* roots is below the biological active range for a putative function in defence (Sahi et al., 1995), hence, benzoxazinoids may have a yet unknown function in *A. squarrosa*. In contrast, DIBOA levels as high as in maize seedlings are present in *C. orientalis* and *L. galeobdolon*. The distribution patterns, however, are distinct: *C. orientalis* is characterized by high DIBOA levels in flowers and flower buds, benzoxazinoids are not detectable in roots. *L. galeobdolon* has highest DIBOA concentrations in green tissues, and low but clearly detectable levels in adventitious roots. None of the dicot plants shows the restriction of benzoxazinoids to young developmental stages characteristic for maize and other grasses (Sicker et al., 2000). In the adult plants and during flowering DIBOA-concentrations are present that are sufficient to function in chemical defence. In conclusion, the expression of benzoxazinoids in plants follows diverse patterns and hence the biosynthetic genes may be connected to different regulatory regimes.

All plant tissues that accumulate benzoxazinoids are capable to synthesise these secondary metabolites. No discrepancy between DIBOA concentration and *de novo* biosynthesis measured by integration of exogenously applied indole, is seen. Only *C. orientalis* open flowers, being terminal organs, seem to have a reduced *de novo* biosynthesis rate. In contrast to e.g. pyrrolizidine alkaloids in *Senecio vernalis* and nicotine in *Nicotiana* sp. where the site of biosynthesis and of accumulation are different and the metabolite is transported (Baldwin, 1989; Hartmann et al., 1989), transport processes are not required for the distribution of benzoxazinoids.

3.2. Evolution of DIBOA-biosynthesis

The dicot plants that synthesise benzoxazinoids are not model plants; no genetically defined lines are available. The ornamentals *A. squarrosa* and *L. galeobdolon* require specific pollinators and are propagated vegetatively; *C. orientalis* is according to our experience an obligate outcrosser. It is not known how many alleles of a given gene are present in the plant species analysed. Transformation protocols and reverse genetic tools are not yet available for any of the three plant species. Therefore, a feasible starting point for the analysis of the biosynthetic pathway and the genes involved, are biochemical studies and the analysis of candidate genes.

Tracer and inhibitor studies indicate that indole is an intermediate in benzoxazinoid biosynthesis in dicots and that P450 enzymes participate in DIBOA-biosynthesis in *C. orientalis* and *L. galeobdolon*. The demonstration that indole is converted to indole-2-one in an NADPH-dependent manner by isolated microsomes suggests that the first two biosynthetic steps in the pathway are congruent in grasses and in dicots: indole serves as substrate that is oxygenated by a P450 enzyme (Fig. 6). *Bx2* to *Bx5*, members of the P450 gene family *Cyp71*, catalyse the four oxygenation-steps of DIBOA-biosynthesis in the grasses. The *Cyp71* family includes further enzymes with substrates related to indole (Nafisi et al., 2007; Schuhegger et al., 2006). It remains to be shown whether the dicot P450s of the benzoxazinone biosynthesis belong to the same P450 family or have a similar evolutionary origin as the genes of the grasses.

Preliminary results (data not shown) indicate that, like in the grasses, an UDP-glucosyltransferase generates the less toxic benzoxazionoid-glucoside. Hence, benzoxazinoid biosynthesis would, in principle, appear to follow the same chemical route in dicots and monocots (Fig. 6). The question whether monophyletic or independent evolution underlies this analogy can only be answered by isolation of the genes of the pathway. Since indole is the common starting point for benzoxazinoid-biosynthesis in all plants, it seems to be straightforward to isolate the branch point gene that provides indole to study the molecular evolution of the pathway. It has been shown recently that bacteria synthesise substantial amounts of indole and use free indole for a variety of processes (e.g. biofilm production and plasmid replication; Collet et al., 2007; Chant and Summers, 2007). In bacteria, indole is delivered *via* tryptophanase, the bacterial TSA is only effective in tryptophan biosynthesis. Genes with homology to tryptophanase have not been identified in plants. In contrast, in plants the *Igl* gene family includes enzymes that efficiently catalyse the formation of indole. Hence, it can be supposed that the branch point enzyme in plants is an *Igl*. Therefore, the strategy to isolate the gene was to identify an as complete as possible set of expressed *Igl*-genes and to assay the encoded enzymes for indole production. Considering the above-mentioned limitations of the plant systems, it is obvious that candidates can be identified, further characterisation of the *in planta* function has to await reverse genetic methods.

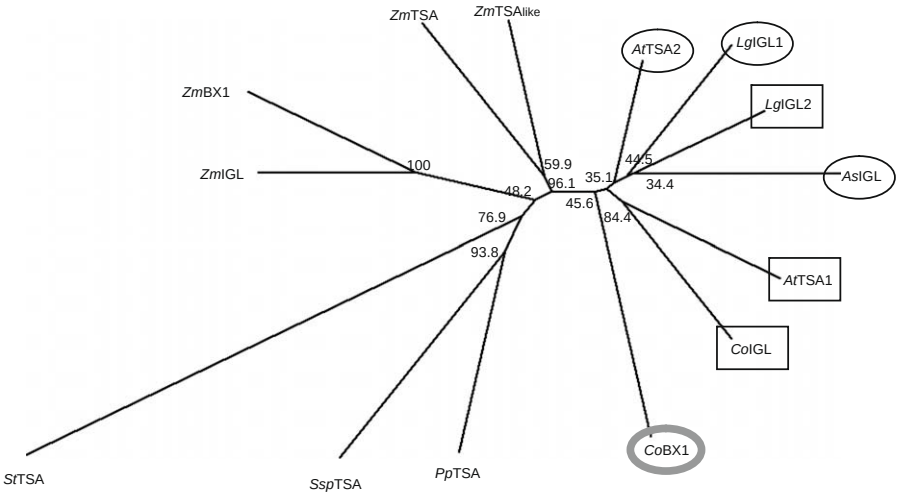

Fig. 4. Phylogenetic analysis of plant *Igl*-sequences. Maize and dicot sequences have distinct roots. No pronounced relationships are displayed between dicot sequences with similar catalytic activities (low activity encircled, medium activity in square box, high activity encircled with thick grey line). Maize ZmBX1 and *Consolida orientalis* CoBX1 have no common phylogenetic root. In contrast the two efficient IGLs of maize ZmBX1 and ZmIGL have a common ancestor gene and are products of duplication and modification of the progenitor gene. According to the definition given in 2.3 ZmIGL is a BX1 enzyme, ZmTSAlike and AtTSA2 are IGLs.

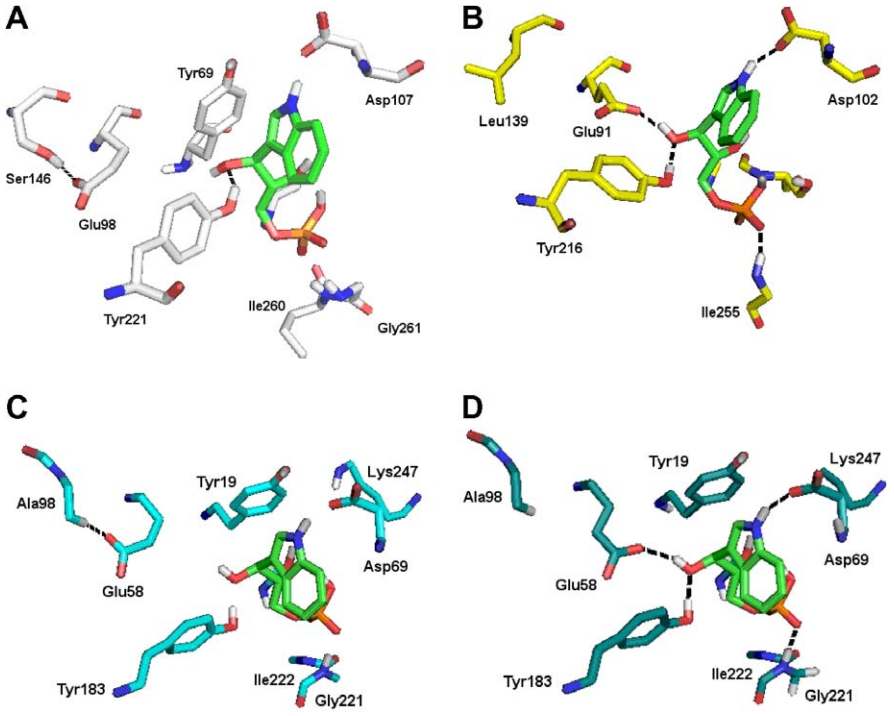

Fig. 5. Modelling the conformation at the catalytic centre for plant IGL-enzymes. The glutamate in the active centre is essential for catalysis (retro-aldol-reaction). In the active conformation glutamate and tyrosin form hydrogen bonds with the substrate indole-3-glycerol phosphate, shown in green. (A) *Arabidopsis thaliana* AtTSA1, (B) *Consolida orientalis* CoBX1, (C) and (D), *Arabidopsis thaliana* AtTSA2, in C the enzyme is in an inactive conformation, in D the active conformation is shown. The ligand is positioned based on the X-ray structure of 1tjp from *Salmonella typhimurium*.

The screening of the cDNA libraries for the *Igl*-sequences was comprehensive using 500.000 recombinant phages for each plant species. More than one *Igl* sequence per plant species was identified, most probably representing one gene with two alleles in *A. squarrosa* and two genes in the case of *C. orientalis* (and one of these genes with two alleles) and *L. galeodolon*. If the presence of several alleles of one gene is assumed, the picture given in Southern-analysis for the three plants can be explained with the isolated *Igl* genes. The seven characterized *Igl*-sequences may largely present the arsenal of *Igl* genes in these plants. The encoded IGLs display a wide range for the kinetic constant k^{cat}/K_m^{IGP} that can be taken as a parameter for catalytic efficiency in the catalysis, the value of which spans four orders of magnitude for the enzymes studied here; enzymes at the lower edge have similar parameters as the *E. coli* TSA enzyme (Weischet and Kirschner, 1976). When the IGLs are divided into enzymes with low, medium and high, *viz.* BX1-type, catalytic activity (Table 2), the two characterized plant TSAs from *Arabidopsis* and *Zea mays* (Kriechbaumer et al., 2008) are located in the lower group together with CoIGL and LgIGL2. However, in addition to this putative TSA, every plant investigated, including *A. thaliana*, has an IGL with significantly higher k^{cat}/K_m^{IGP}. This enzyme may catalyse the biosynthesis of indole

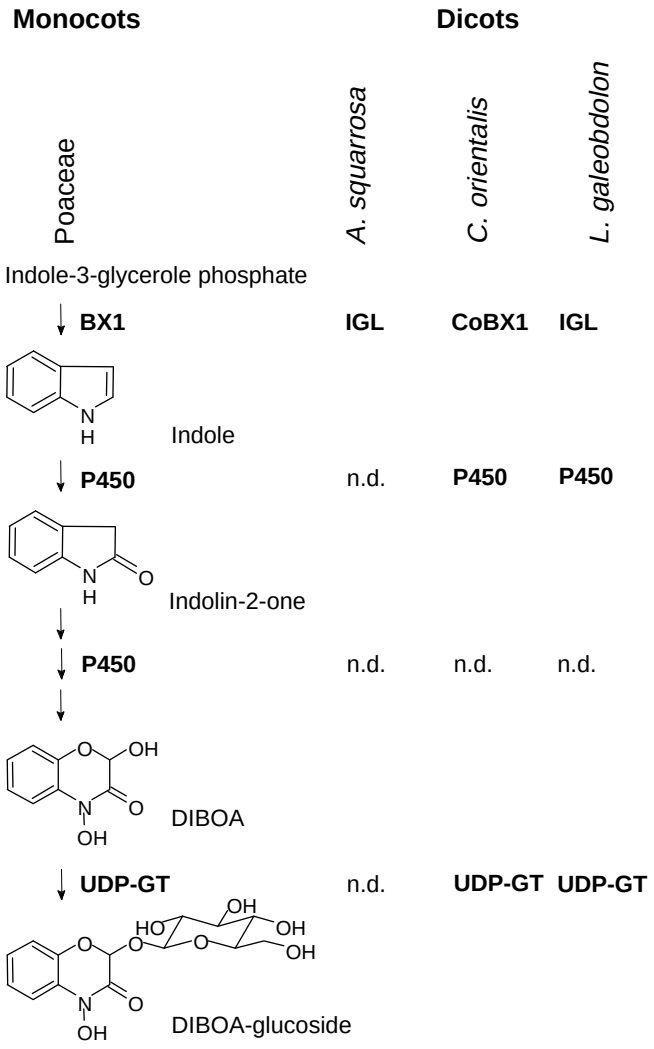

Fig. 6. DIBOA-biosynthesis in plants. The first specific intermediate indole is common in all plant species. The enzyme involved in generation of indole is an indole-glycerole phosphate lyase (IGL). The IGLs in the different plants evolved independently from the alpha-subunit of tryptophan synthase (TSA). A cytochrome P450 enzyme introduces an oxygen atom to yield indole-2-one in the grasses, *Consolida orientalis* and *Lamium galeobdolon*. DIBOA is glucosylated by an UDP-glucosyltransferase in all plant species. n.d., not determined.

independent of the formation of a tryptophan synthase complex. The delivered free indole may be used as a substrate in different biosynthetic pathways, in *A. squarrosa*, *C. orientalis* and *L. galeobdolon* it may enter benzoxazinoid biosynthesis. The genes *AsIgl*, *CoBx1* and *LgIgl2* may represent the committed enzymes for benzoxazinoid biosynthesis. It cannot be excluded that the branch point IGL has not been isolated in the approach. However, CoBX1 has kinetic constants that classify this enzyme in one efficiency group with BX1 (Table 2) and the expression profile of *CoBx1* is in good correlation with the DIBOA distribution in *C. orientalis* (Fig. 3). Hence, all data available are in line with CoBX1 being the branch point enzyme of benzoxazinoid biosynthesis in *C. orientalis*. The comparison of the BX1 enzymes of the grasses and CoBX1 reveals that the enzymes have no apparent monophyletic origin and were recruited independently. Hence benzoxazinoid biosynthesis arose independently in these lineages. A similar result was reported for the first committed enzyme in pyrrolizidine alkaloid-biosynthesis, homospermidine synthase in different plant species (Reimann et al., 2004).

Duplication of *Igl*-sequences seems to be a frequent event in plants. Phylogenetic analysis shows that these duplications are independent events in monocots and dicots (Fig. 4). Two genes are present in *A. thaliana*, at least two in *L. galeobdolon*, *C. orientalis*, and four genes are isolated for *Zea mays*. *ZmTSA*, *ZmTSAlike*, *ZmBx1* and *ZmIgl* encode enzymes with distinguished catalytic properties and biological functions (Kriechbaumer et al., 2008; Frey et al., 2000, 1997). The BX1-type enzyme of grasses (ZmBX1, TaBX1, HlBx1, ZmIGL) can be traced back to a single duplication event. Within the dicot sequences no subgroups forming distinct branches are evident, supposing an independent evolution of the analysed dicot *Igl* genes (Fig. 6). However, although apparently no correlations between the overall sequence-similarity of the IGLs and the respective catalytic properties are given, a consistent theme that can explain the different efficiencies is revealed by modelling of the enzyme structures. The positioning of the catalytically active glutamate relative to the substrate IGP seems to be essential for the activity. A productive conformation appears to be greatly influenced by interactions of the glutamate with neighbouring amino acids, neutral-nonpolar amino acids with a moderate sized side chain (e.g. leucin and valine) are most effective. It is an open question if the ancestral *Igl* gene in plants was a typical TSA subunit. Alternatively, the ancestor could be an IGL with moderate catalytic properties that functioned both in tryptophan biosynthesis and indole production. Gene modification then would have taken two routes, towards efficient tryptophan biosynthesis *via* interaction with the TSB subunit in the tryptophansynthase complex, and in some plants, e.g. the grasses and *C. orientalis*, towards efficient indole production by monomeric IGLs.

4. Experimental

4.1. Chemicals

Indole and indolin-2-one were purchased from Fluka Chemie, Buchs, CH, U-$^{13}C_8$; ^{15}N-indole from Cambridge Isotope Laboratories, Inc., 50 Frontage Road Andover, MA, USA, [^{14}C]-indole (50 mCi/mmol) from American Radiolabeled Chemicals, Inc. St. Louis, MO, USA, and [^{14}C]-tryptophan (50 mCi/mmol) from Perkin–Elmer, Boston, MA, USA.

Oligonucleotides were purchased from Biomers.net GmbH, Ulm, Germany. DNA-sequence analysis was by Eurofins MWG Operon, Ebersberg, Germany.

4.2. Plant material and growth conditions

Aphelandra squarrosa plants and *Consolida orientalis* seed were a kind gift of Dr. M. Schulz, Institut f. Landwirtschaftliche Botanik, Univ. Bonn.

A. squarrosa was propagated by shoot culture, roots were induced by incubation of the cut surface with Rhizopon AA powder (Rhizopon, Netherlands) and planting in wet sand. Plants were grown at 18 °C under natural light conditions in the rooms of the institute.

C. orientalis was grown at 19 °C and at 12 h light/day in growth chambers. Flowering plants were grown in 16 h light/day light regime in growth chamber or in the field.

Lamium galeodolon shoots with adventitious roots, flowers and buds were harvested at the Staudengarten Weihenstephan, FH Freising; root formation on explants was induced as described for *A. squarrosa*, the plantlets were grown under the same conditions as *C. orientalis*.

4.3. Isolation of substrates and standards

Plant extracts and enzyme assays were analyzed by reverse phase high performance liquid chromatography (HPLC) using LiChroCart RP18e columns (5 mm, 250-4, flow rate 1 ml/min) and

the system "Gold" Beckman. Unless stated elsewhere, conditions were as described by Glawischnig et al. (1999).

DIBOA was purified according to Bailey and Larson (1991) and Glawischnig et al. (1999). DIMBOA was purified according to Hartenstein et al. (1992). DI(M)BOA-glc from *A. squarrosa* roots was isolated as described by Baumeler et al. (2000).

Tryptophan was isolated from plant tissue after grinding in liquid nitrogen by extraction with aceton, which was repeated twice. For the analysis of protein-bound tryptophan the ground plant tissue was incubated in 2 M $Ba(OH)_2$ at 90 °C over night, and neutralised by addition of dry ice. The hydrolysate was applied to HPLC (LiChrosphere RP18e 5 mm column 250-4, 20% to 60% MeOH in 0.3% formic acid in 14 min and 100% MeOH, 1 min). Fractions containing tryptophan were collected and the liquid evaporated.

Indole-3-glycerol phosphate (IGP) was synthesised by incubation of the purified enzyme CoBX1 with the substrates 1 mM indole and 0.5 mM glyceraldehyde phosphate in 50 mM Tris and 1 mM EDTA, pH 8.0, over night at room temperature. The reaction was terminated and the protein precipitated by addition of 1 volume MeOH. After centrifugation the supernatant was applied to an Alusphere RT100 (5 mm column, 250-10, Merck) and IGP eluted with a gradient of 10–100% MeOH in 0.125% ammonia, in 15 min, flow rate 1 ml/min. The fractions containing IGP were collected and the liquid evaporated. IGP was resolved in 5 mM Tris, pH 8.0.

4.4. Determination of the incorporation of radioactive substrates

Radioactively labelled indole and tryptophan (25 nCi/ incubation) were applied to the shoots in 50 mM MES, pH 5.7. After incubation, isolation of benzoxazinoids and tryptophan were as described. The plant extracts were dissolved in methanol and applied to silica gel 60 F_{254} thin layer chromatography (TLC) plates (Merck). Methanol:chloroform (3:2, v/v) (benzoxazinoids) or butanol:H_2O:acetic acid (5:3:2, v/v) (tryptophan) were used as solvents. The plates were analysed with the Storm phosphoimager (GE Healthcare Life Sciences). Calibration curves were generated on the basis of defined amounts of radioactive indole or tryptophan spotted on to the TLC plates.

4.5. Isolation of microsomes and assay conditions

Fifteen to twenty gram leaf material were homogenized in 20 ml extraction buffer (250 mM Tris, 250 mM saccharose, 100 mM ascorbic acid, 2 mM DTT, 5 mg/ml BSA, 2 mM EDTA, at pH 8.0) with 0.3 g/g plant material PolyKlar AT and sea sand. After centrifugation at 24,000*g* the supernatant was subjected to centrifugation at 200,000*g* for 90 min. The pellet was resuspended in 100 mM Tris, 25 mM saccharose, 50 mM NaCl, 2 mM DTT at pH 8.0, and microsomes were pelletised by centrifugation at 200,000*g* for 30 min. The microsomes were resuspended in 100 mM Tris, 25 mM saccharose, 50 mM NaCl, 2 mM DTT, 15% glycerole at pH 8.0. The integrity of the isolated microsomes was tested by hydroxylation of cinnamic acid because this P450-enzyme catalyzed reaction is functioning in all plant species tested so far. The conversion of indole and U-$^{13}C_8$;^{15}N-indole to the respective indolin-2-one was tested by incubation of microsomal protein in 50 mM potassium phosphate buffer, pH 7.4, 0.7 mM NADPH and 0.2 mM indole for 2 h at room temperature. The reaction was terminated by the addition of 1 volume methanol. After addition of 1.3 volumes 0.1 M acetic acid, the reaction was extracted three times with 1 volume ethyl acetate. The combined ethyl acetate fractions were evaporated and the residue dissolved in methanol. Analysis was by HPLC on a LiChrosphere RP18e 5 mm column. A gradient of 20–100% acetonitrile in 0.3% formic acid was applied (15 min, 1 ml/min).

4.6. LC–MS analysis

A Bruker Daltonics esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a variable wavelength detector was utilized. Components were separated with a Phenomenex (Aschaffenburg, Germany) Luna C-18 column (150 mm long × 2.0 mm inner diameter, particle size 5 μm) which was held at 25 °C. A linear gradient from 80% A (0.1% formic acid in water) to 80% B (0.1% formic acid in acetonitrile) in 30 min was used with a flow rate of 0.2 ml/min. The detection wavelength was 280 nm. The electrospray ionization voltage of the capillary was set to −3811 V and the end plate to −500 V. Nitrogen was used as nebulizer gas at a pressure of 30 psi and dry gas at a temperature of 330 °C and a flow rate of 9 l/min. The full scan mass spectra were measured in a scan range from *m/z* 50 to 800 with a scan resolution of 13,000 *m/z*/s until the ICC target reached 30,000 or 200 ms, whatever was achieved first. Tandem mass spectrometry was carried out using helium as collision gas (3.56×10^{-6} mbar) with the collision voltage set at 1 V and precursor selection at 131, 134 and 140 *m/z*. Spectra were acquired in the positive ionization mode with SPS at 138 *m/z*. Data analysis was performed using the DataAnalysis 3.1 software (Bruker Daltonics, Bremen, Germany).

4.7. Molecular biology methods and construction and screening of the cDNA library

Unless stated elsewhere, all molecular biology methods were as described by Frey et al. (1995).

PCR and quantitative RT–PCR were as described by v. Rad et al. (2001). RNA was isolated using the NucleoSpin RNA plant kit from Macherey and Nagel (Düren, Germany) and single stranded cDNA for quantitative PCR-analysis was synthesised from total RNA with the TagMan Kit (Perkin-Elmer). The primer pairs used for amplification of hybridization probes and quantitative PCR are given in Supplemental Table S1.

For the generation of the cDNA libraries, RNA was isolated from *A. squarrosa* roots, *C. orientalis* and *Lamium galebodolon* plantlets according to Logemann et al. (1987). Poly(A)$^+$ RNA was prepared using Oligotex Direct mRNA Midi Kit (Qiagen). The cDNA libraries were constructed using the Lambda-ZAP system (Stratagene) according to the manufacturer's specifications. The primary libraries (about 5×10^5 pfu each) were amplified and 5×10^5 pfu of the amplified library were screened. Lambda-ZAP-clones were converted to pBluscript plasmids and the cloning of full-size cDNA was verified by sequence analysis.

For the screening of the libraries and for Southern analysis, DNA fragments were labeled with ^{32}P by oligo-primed labeling with the Klenow polymerase. Hybridization of the cDNA library was under low stringency conditions (2 × sodium–sodium phosphat-EDTA-Buffer (SSPE), 55 °C). High stringency conditions in Southern analysis were 0.2 × SSPE and 65 °C.

4.8. Heterologous expression of plant Igl-genes in E. coli

For heterologous expression the *Igl*-gene cDNAs were fused to the N-terminal his-tag of pET28 via introduction of an *NdeI* restriction cut comprising the start codon. Induction and purification of the proteins under native conditions was as described by the manufacturer of the Ni–NTA agarose (Quiagen, Hilden, Germany). Transit peptide sequences at the amino terminus were not included in the expression clones. Storage at −70 °C and 4 °C did not influence the catalytic activity of the enzymes during a period of several months.

4.9. DNA sequencing and computer analysis

DNA Phylogenetic trees were generated using CLUSTALW (http://clustalw.genome.jp/), based on the Neighbor Joining (NJ) algorithm (Saitou and Nei, 1987).

4.10. Modelling

Proteins homologous to TSA-proteins with a resolved X-ray structure were identified by BLASTP search in the Brookhaven Protein Data Bank (PDB). The X-ray structure 1rd5 (Kulik et al., 2005) was found most similar and useful for homology modeling with an amino acid sequence homology of about 64%. Using the molecular modeling program MOE (Molecular Operating Environment, Chemical Computing Group Inc., Montreal, Canada) the TSA sequences were aligned to that of 1rd5 with the BLOSUM60-substitution matrix (Henikoff and Henikoff, 1992, 1993). For each protein ten gas phase models were generated and the structures were minimized using CHARMM22 force field (gradient below 0.05) (MacKerell et al., 1998) and Born solvation (Pellegrini and Field, 2002). The stereochemical quality of all structures was examined by PROCHECK (Laskowski et al., 1993) and native folding by PROSAII (Sippl, 1990). All parameters for good stereochemical quality were fulfilled for all modelled proteins, for each case, some 92% of all amino acids are located in the most favoured region, and 8% of the amino acids in the additionally allowed region (by PROCHECK). All other stereochemical parameters were within the quality range expected for a structure with a 2.0 Å resolution. PROSA analysis showed all residues in the negative energy area except of a small N-terminal loop region. The sum of these results indicates the prediction of a native fold of the proteins. The homologous protein from *Salmonella typhimurium* (pdb 1tjp, Kulik et al., 2005) was crystallized with the ligand 1-[(2-hydroxyphenyl)amino]3-glycerolphosphate. By superposition of this structure with the modelled ones the ligand was accordingly placed to the active site and subsequently minimized within the enzymes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.08.023.

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