Comparative Analysis of Benzoxazinoid Biosynthesis in Monocots and Dicots: Independent Recruitment of Stabilization and Activation Functions

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Benzoxazinoids represent preformed protective and allelophatic compounds that are found in a multitude of species of the family Poaceae (Gramineae) and occur sporadically in single species of phylogenetically unrelated dicots. Stabilization by glucosylation and activation by hydrolysis is essential for the function of these plant defense compounds. We isolated and functionally characterized from the dicot larkspur (Consolida orientalis) the benzoxazinoid-specific UDP-glucosyltransferase and ß-glucosidase that catalyze the enzymatic functions required to avoid autotoxicity and allow activation upon challenge by herbivore and pathogen attack. A phylogenetic comparison of these enzymes with their counterparts in the grasses indicates convergent evolution by repeated recruitment from homologous but not orthologous genes. The data reveal a great evolutionary flexibility in recruitment of these essential functions of secondary plant metabolism.

INTRODUCTION

Plants produce a vast array of secondary metabolites. Many of these compounds are toxic to plants and other organisms and are involved in defense against microbial attack or herbivore predation. Defense compounds may be synthesized de novo in response to pathogen attack (e.g., phytoalexins that are synthesized in plant–microbe interactions). Alternatively, protective compounds may be produced without challenge during normal development and are then termed constitutive or preformed defenses. To mitigate autotoxicity, these preformed compounds are frequently stored in special plant organs or organelles and are often inactivated by conjugation. Biosynthesis of preformed defenses can be distinguished by the following steps: (1) the branch point reaction committing the flux from primary to secondary metabolism, (2) biochemical modification of the initial intermediate leading to a biological active (toxic) secondary metabolite, (3) detoxification (stabilization) and storage of this metabolite, and (4) activation of the compound upon pathogen or herbivore attack. Individual classes of constitutive defenses occur with quite different frequencies within the plant kingdom.

Cyanogenic glycosides are widespread and are present in more than 2650 plants from ferns to angiosperms (Bak et al., 2006). Contrarily, avenacins are restricted to the genus Avena (Hostettmann and Marston, 1995), and glucosinolates are found in the order Capparales and as an exception in the genus Drypetes (Rodman et al., 1998). Furthermore, the biosynthesis of specific preformed defense compounds can be characteristic for one plant family but additionally occur sporadically in single species of phylogenetically unrelated families. Such a distribution pattern is described for pyrrolizidine alkaloids (Hartmann and Witte, 1995) and benzoxazinoids (Sicker et al., 2000).

Benzoxazinoids represent protective and allelophatic defense compounds that are found in a multitude of species of the family Poaceae (Gramineae) of the monocot plants, including the major agricultural crops maize (Zea mays), wheat (Triticum aestivum), and rye (Secale cereale). Benzoxazinoid biosynthesis is fully elucidated in maize (Frey et al., 1997, 2003; von Rad et al., 2001; Jonczyk et al., 2008; Figure 1) and is characterized in part for wheat (Nomura et al., 2002, 2003, 2005; Sue et al., 2011), diploid Triticales (Nomura et al., 2007), and wild barley (Hordeum lechiarii; Grün et al., 2005). DIBOA [2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one] and its C7-methoxy derivative DIMBOA are the predominant benzoxazinoids (Niemeyer, 1988). DIMBOA is the major benzoxazinoid in maize and wheat; DIBOA is prevalent in rye aboveground tissue, while DIMBOA is found in the root (Rice et al., 2005). Most of the benzoxazinoid-containing dicot species, including larkspur (Consolida orientalis), possess DIBOA.

In the grasses, a series of five genes is sufficient to encode the enzymes for DIBOA biosynthesis (Frey et al., 1997; Nomura et al., 2002; Grün et al., 2005; Figure 1). The committing enzyme of benzoxazinoid biosynthesis, BX1, links primary to secondary
metabolism. It evolved via gene duplication and neofunctionalization from the gene for the α-subunit of Trp synthase (TSA). Both enzymes are functionally indole-3-glycerol phosphate lyases that cleave indole-3-glycerol phosphate into indole and glyceraldehyde-3-phosphate. The following reactions in biosynthesis comprise the conversion of indole to DIBOA by the introduction of four oxygen atoms. These reactions are catalyzed by four cytochrome P450-dependent monooxygenases, termed BX2 to BX5, which are members of the CYP71C subfamily.

Inhibitor studies indicate that in dicot benzoxazinoid biosynthesis, these reactions are also catalyzed by cytochrome P450 enzymes (Schullehner et al., 2008). Glucosylation by UDP-glucosyltransferases (UGTs) chemically stabilizes and increases the solubility of the constitutive defense compound rendering it suitable for storage in the vacuole. The two UGTs BX8 and BX9 catalyze the formation of the benzoxazinoid-glucoside. In maize, DIBOA-glucoside (GDIBOA) is converted into DIMBOA-glucoside (GDIMBOA) by the 2-oxoglutarate–dependent dioxygenase BX6 (Frey et al., 2003) and the O-methyltransferase BX7 (Jonczyk et al., 2008). In maize, as well as in other grasses, DIMBOA is exuded into the rhizosphere and has in addition to activity against nematodes and root-feeding insects (Xie et al., 1992; Zasada et al., 2005), allelopathic function (Sicker et al., 2000). Since DIMBOA is the preferred substrate for BX9, this UGT is rather involved in protecting root tissue from exogenous DIMBOA than in formation of GDIBOA during biosynthesis (von Rad et al., 2001). Hydrolysis of the glucosidic linkage by β-glucosidases (β-GLUs) activates the defense compound. In the intact maize plant, benzoxazinoid-glucosides and two specific-glucosidases (Zm-GLU1 and Zm-GLU2; Babcock and Esen, 1994) are kept in two different cellular compartments, the vacuole and the plastid, respectively. The toxic aglucone is produced upon disintegration of the cell due to pathogen or herbivore attack.

Outside the Poales, benzoxazinoids are detected in two distant orders of the eudicots, the Ranunculales and the Lamiales. Benzoxazinoid biosynthesis in these orders is restricted to single isolated species (Sicker et al., 2000). Two explanations exist for the parallel occurrence of the same metabolite in unrelated species: a monophyletic origin or convergent evolution. In the grasses, all DIBOA biosynthetic genes, including the UGT and the β-GLU, are of monophyletic origin (Nikus et al., 2003; Nomura et al., 2002, 2003; Frey et al., 2009; Sue et al., 2000, 2011). However, the branchpoint enzyme BX1 was independently recruited in three dicotyledonous species (with respect to each other and to grasses), although the orthologous enzyme of primary metabolism, TSA, was employed (Schullehner et al., 2008). TSA homologs in maize and rice (Oryza sativa) (indole-3-glycerol phosphate lyase; Frey et al., 2000; Zhuang et al., 2012), which catalyze the formation of indole as a volatile signal in tritrophic interaction, represent additional cases of independent recruitment and neofunctionalization of TSA.

Plant UGTs and β-GLUs form large gene families (e.g., 122 family 1 UGTs and 40 family 1 β-GLUs) are annotated in Arabidopsis thaliana. We were therefore interested in determining whether the functions that are responsible for the stabilization of benzoxazinoids and for the release of the toxic aglucone have a monophyletic origin in monocots and dicots or were established by recruiting different members from UGT and β-GLU gene pool. In the evolution of preformed defenses, the stabilization and activation of the reactive intermediate(s) can be considered as crucial points. To benefit from the biosynthesis of a toxin, the plant needs a tool to avoid autotoxicity and, complementarily, a function to release the toxic aglucone. Therefore, these functions may be required at the origin of an evolving pathway. Phylogenetic analysis of the glucosyltransferase and the glucosidase of sporadically occurring pathways can shed light on the specific constraints for these functions in constitutive defense compound biosynthesis.

Figure 1. Benzoxazinoid Biosynthesis in Maize.
GDIMBOA is stored in the vacuole. Hydrolysis of the glucoside by Zm-GLU generates the reactive aglucone.
We isolated and functionally characterized the DIBOA-specific glucosyltransferase and glucosidase from the dicot larkspur (*C. orientalis*; Ranunculaceae). These functions seem to have evolved independently from their counterparts in the Gramineae. Specific biochemical features of the dicot enzymes corroborate the phylogenetic analysis based on amino acid sequences.

**RESULTS**

**Isolation of the *C. orientalis* UDPG: DIBOA-Glucosyltransferase Co-Bx8 and the GDIBOA-Glucosidase Co-Glu**

To identify the functions for stabilization (BX8) and activation (termed BxGLU in the following) of benzoazoxinoid biosynthesis in a dicot species, we isolated the specific glucosyltransferase and β-GLU from *C. orientalis*. The isolation was based on transcriptome data of 7- to 10-d-old seedling shoots. This plant material was chosen since it is available in large quantities and has high benzoazoxinoid content and DIBOA biosynthesis activity (Schullehner et al., 2008). A total of 250,000 reads of the normalized cDNA were assembled using the Phred software (Ewing et al., 1998; Ewing and Green, 1998), which delivered 6.8 Mb of singlet sequences and 15.5 Mb of contigs.

Co-BX8 was identified by comparison of the transcriptome with peptide sequences of the DIBOA:UDP-glucosyltransferase (UDPG) activity that was purified almost to homogeneity from crude soluble protein extracts by ammonium sulfate precipitation and four chromatographic separations similar to the purification of the corresponding maize UGTs (von Rad et al., 2001; see Supplemental Table 1 online). Analysis of individual fractions on SDS-PAGE gels revealed a band with a molecular mass of ~55 kD that correlated with UDPG:DIBOA-glucosyltransferase activity in the enzyme assay (see Supplemental Figure 1 online). This band was excised and digested following the protocol of Schäfer et al. (2001). The sequence of several fragments of the trypsin digest was determined by mass spectroscopy.

In the *C. orientalis* transcriptome, 25 singlets and 37 contigs were assigned to UGT domain proteins due to the presence of the PFAM signature PF00201 (Finn et al., 2010; see Supplemental Table 2 online). Five contig sequences were assembled from at least 10 sequence reads each (see Supplemental Table 2 online) and thus represent abundant transcripts. The deduced amino acid sequences of these contigs were compared with the data from mass spectroscopy of the purified CoBX8. One contig with a size of 1174 bp included seven peptides of CoBX8 (see Supplemental Figure 2 online); no other significant similarities were detected in the transcriptome. The full-size cDNA corresponding to the candidate contig was isolated by screening a *C. orientalis* seedling cDNA library (Schullehner et al., 2008). The open reading frame of Co-Bx8 comprises 1428 bp; the deduced amino acid sequence includes the highly conserved plant secondary product glucosyltransferase box consensus sequence that characterizes the family 1 UGTs involved in plant secondary metabolism (Hughes and Hughes, 1994). The closest UGT homologs with defined biochemical functions are the cytokinin-O-glucosyltransferase At-COGT2 from *Arabidopsis* and the hydroxymandelonitril UGT Sb-HMNGT from *Sorghum bicolor* (49 and 43% identity on the amino acid level, respectively). For heterologous protein expression, the cDNA was cloned in the *Escherichia coli* expression vector pET28a as an N-terminal His-tag fusion protein. The recombinant enzyme had UDPG-DIBOA-glucosyltransferase activity and hence BX8 function (see below for detailed analysis).

The rationale for the isolation of the β-GLU enzyme was based on our experience that Bx genes are generally highly expressed in tissues with high benzoazoxinoid content (Frey et al., 1995; von Rad et al., 2001; Jonczyk et al., 2008). The β-GLU genes present in 454 transcriptome data from *C. orientalis* shoot were identified by a BLAST search using Zm-GLU1 (Esen, 1992) and the *Arabidopsis* myrosinase 1 At-TGG1 (Barth and Jander, 2006), two quite diverge members of the β-GLU family 1 (42% identity and 60% similarity), as query sequences (see Supplemental Table 2 online). Two abundant homologs were represented in full length in the seedling phage cDNA library. These genes have open reading frames of 1536 and 1527 bp and encode enzymes with 65% amino acid identity to each other. Both enzymes contain the sequence motifs ITENG and TFNEP that are characteristically conserved in glycoside hydrolases of family 1 (Davies and Henrissat, 1995). A signal peptide for targeting the proteins into the secretory pathway is predicted for both enzymes, and transit peptide sequences for localization to the plastid are not detected (TargetP 1.1, Emanuelsson et al., 2000; iPSORT, Bannai et al., 2002). The enzymes have ~45% amino acid identity to the query sequences Zm-GLU1 and At-TGG1 and ~60% amino acid identities to the closest homologous enzymes with defined function, the exoglucanase Os-4BGLU12 (Opassiri et al., 2006) and the hydroxynitril-glucoside hydrolyzing enzyme Lj-BGD2 (Morant et al., 2008a; Takos et al., 2010). Expression of the β-GLUs in *E. coli* failed probably due to instability caused by lack of plant-specific protein modification (Zhao et al., 2002; Morant et al., 2008b). Alternatively, transient expression of the proteins by magnification (Marillonnet et al., 2005) with a *Tobacco mosaic virus*-based vector system was used. The vector system (Engler et al., 2008) was transferred via *Agrobacterium tumefaciens* into *Nicotiana benthamiana* leaves. Both enzymes were the major proteins in leaves 4 to 10 d after infection (see Supplemental Figure 3 online). The functionality of the heterologously expressed β-GLUs was verified by the detected hydrolysis of the general artificial substrate p-nitrophenyl-β-glucopyranoside (pNPG). One of the enzymes hydrolyzed GDIBOA and hence is functionally analogous to Zm-GLU1 (see below for detailed analysis). This enzyme is termed Co-GLU in the following. The second enzyme had no activity toward the benzoazoxinoid substrate and thus was not further analyzed. The expression pattern of the other highly expressed β-GLUs differs substantially from the distribution of DIBOA in *C. orientalis* (see Supplemental Figure 4 online); therefore, it is unlikely that they encode benzoazoxinoid-specific enzymes.

**Expression Profiles of Co-Bx8 and Co-GLu Imply a Function in Benzoazoxinoid Metabolism.**

The expression pattern of hitherto characterized benzoazoxinoid pathway genes in grasses and dicots (Frey et al., 2009) is largely
connected to the accumulation of the metabolite in the plant. In *C. orientalis*, DIBOA is synthesized and present in significant concentrations in leaves of seedlings and mature plants and in flowers but has concentrations below the detection limit in the roots (Schullehner et al., 2008). The transcript levels of Co-Glu and Co-Bx8 were determined by quantitative RT-PCR for these tissues (Figure 2). Co-Glu transcript levels close to the values of the housekeeping gene GAPDH were found in leaves, flower buds, and open flowers; the level is below the detection limit in the root. Co-Glu displays essentially the same expression pattern as Co-Bx7, the first gene in the pathway (Schullehner et al., 2008). By contrast, the UGT Co-Bx8 is not only expressed in the shoot, leaves, and flower, but also in the root (Figure 2). This implies an additional function of the UGT, since no benzoxazinoid biosynthesis takes place and benzoxazinoids are not detectable within the root. Co-Bx8 may protect the root tissue against exogenous benzoxazinoids that can originate from plant debris (Blum, 1999). In addition to the role in biosynthesis, Co-Bx8 could function in protection against exogenous benzoxazinoids such as proposed for Zm-Bx9. Zm-Bx9 is also expressed in older root tissue where benzoxazinoid biosynthesis is nearly absent (Schmädl, 2002). To determine the potential of Co-Bx8 to restrict allelopathic effects of exogenous benzoxazinoids, transgenic Arabidopsis plants expressing Co-Bx8 were generated. BX8 activity was assayed in crude extracts of six independent transgenic plant lines. All transgenic but no wild-type control plants glucosylated DIBOA. Homozygous progeny of two transgenic lines with the highest Co-Bx8 activity were tested for tolerance to DIBOA and DIMBOA (Figure 3). The benzoxazinoids were applied in concentrations of 0.1 to 2.0 mM to the two transgenic lines, as well as to wild-type control plants and Zm-Bx8 or Zm-Bx9 expressing homozygous transgenic Arabidopsis plants (von Rad et al., 2001). Even the lowest benzoxazinoid concentration impaired the growth of wild-type plant slightly, while 1.0 and 2.0 mM concentrations resulted in growth arrest and bleaching or browning of these plants. By contrast, all transgenic plants survived the treatment, although the Co-Bx8 enzyme seems to be less effective in detoxification of DIMBOA compared with Zm-Bx8 and Zm-Bx9. This difference may result from the relatively high $K_m$ of Co-Bx8 (see below). However, both *C. orientalis* and maize UDPG: DIBOA-glucosyltransferases were sufficient to overcome DIBOA and DIMBOA phytotoxicity.

**Detoxification and Activation Functions of the Benzoxazinoid Pathway in *C. orientalis* and in the Poaceae Are in Phylogenetic Groups That Separated before the Diversification of Monocots and Dicots**

The expression patterns and enzymatic properties strongly suggest functionality of Co-Bx8 and Co-Glu in benzoxazinoid metabolism. The phylogenetic relationship of the dicot and the respective monocot genes was predicted to reveal the evolutionary origin of these two functions.

Family 1 UGTs and family 1 β-GLUs comprise large gene families in plants (Paquette et al., 2003; Xu et al., 2004). Experimental evidence for enzyme function is only available for a subset of the enzymes. Family 1 UGTs involved in secondary metabolism are subdivided into 14 groups (Ross et al., 2001). UGTs involved in flavonoid metabolism are characterized across a wide phylogenetic range of plant species including maize. The monophyletic origin of the enzymes functioning in this common plant secondary metabolic pathway including UGTs is well established (Rauscher, 2006). The analysis of UGTs for flavonoid metabolism strongly suggests functionality of Co-Bx8 and Co-Glu in benzoxazinoid metabolism. The phylogenetic relationship of the dicot and the respective monocot genes was predicted to reveal the evolutionary origin of these two functions.

**Figure 2.** Pattern of Co-Bx8 and Co-Glu Expression and DIBOA Distribution.

Transcript levels were determined for 2-week-old seedlings (lane 1), hypocotyl and cotyledon (lane 2), and rosette leaves (lane 3) of 4-week-old plants, mature leaves (lane 4), and senescence leaves (lane 5) of 8- to 10-week-old plants, root (lane 6), green flower buds (lane 7), flower buds with violet petals (lane 8), and flowers (lane 9). The transcript levels were normalized with GAPDH expression, and DIBOA content was determined per milligram of fresh weight. Two biological replicates were analyzed in two technical replicates each. Co-Bx8, dark-gray bars; Co-Glu, light-gray bars; DIBOA, black bars. The SD is indicated.
metabolism provides a measure for the phylogenetic relationship of the DIBOA-specific UGTs. To make equivalent data sets for both pathways available, we isolated the flavonoid-O-glucosyltransferases from *C. orientalis* and *Lamium galeobdolon*. In addition, the BX8/BX9 ortholog of wheat was isolated; this gene may be an allelic variant of the wheat Bx8 genes isolated recently (Sue et al., 2011) or represent a closely related duplicate thereof (98 to 96% identity on amino acid level). The genes were identified by screening the *C. orientalis* and *L. galeobdolon* transcriptome and the GenBank wheat EST collection for homologous sequences. The functions of the isolated genes were verified by heterologous expression and enzyme assays (see Supplemental Figure 5 online).

A phylogenetic tree (Figure 4; see Supplemental Data Set 1 online) was constructed that comprises plant secondary product glucosyltransferase box UGTs with defined biochemical functions and close homologs thereof. Family 1 UGTs for steroid metabolism were not included. Sequences from monocot and dicot plants were represented in almost all groups, demonstrating that diversification of the UGTs predates the divergence of monocots and dicots 180 million years ago (Brown et al., 2011). The flavonoid-3-O-glucosyltransferases enzymes (F3GTs) are found in group F; the analysis predicts a common precursor of F3GTs that existed before the separation of monocots and dicots. By contrast with F3GTs, the dicot Co-BX8 and the BX8 enzymes from grasses are found in distinct groups (G and H), and both groups separated before the diversification of dicots as predicted by the presence of dicot and monocot sequences in both groups. According to the standardized nomenclature system (Mackenzie et al., 1997), Co-BX8 is named UGT85N1, while the UGTs of grasses belong to family UGT710E. Except for BX8, group G enzymes are otherwise involved in N-glucosylation of cytokinins (At-COGT1) and biosynthesis of cyanogenic glucosides (Sb-HMNGT) and sweet glucosides (Sr-UGT85C2), while the group H enzymes At-CNGT1/At-CNGT2 are involved with N-glucosylate cytokinins.

The phylogenetic analysis of the BxGLUs was done analogously. The data set included enzymes that display (additional) mannosidase activity and enzymes hydrolyzing cell wall compounds (common substrates), β-GLUs of defense pathways (benzoxazinoids, cyanogenic glucosides, glucosinolates, and saponins; summarized in Morant et al., 2008b), manually reannotated β-GLU sequences from Arabidopsis and rice (Xu et al., 2004; Opasiri et al., 2006), and β-GLUs annotated in the Uniprot database. The tree (Figure 5; see Supplemental Data Set 2 online) can be divided into three clades; one of these comprises common substrate glucosidases (clade 2). Monocot and dicot enzymes with mannosidase activity or cell wall compound-cleaving enzymes are present in separate branches of clade 2, indicating diversification of the respective precursor glucosidases before the separation of monocots and dicots. The two other clades each include enzymes cleaving benzoxazinoid glucosides (Co-GLU in clade 1 and Bx-GLUs of the grasses in clade 3). Interestingly, the BxGLUs are in subbranches with dicot and monocot enzymes that have cyanogenic glucoside as substrates (Co-GLU with β-vicianoside β-glucosidase; BxGLUs of the grasses with dhurrin β-GLU).

The phylogeny indicates independent evolution of UGTs and β-GLU for benzoxazinoid biosynthesis in monocots and dicots. This pattern suggests that these enzymes originated independently.
from enzymes that did not necessarily share functions in plant metabolism.

Monocot and Dicot BX8 and BxGLU Enzymes Display Distinct Differences in Enzymatic Function

To test the idea that the monocot and dicot BX8 and BxGLU functions arose independently, we investigated to what extent the C. orientalis and maize UGTs and β-GLUs exhibit distinct enzymatic specificities that may reflect different phylogenetic origins.

The steady state kinetic constants of E. coli–expressed Co-BX8 were determined for the substrates DIBOA, DIMBOA, and UDPG (Table 1). The reaction followed Michaelis-Menten kinetics for the three substrates (see Supplemental Figure 6A online). The $K_m$ values for DIBOA and UDPG are in the same range. Compared with Zm-BX8, the values are increased, but $k_{cat}$ values are similar (Table 1). Since the DIBOA concentrations in C. orientalis tissues (17 to 36 μmol/g fresh weight) exceed this concentration by at least a factor of 40, this enzymatic property is not contradictory to BX8 function of the enzyme. In addition, analysis of the DIBOA-glucosyltransferase activity in protein crude extract revealed a $K_m^{DIBOA}$ similar to the heterologously expressed enzyme has a reduced affinity to DIBOA (Table 1). Further differences between Co-BX8 and the maize UGTs were found for the substrates HBOA and HMBOA. The presence of the 7-N-OH group and has significantly reduced substrate specificity for HBAO and HMBOA. The presence of the 7-O-methyl group seems suspected as substrates since benzoxazinoid biosynthesis is connected with auxin biosynthesis via the Trp biosynthetic pathway. The phylogenetic analysis for the dicot and monocot BX8 enzymes displays a close homology to cytokinin-glucosylating enzymes. However, BX8 enzymes do not glucosylate the hormones (see Supplemental Table 3 online); likewise, flavonoids are not substrates. Hence, the dicot and the monocot BX8 enzymes are specific for benzoxazinoids but require different modifications of the core benzoxazinoid structure for highest activity.

To characterize the biochemical activity of Co-GLU, a range of β-glycosides was tested as substrates (see Supplemental Figure 9 and Supplemental Table 4 online). BxGLUs are phylogenetically
related to cyanogenic glucoside-cleaving enzymes, and the cyanogenic glucoside dhurrin has been shown to be a competitive nonmetabolized inhibitor of Zm-GLU (Babcock and Esen, 1994). Furthermore, Zm-GLU (isoform Zm-p60.1) activity toward cytokinin glucosides has been demonstrated (Brzobohaty et al., 1993). These nonbenzoxazinoid substrates were included in our analysis of Co-GLU. Our results show that Co-GLU accepts the benzoxazinoids GDIBOA and GDIMBOA, the cyanogenic glucoside dhurrin, the cytokinin glucoside trans-zeatin-O-glucoside (tZOG), and pNPG as substrates. Hydrolysis of tZOG can be considered as a side reaction being 10 times lower in specific activity compared with the artificial glucoside pNPG (specific activity 61±2 nmol-min⁻¹·mg⁻¹ compared with values of 550±14 nmol-min⁻¹·mg⁻¹ for pNPG, at substrate concentrations of 500 μM). Kₘ values and kₕ values were determined for GDIBOA, GDIMBOA, and dhurrin (Table 2; see Supplemental Figure 6B online). A clear preference for the substrates GDIBOA and dhurrin was found. GDIBOA and dhurrin have similar catalytic values, while GDIMBOA is significantly less efficiently cleaved having a Kₘ value ~6 times higher and a kₕ value 8 times lower compared with GDIBOA (Table 2). The Kₘ values for GDIBOA (5.2 mM) and dhurrin (4.8 mM) hydrolysis were determined with C. orientalis crude extract and fit with the data from the heterologously expressed enzymes (see Supplemental Figure 7 online). In the grasses, GDIMBOA is the most efficiently hydrolyzed benzoxazinoid (Babcock and Esen, 1994; Sue et al., 2006); only in rye are the catalytic data for GDIBOA similar to the values for GDIMBOA (Nikus et al., 2003; Sue et al., 2006). Hence, the benzoxazinoid β-GLU functions of Co-GLU and monocot BxGLUs are distinct. Further substantial differences were displayed with respect to the enzyme activity toward dhurrin. While the maize enzyme does not hydrolyze dhurrin but GDIMBOA hydrolysis is inhibited by the cyanogenic glucoside, Co-GLU hydrolyzes dhurrin efficiently (Table 2), and the presence of dhurrin inhibits GDIBOA hydrolysis in the assay with heterologously expressed Co-GLU and in the crude extract (see Supplemental Figure 10 online).
DISCUSSION

Plants use chemical defense as a major barrier to protect against herbivores and microbial pathogens. The large chemical and structural diversity of the reactive natural products produced by the plant kingdom assures that the development of generalists is restricted. On a genomic level, the biosynthetic potential of plants is reflected by the expansion of genes encoding enzymes functioning in secondary metabolic pathways, such as oxygenases, methyltransferases, UGTs, etc., into large gene families. The proliferation of the P450 monooxygenase gene family in plants dates to 450 million years ago, when the first vascular plants, mosses, colonized land (Gonzalez and Nebert, 1990; Nelson, 2006). In the case of UGTs, vascular plant speciation was accompanied by an increase in the number of ancestral genes and a remarkable expansion occurring in a lineage-specific manner (Yonekura-Sakakibara and Hanada, 2011). Here, we were interested in the evolution of the UGTs and β-GLUs that are required for stabilization and activation of the benzoxazinoids.

The UGT Co-BX8 and the β-GLU Co-GLU catalyze the ultimate steps of benzoxazinoid metabolism in C. orientalis. The data from protein purification and transcriptome analysis identified CoBX8 as the UDPG:DIBOA-glucosyltransferase present in shoots. The analysis of Co-BX8 in transgenic Arabidopsis demonstrated in planta the potential of the enzyme to glucosylate benzoxazinoids. Evidence for Co-GLU representing a BxGLU was revealed by the ability to hydrolyze GDIBOA in vitro and its glucosylation in planta was determined for the protein extracts (von Rad et al., 2001). Here, we cannot exclude by genetic analysis that additional genes with DIBOAGlucosylation or DIBOA-hydrolyzing function exist in C. orientalis. However, since the $K_m$ values determined for the protein extracts and heterologously expressed proteins correspond and the genes are highly expressed in benzoxazinoid-rich tissue, Co-BX8 and Co-GLU are concluded to be UGT and β-GLU enzymes catalyzing in planta the respective steps in benzoxazinoid metabolism.

Catalytic data for BxGLUs have been determined for maize (Cicik et al., 2000), wheat (Sue et al., 2006), and rye (Nikus et al., 2003; Gruen et al., 2005; Frey et al., 2009; Sue et al., 2011; this article), but the analysis of the first gene of the Bx1 family in grasses and benzoxazinoid-producing dicots revealed an evolution by repeated independent duplications of the TSA gene (Schuellehner et al., 2008). For the evolution of sporadically occurring biosynthetic pathways, further data are available for pyrrolizidine alkaloids (Reimann et al., 2004). Like in benzoxazinoid biosynthesis, the branchpoint enzyme homospermidine synthase evolved by repeated independent recruitment of nonorthologous members of the gene families.

Benzenzoazinoid biosynthesis is monophyletic in the grasses (Nomura et al., 2003; Grun et al., 2005; Frey et al., 2009; Sue et al., 2011; this article), but the analysis of the first gene of the pathway, Bx1, in grasses and benzoxazinoid-producing dicots revealed an evolution by repeated independent duplications of the TSA gene (Schuellehner et al., 2008). For the evolution of sporadically occurring biosynthetic pathways, further data are available for pyrrolizidine alkaloids (Reimann et al., 2004). Like in benzoxazinoid biosynthesis, the branchpoint enzyme homospermidine synthase evolved by repeated independent recruitment of a primary function, deoxyhypusine synthase. The analysis of widespread plant secondary pathways, such as flavonoid and cyanogenic glucoside biosynthesis, demonstrated recruitment of genes from the same gene family for the respective enzymatic steps in the pathways, such as the CYP75 flavonoid hydroxylases in angiosperm flavonoid biosynthesis (Nelson and Werck-Reichhart, 2011), CYP79 and CYP71E monooxygenases (Jorgensen et al., 2011), and UGT85 glucosyltransferases in cyanogenic glucoside biosynthesis (Jones et al., 1999; Franks et al., 2008; Kannangara et al., 2011). However, the recruited genes are not necessarily orthologous.

### Table 1. Steady State Constants of the UDP-Glucosyltransferases Co-BX8, Zm-BX8, and Zm-BX9

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (μM⁻¹ s⁻¹)</th>
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<tbody>
<tr>
<td>UDPG</td>
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<tr>
<td>DIBOA</td>
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<tr>
<td>DIMBOA</td>
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<tr>
<td>Co-BX8</td>
<td>743 ± 77</td>
<td>8.5 ± 0.2</td>
<td>11.4</td>
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<tr>
<td>Zm-BX8a</td>
<td>81</td>
<td>22.6</td>
<td>280</td>
</tr>
<tr>
<td>Zm-BX9a</td>
<td>96</td>
<td>22.6</td>
<td>117</td>
</tr>
</tbody>
</table>

*aThe data for Zm-BX8 and Zm-BX9 are from von Rad et al. (2001).

### Table 2. Steady State Constants of Heterologously Expressed Co-GLU with the Substrates GDIBOA, GDIMBOA, and Dhurrin

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (μM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDIBOA</td>
<td>4.6 ± 0.4</td>
<td>32.0 ± 1.55</td>
<td>7.00</td>
</tr>
<tr>
<td>GDIMBOA</td>
<td>28.4 ± 4.4</td>
<td>4.2 ± 0.5</td>
<td>0.15</td>
</tr>
<tr>
<td>Dhurrin</td>
<td>4.8 ± 1.0</td>
<td>52 ± 6.4</td>
<td>10.80</td>
</tr>
</tbody>
</table>
as shown recently for cyanogenic glucoside biosynthesis by Takos et al. (2011). It has been suggested that repeated recruit-
ment might represent the majority of convergent evolution events in plant secondary metabolism (Cseke et al., 1998; Pichersky and
Lewinsohn, 2011; Takos et al., 2011), and the elucidation of
cyanogenesis in insects revealed convergent evolution even
across the plant and animal kingdoms by recruitment of mem-
ers of the same gene functions (Jensen et al., 2011). Although
the encoded enzymes, two multifunctional P450 monoxygen-
ases, and one UGT in plant and insect use identical substrates to
synthesize the same pathway intermediates, all three genes evolved independently. To investigate the evolutionary origin of
the UGTs and β-GLUs of benzoxazinoid biosynthesis, we
reconstructed phylogenies using representative amino acid se-
quences and rigorous statistical evaluation of the results (Figures
4 and 5). In principle, the number of characters in the alignment
limits the resolution of any gene tree. In our trees, this is reflected
by high bootstrap support for most of the nodes but poor
bootstrap values for the most ancient groupings. However,
comparisons of these topologies with trees obtained by an
alternative tree reconstruction method (Bayesian approach)
resulted in good overall agreement. The C. orientalis F3GT is
found together with the respective monocot and dicot enzymes in
the group F branch of UGT in a position that reflects the
phylogeny of the Ranunculaceae (Figure 4). Like UGTs of flavo-
noid biosynthesis, UGTs of cyanogenic glucoside pathways have
been recruited from the same UGT group. By contrast,
benzoxazinoid-specific UGTs belong to different groups, UGT85
and UGT710. The analysis locates the BxGLUs in two distinct
clades of the β-GLU tree; therefore, our phylogenetic data
indicate independent recruitment of stabilization and activation
function from homologous but clearly not orthologous genes in
the dicot C. orientalis compared with the monocot lineage.

Substrate specificity of UGTs can be relaxed, and enzymatic versa-
tility of UGT family members enables glucosylation of broad
spectra of endogenous and nonendogenous substrates (Jones et al., 1999; Schwab 2003). For example, linalool produced by
heterologous expression of S-linalool synthase in petunia (Petu-
nia hybrida) was readily glucosylated by an endogenous gluco-
yltransferase (Lücker et al., 2001). The sensitivity of Arabidopsis
to exogenous benzoxazinoids indicates that there is no universal
efficient protection by plant UGTs against this nonendogenous
substrate. Similarly, no Arabidopsis UGT was capable to convert
p-hydroxy-mandelonitrile into dhurrin (Tattersall et al., 2001).
Hence, distinct secondary metabolites might require specific
UGTs to be glucosylated. Our results for UGTs involved in
benzoxazinoid biosynthesis support the notion that these
UGTs independently acquired their ability to recognize the spe-
cific substrates after the expansion that occurred in vascular
plant lineages (Yonekura-Sakakibara and Hanada, 2011). The
BX8 UGTs of maize and C. orientalis have narrow substrate
spectra; activity is restricted to benzoxazinoids, a rather unique
class of metabolites (see Supplemental Table 3 online). However,
within the benzoxazinoids, maize and C. orientalis BX8 functions
discriminate differentially with respect to modifications of the
benzoxazinoid basic structure (see Supplemental Table 3 and
Supplemental Figure 8 online). C. orientalis BX8 has optimal
catalytic properties for DIBOA, the principal benzoxazinoid-
aglucone in this plant. Zm-BX8, on the other hand, does not
differentiate between DIBOA and DIMBOA, although DIBOA is
glucosylated prior to the modifications at C-7 to yield DIMBOA
(Figure 1). The difference in specificity may reflect intrinsic
enzymatic properties of the distinct progenitor UGTs or may
have evolved in maize as a consequence of the demand to
detoxify exogenous DIMBOA exuded by neighboring plants.
The closest characterized relatives of the monocot and dicot BX8
functions have substrate specificities ranging from cytokinin to
steviol glycosides (Figure 4). It can be speculated that the
progenitor enzymes of benzoxazinoid biosynthesis and cytokinin
homeostasis had activity toward both classes of substrates.
However, the fraction of UGT sequences with biochemically
defined function is relatively small and thus biases the phyloge-
etic analysis. There is no experimental evidence for an overlap
of substrates of UGTs of benzoxazinoid biosynthesis and cyto-
inin metabolism.

As discussed above, the phylogenetic analysis demonstra-
respective progenitors of the BxGLUs are not orthologous (Figure 5).
Similar to the finding for the UGTs of the pathway, the specificity of the BxGLUs reflects the
predominant benzoxazinoid present in a particular plant spe-
cies, and specificity probably has been selected during evo-
lution of the genes. GDIMBOA is the main benzoxazinoid and
the preferred substrate for the maize and wheat enzymes
(Babcock and Esen, 1994; Sue et al., 2006). In S. cereale, both
GDIBOA and GDIMBOA are prevalent, and Sc-GLU uses both
these substrates with similar efficiencies (Nikus et al., 2003;
Sue et al., 2006), while for Co-GLU, only GDIBOA is a suitable
substrate.

**Promiscuity of Monocot and Dicot BxGLUs**

The substrate specificity of plant β-GLUs ranges from highly
selective to broadly general substrate spectra (Morant et al.,
2008b). BxGLUs are promiscuous enzymes. According to cat-
ylytic data, the maize, wheat, and C. orientalis β-GLUs each
each a second substrate as efficiently as the benzoxazinoid,
the cytokinin izOG (Campos et al., 1992; Brzobohaty et al.,
1993), the isoflavonoid genistein glucoside (Sue et al., 2000),
and dhurrin (this work), respectively. The relevance of these activities
on dhurrin and genistein in C. orientalis and wheat is unclear
since the compounds have not been detected in these species. A
function in cytokinin homeostasis, as proposed for the maize
enzyme, can be excluded in C. orientalis since Co-GLU has only
minor activity on izOG. Remarkably, the cyanogenic glucoside
dhurrin represents an inhibitor for Zm-GLU but functions as a
substrate for Co-GLU. Similarly, β-GLUs of cyanogenesis also
show notable second activities. The enzyme mixture of the
purified β-GLUs Lj-BGD2 and Lj-BGD4 that are involved in
activation of cyanogenic and noncyanogenic hydroxynitril glu-
cosides in Lotus japonicas (Takos et al., 2010) hydrolyze the
isoflavonoid-glucoside daidzin (Morant et al., 2008a), although
isoflavonoids are not natural substrates for these enzymes.
Based on phylogenetic analysis and the promiscuity of the
β-GLUs, it has been hypothesized that isoflavonoid glucoside-
cleaving enzymes have evolved from cyanogenic glucoside
activating β-GLUs (Chuankhayan et al., 2007; Morant et al.,
2007).
2008a). Parallels exist for benzoxazinoids: Cyanogenic glucosides and benzoxazinoids are found in grasses and in Ranunculaceae (Shariples et al., 1972; Vetter, 2000). Our phylogeny (Figure 5) suggests that the BxGLUs and an enzyme involved in cyanogenic glucoside cleavage (Sb-DHR or Va-VH) have a close common ancestor. However, since repeated independent recruitment has been shown for cyanogenic glucoside biosynthesis (Takos et al., 2011), this may reflect promiscuity of β-GLUs rather than a direct origin of secondary metabolite β-GLUs from functions of cyanogenic glucoside biosynthesis. The fact that the phylogeny of grass β-GLUs in clade 3 (Figure 5) mirrors the plant phylogeny independent of specific enzyme function underlines the notion that an enzyme with broad substrate specificity might have been recruited and evolved toward β-GLUs with specificities for such diverse substance classes as benzoxazinoids, cyanogenic glucosides, and saponins.

**Required Settings at the Advent of Evolution of Constitutive Defense Compounds**

The evolution of a pathway leading to the accumulation of an autotoxic metabolite seems only realizable in the presence of a suitable stabilization function, as it would be otherwise deleterious for the organism. This idea is reminiscent of the original concept of retrograde evolution of a biosynthetic pathway that was developed by Horowitz (1945). Stabilization by glucosylation can be considered the endpoint in the biosynthesis of a preformed defense compound. In order to become fixed in evolution, expression of a pathway should confer a selective advantage to the plant. In this respect, the β-GLU function that activates the stabilized compound in response to pathogens and herbivore attack is equally important. Such a selective advantage has been shown by the transgenic expression of Sb-DHR from sorghum in barley. Barley normally synthesizes the Leu-derived cyanogenic glucoside epis heterodendrin but lacks an activating β-GLU (Nielsen et al., 2002). Transgenic expression of Sb-DHR established cyanogenesis and increased the resistance of the plant against the pathogen Blumeria graminis (Nielsen et al., 2006).

Mechanisms for the evolution of metabolic pathways have been a matter of debate for a long time. Jensen (1976) proposed proliferation of pathways based on the substrate ambiguity of enzymes. Promiscuous functions are latent and not under selection pressure. Experimental data (e.g., Aharoni et al., 2005) demonstrated the possibility for generation of a new enzyme activity based on promiscuity. Interestingly, additional enzyme function is gained without loss of the original activity, demonstrating a plasticity that can serve as a starting point in evolution. Substrate ambiguity of UGTs and β-GLUs may provide an easily accessible starting point for the establishment of suitable stabilization and activation functions and hence enable repeated evolution of benzoxazinoid biosynthesis. Subsequently, gene duplication and functionalization can deliver more specific enzymes.

Is the finding that the enzymes for stabilization and activation of benzoxazinoids have been recruited independently in the grasses and in C. orientalis an indication of convergent evolution of the pathway? Two scenarios are conceivable: convergent evolution of the whole pathway in these taxa or monophyletic establishment followed by loss of individual functions and consecutive secondary recruitment of suitable functions from their respective gene pools. Repeated recruitment of the branchpoint enzyme BX1 of the pathway has been shown (Schullehner et al., 2008). Hence, the module that consists of the modifying P450 enzymes remains the only part of the pathway that might be of monophyletic origin. At present, members of the P450 Cyp71C subfamily to which Bx2 to Bx5 belong have not been detected in the genomes of dicot plants (cytochrome p450 homepage D.R. Nelson, http://drnelson.uthsc.edu/CytochromeP450.html). This finding suggests that the P450 enzymes required in DIBOA biosynthesis and, thus, the complete pathway evolved by repeated evolution in the dicot and grass lineages. Final proof, however, requires isolation of the respective P450 genes from C. orientalis. In any case, the independent origin of the enzymes for the branchpoint reaction, for stabilization, and for activation in the grasses and in C. orientalis indicates a great evolutionary flexibility in recruitment of these essential functions for secondary plant metabolism.

**METHODS**

**Standards and Reference Chemicals**

Benzoxazinoids were gifts from Dieter Sicker (University of Leipzig, Germany) or prepared as described by von Rad et al. (2001). Trans-zeatin and zeatin-riboside were purchased from Duchefa Biochemie, trans-zeatin-O-glucoside from OIChemim, and pNPG from Sigma-Aldrich.

**Plant Materials and Growth Conditions**

Plants were grown at 19°C at a 12-h-light/12-h-dark day cycle. Larkspur (Consolida orientalis) seed was provided by Margot Schulz (Institut für Molekulare Physiologie und Biotechnologie der Pflanzen, University Bonn, Germany). Seeds were vernalized at 4°C in soil. Arabidopsis thaliana seed, ecotype Columbia-0, was vernalized at 4°C and grown on half-strength Murashige and Skoog solidified medium or on soil. For selection, the medium was supplemented with 25 mg/L 3,4-phosphonitricine (Duchefa Biochemie). Nicotiana benthamiana was grown on soil and used for infiltration 5 to 6 weeks after germination.

**Generation of Transgenic Plants**

Arabidopsis was transformed by floral dip and selected as described by von Rad et al. (2001). Transient transgenic expression of β-GLU in N. benthamiana was as described by Marillonnet et al. (2005) with vectors described by Engler et al. (2008).

**Molecular Biology Methods**

DNA and RNA isolation, cDNA synthesis, cloning, and PCR amplification were as described by Schullehner et al. (2008). To determine the C. orientalis transcriptome, RNA of seedling shoots was prepared and first-strand cDNA was synthesized using degenerate primers. After normalization and ligation of adaptor sequences (Vertis Biotechnology), the data were collected by 454 sequencing (Eurofins MWG Operon). The sequence reads were assembled using Phred software (Ewing et al., 1998; Ewing and Green, 1998). The transcriptome average sequence size was 340 bp. A.C. orientalis cDNA library (young shoot; Schullehner et al., 2008) was screened for full-size transcripts of the respective gene. Probes for screening were partial gene sequences amplified by PCR using the sequence information from the transcriptome.
Isolation of His-tagged proteins and enzyme assays were as described by von Rad et al. (2001).
A list of primers used for cloning and expression analysis is given in Supplemental Table 5 online.

**Enzyme Purification**

Co-BX8 purification was essentially as described by von Rad et al. (2001) using 7- to 14-d-old leaves (see Supplemental Table 1 online). After ammonium sulfate precipitation (45 to 65% saturation), the protein was dissolved in 20 mM HEPES, 14 mM 2-mercaptoethanol, 0.5 mM EDTA, and 20% glycerol, pH 7.5 (buffer R), and the ammonium sulfate concentration was adjusted to 1 M. The protein solution was loaded to a HiTrap Butyl FF column (2.5 × 16 cm, 5 mL; GE Healthcare). Elution was with a linear gradient of 25 column volumes at 0.5 mL/min to reach 100% buffer R. Fractions with Co-BX8 activity were pooled, and KCl was added to a final concentration of 0.1 M. The protein solution was applied to a sizing column (HiLoad 16/60 Superdex 200 prep grade, 60 final concentration of 0.1 M. The protein solution was applied to a sizing linear gradient of 25 column volumes at 0.5 mL/min to reach 100% buffer was used, and of indole-3-acetic acid glucosylation, the protocol of Jackson et al. (2001) delphinidin was assayed as described by Griesser et al. (2008). For analysis regression (see Supplemental Figure 6A online).

**Determination of Catalytic Properties**

Glucosylation of benzoxazinoids was assayed as described by von Rad et al. (2001). UDPG was used as glucose donor with either benzoxazinoid as substrate. For determination of enzymatic parameters with DIBOA and DIMBOA as acceptor substrates, 2 mM UDPG was used and the aglucone concentration varied from 0.175 to 21 mM and 0.175 to 10.5 mM, respectively. Determination was in three replicates. Enzyme parameters were determined using GraphPad Prism Version 4.03 with nonlinear regression (see Supplemental Figure 6A online). Plant protein extracts were prepared as described by Marillonnet et al. (2005). Protein crude extracts used for the determination of $K_m$ values were passed through gel filtration columns (Illustra NAP-10/25 columns; GE Healthcare) to remove endogenous substrates and products. Boiled protein extracts were used as negative controls.

**Phylogenetic Analysis**

The initial files containing manually selected UGT and β-GLU protein sequences were aligned using the multiple alignment program MUSCLE 3.8.31 (Edgar, 2004). The alignments were manually trimmed to remove poorly conserved terminal regions and signal peptides (see Supplemental Data Sets 1 and 2 online). Phylogenetic trees were calculated using the program RAxML 7.0.4 (Stamatakis, 2006) using the PROTGAMMAJTT model, fast bootstrap mode (-f a option), and 1000 bootstrap samples. As the comparison of the derived topologies with trees generated by an alternative method (Bayesian approach) did not result in major differences, we used the RAxML trees for further analysis. The trees were visualized and colored by organism (monocots in green and dicots in red) using the iTOL software (Letunic and Bork, 2007). Internal nodes found in more than 500 of the bootstrap samples were visualized by circles, which were scaled according to their bootstrap values.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: HM559229 (Co-Bx8, UGT85N1), HM559224 (Co-F3GT, UGT78B3), HM559224 (Lg-F3GT, UGT78U1), HM559230 (Ta-Bx8, cv Kanzler UGT7710E8), HM559225 (Co-Glu), and HM559226 (C. orientalis β-Glu with unknown function).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Purification of Co-BX8.

**Supplemental Figure 2.** Amino Acid Sequence of Co-BX8.

**Supplemental Figure 3.** Heterologous Expression of Proteins by Magnification.

**Supplemental Figure 4.** Gene Expression Pattern of Candidate β-Glucosidases.

**Supplemental Figure 5.** Substrate Specificity of Co-F3GT and Lg-F3GT and Michaelis-Menten Kinetics of Ta-BX8.

**Supplemental Figure 6.** Michaelis Menten Kinetics of Co-BX8 and Co-GLU.

**Supplemental Figure 7.** Kinetics of DIBOA Glucosylation, GDIBOA, and Dhurrin Hydrolysis in C. orientalis Protein Crude Extract.

**Supplemental Figure 8.** Substrates Analyzed for Glucosylation by Co-BX8.

**Supplemental Figure 9.** Substrates Analyzed for Hydrolysis by Co-BX8.

**Supplemental Figure 10.** Kinetics of GDIBOA Cleavage in the Presence of Dhurrin.

**Supplemental Table 1.** Purification of Co-BX8 from C. orientalis.

**Supplemental Table 2.** Transcriptome Statistics.

**Supplemental Table 3.** Substrate Specificity of Co-BX8, Zm-BX8, and Zm-BX9.

**Supplemental Table 4.** Determination of Enzyme Specificity of Co-GLU.

**Supplemental Table 5.** Lists of Primers.


Supplemental Data Set 1. Amino Acid Alignment Used to Generate the Phylogenetic Tree of Plant Family 1 UDP-Glucosyltransferases Shown in Figure 4 (Phylip File).

Supplemental Data Set 2. Amino Acid Alignment Used to Generate the Phylogenetic Tree of Plant β-glucosidases Shown in Figure 5 (Phylip File).

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AUTHOR CONTRIBUTIONS

R.D. performed the research. T.R. contributed new computational tools for phylogenetic analysis. M.H. provided the peptide sequence data for phylogenetic analysis. M.F. designed the research and wrote for the manuscript. T.R. contributed new computational tools and application of cytochromes P450. Phytochem. Rev. 15: 1481–1498.

REFERENCES


Ciccek, M., Blanchard, D., Bevan, D.R., and Esen, A. (2000). The glycosyl specificity-determining sites are different in 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-glucosidase (maize beta-glucosidase) and dhurrinase (sorghum beta-glucosidase). J. Biol. Chem. 275: 20002–20011.


Evolution of Benzoazoxinoid Biosynthesis


Comparative Analysis of Benzoxazinoid Biosynthesis in Monocots and Dicots: Independent Recruitment of Stabilization and Activation Functions
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