Elucidation of the Final Reactions of DIMBOA-Glucoside Biosynthesis in Maize: Characterization of Bx6 and Bx7[1][W][OA]

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Benzoxazinoids were identified in the early 1960s as secondary metabolites of the grasses that function as natural pesticides and exhibit allelopathic properties. Benzoxazinoids are synthesized in seedlings and stored as glucosides (glcs); the main aglucone moieties are 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA). The genes of DIBOA-glc biosynthesis have previously been isolated and the enzymatic functions characterized. Here, the enzymes for conversion of DIBOA-glc to DIMBOA-glc are identified. DIBOA-glc is the substrate of the two enzymes BENZOXAZINLESS6 (BX6) and exhibit moderate enzymatic functions. Both enzymes exhibit moderate Km values (below 0.4 mM) and kcat values of 2.10 s⁻¹ and 0.25 s⁻¹, respectively. Although BX6 uses a glucosylated substrate, our localization studies indicate a cytoplasmic localization of the oxygenase. Bx6 and Bx7 are highest expressed in seedling tissue, a feature shared with the other Bx genes. At present, Bx6 and Bx7 have no close relatives among the members of their respective gene families. Bx6 and Bx7 map to the cluster of Bx genes on the short arm of chromosome 4.

Plants produce a remarkably diverse array of over 200,000 low-mass natural products, known as secondary metabolites. This rich diversity results in part from an evolutionary process driven by selection for acquisition of improved chemical defense against microbial attack and herbivore predation. Related plant taxa generally make use of related chemical structures for defense. Benzoxazinoids are abundant in grasses, including the major agricultural crops maize (Zea mays), wheat (Triticum aestivum), and rye (Secale cereale). Outside the Gramineae these secondary metabolites are found dispersed in isolated dicotyledonous species.

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The conversion of DIBOA to DIMBOA requires hydroxylation and methylation at C-7 of DIBOA. A 2-oxoglutarate-dependent dioxygenase (2ODD) was identified by transposon tagging in maize (Frey et al., 2003). Hydroxylation at C-7 was defective in the transposon-induced mutants, and the gene was termed Bx6. Here, we show in vitro that BX6 accepts only DIBOA-glc as substrate producing the hydroxy derivative 2,4,7-trihydroxy-2\(^{-}\)H,1,4-benzoxazin-3-(4\(^{-}\)H)-one-glc (TRIBOA-glc). In addition, an O-methyltransferase (OMT) named BX7 was isolated and characterized. BX7 catalyzes the formation of DIMBOA-glc from TRIBOA-glc. Thus, DIMBOA biosynthesis in maize is now completely described on the gene and enzyme level.

**RESULTS**

**The Substrate of BX6 Is DIBOA-glc**

Recently, it has been shown by reverse genetic analysis that the 2ODD BX6 is responsible for the hydroxylation in position C-7 of the benzoxazinoids in maize (Fig. 1; Frey et al., 2003). We have now determined the biochemical function of BX6 by expression in *Escherichia coli* and analysis of the recombinant protein. Incubation of DIBOA-glc with BX6 protein under standard conditions for 2ODDs (see “Materials and Methods”) led to the formation of a new product. This product has the same retention time in HPLC analysis and the same absorption spectrum as enzymatically produced TRIBOA-glc from chemically synthesized TRIBOA glucosylated by the glucosyltransferase BX8. The product identity was further verified by mass spectrometry (MS; see “Materials and Methods”). The TRIBOA-glc appears predominantly as (M-H + HOAc) adduct ion (mass-to-charge ratio = 418 D) beside (M-H)\(^{-}\) ion (mass-to-charge ratio = 358 D). The adduct ions are formed exclusively with acetic acid, not with isopropanol (same nominal mass), which was demonstrated independently in a second experiment.

DIBOA was not hydroxylated by BX6 (Fig. 2). The result is consistent with the accumulation of DIBOA-glc in Bx6 mutant plants (Fig. 3). A pH optimum of 6 was determined for the DIBOA-glc hydroxylation reaction. The steady-state kinetic constants for the substrates DIBOA-glc and 2-oxoglutarate (Table I) were determined. The reaction is characterized by a high \(k_{cat}\) for both substrates and a significant difference in \(K_m\) values for the two substrates \(K_m\) DIBOA-glc (373 \(\mu\)M) is about 6 times higher than \(K_m\) 2-oxoglutarate (70 \(\mu\)M).

**BX6 Is Localized in the Cytoplasm**

In plants, glucosylation plays a key role in the detoxification of reactive secondary metabolites, with their glucosides often accumulating in the vacuoles. Multi-drug resistance-associated protein (MRP)-type ATP-binding cassette transporters are reported to be involved in the vacuolar sequestration (Rea, 2007). In plants, where DIBOA-glc is the final product of...

![Figure 1](image.png)
benzoxazinoid biosynthesis, this secondary metabolite is stored in the vacuole. Therefore we tested the possibility that the conversion of DIBOA-glc to TRIBOA-glc would occur in this cellular compartment. From in silico analysis, no clear indication of the subcellular location of BX6 by prediction algorithms (PREDOTAR, iSPORT, and TargetP) was obtained. However, signal peptides and the requirements for protein targeting to the vacuole are poorly defined (Carter et al., 2004). These targeting signals can be located either at the amino or the carboxy terminus of the protein. We therefore sandwiched the GFP gene between the amino-terminal half of BX6 and the complete BX6 sequence (see “Materials and Methods”). BX6 is highly expressed in young plants and transcript levels exceed the levels of the housekeeping gene GAPC (Fig. 6). Since the native promoter of BX6 is not yet defined, the 35S promoter, conferring moderate to high transcript levels in monocot plants (Schledzewski and Mendel, 1994), was employed to drive the gene. The chimeric gene BX6part-GFP-BX6 was expressed transiently in maize protoplasts and transformed into maize plants. In transient
and stably transformed cells, GFP was consistently detected in the cytoplasm and there is no indication for a vacuolar localization of BX6 (Fig. 4). Therefore, hydroxylation of DIBOA-glc most likely takes place in the cytosol.

**Isolation and Characterization of Bx7**

TRIBOA-glc was used as substrate to purify the putative OMT that catalyzes the last step in benzoxazinoid biosynthesis of maize. Benzoxazinoid-glc's are not stable in raw protein extracts from maize plantlets under standard OMT conditions. Therefore, affinity chromatography on adenosine agarose was adopted as a first purification step to get rid of unfavorable enzyme activities. Protein fractions eluting during application of a gradient of S-adenosyl-Met (SAM) were tested for the conversion of TRIBOA-glc to DIMBOA-glc. Active fractions were pooled and applied to anion-exchange chromatography. Analysis of individual fractions on SDS-PAGE gels revealed a band with a molecular mass of about 40 kD that correlated with TRIBOA-OMT activity in the enzyme assay (Supplemental Fig. S1). This band was excised and digested following the protocol of Scha¨fer et al. (2001). The sequence of several fragments of the trypsin digest was determined by mass spectroscopy (Supplemental Fig. S2). The deduced peptide sequences were compared with a set of predicted maize OMT sequences. The genes were selected from the Pioneer Hi-Bred EST collection by the criterion of high expression in seedling tissue. One gene demonstrated significant similarity to the deduced peptides, especially in a unique sequence stretch of 14 amino acids at the amino terminus. The corresponding peptide is not present in other OMT sequences; the other peptides are located in more conserved regions of the protein and are less indicative.

cDNA was isolated for the candidate gene and expressed in *E. coli* as a His-tagged protein. Subsequently, trypsin-digested purified protein was subjected to matrix-assisted laser-desorption ionization (MALDI)-MS analysis. The generated peptide pattern was congruent with the pattern of the protein isolated from maize (Supplemental Fig. S2B). The recombinant enzyme was tested in vitro for the methylation of different substrates. The only substance that served as a substrate was TRIBOA-glc, neither the aglucone TRIBOA nor well-known substrates of plant OMTs (Table I) were efficiently converted by the enzyme. In case of quercetin, a minor activity was detected (just above detection level). The reaction was independent of Mg²⁺. The steady-state kinetic constants of BX7 for the substrate TRIBOA-glc are in a reasonable range (Table I); *V*ₘₐₓ values of 0.45 μkat g⁻¹ (Christensen et al., 1998) to about 80 μkat g⁻¹ (Gauthier et al., 1998) have been reported for plant OMTs. The *K*ₘ of the isolated BX7 is in the higher range, but similar values were determined.

**Figure 4.** Subcellular localization of BX6. The fusion protein BX6part-GFP-BX6 is located in the cytoplasm of the cell. No GFP signal is detected in the vacuole. A and C, Control 2 × 35S promoter-GFP. B and D, BX6part-GFP-BX6 fusion. A and B, Transient expression. C and B, Transgenic plants. The bar equals 10 μm.
Phylogenetic Relationship of Bx7 in the OMT Gene Family

Plant OMTs have been categorized into two classes, based primarily on protein sequences (Joshi and Chiang, 1998). According to the occurrence and spacing of conserved amino acid sequence motifs, BX7 belongs to class II OMTs. The most comprehensively studied representatives of this class are caffeic acid OMTs (COMTs), which are involved in the biosynthesis of lignin, the preferred substrates being caffeoyl aldehyde and 5-hydroxyconiferaldehyde (Osakabe et al., 1999; Parvathi et al., 2001). Other class II OMTs catalyze the methylation of flavonoids and phenolics. In the phylogenetic network, Bx7 is joined to a web that connects OMTs of berberine alkaloid, isoflavone, and phenylpropene biosynthesis (Fig. 5). BX7 has no catalytic activity toward phenylpropanoids and flavonoids (Table I). Especially, apigenin, the substrate of a flavonoid-7-OMT from barley, is no substrate, although this gene is the closest relative in phylogenetic analysis (Fig. 5). BX7 shares the origin in the net with this OMT from barley and the maize OMT ZRP4. It has been proposed that ZRP4 is involved in suberin biosynthesis (Held et al., 1993); however, no experimental evidence is available to our knowledge. The common root may simply reflect the fact that three enzymes of grasses are compared and does not indicate related functions.

A comprehensive survey of TUSC, the Pioneer reverse genetic resource (Benson et al., 1995), and public collections of maize mutants did not result in the identification of a Bx7 mutant. Hence, the genetic proof that Bx7 is not only capable to perform the last step in DIMBOA-glc biosynthesis but is the only enzyme in maize that catalyzes this step is not yet given.

Bx6 and Bx7 Are Predominantly Expressed in the Maize Seedling

The benzoxazinoid content is highest in the young maize plant and correlating with this distribution, major amounts of Bx1 to Bx5 transcripts are present in seedling tissue (von Rad et al., 2001; Frey et al., 2003). The same expression pattern is displayed for the 2ODD BX6 and the methyltransferase BX7 (Fig. 6). Peak levels of transcripts are detected in 3- and 4-d-old plantlets. Highest transcriptional expression levels of Bx6 and Bx7 are displayed in the scutellar node. In this tissue, the amount of mRNA encoding the housekeeping gene GAP C is exceeded by each of the Bx gene transcripts. Mature organs like leaf and husk of 10-week-old plants, cob, and tassel show low to nondetectable levels of the Bx gene transcripts. While primary and adventitious roots of plants 3 weeks after imbibition display low transcript levels, the newly formed crown roots of 10-week-old plants reach almost the Bx gene transcript levels present in seedling roots.

All DIMBOA-glc Biosynthetic Genes Are Located on the Short Arm of Chromosome 4

It was previously shown that the Bx genes are clustered at the short arm of chromosome 4 (Frey et al., 1997; von Rad et al., 2001; Frey et al., 2003). The set of recombinant inbred lines based on the cross CM37xT232 (Burr and Burr, 1991) was used for mapping of the Bx7 gene employing a PCR polymorphism. Bx7 is located 9 cM distal to the marker adh2 at position 47 (Fig. 7); the complete set of DIMBOA-glc biosynthetic genes is found within 41 cM. Linkage of Bx7 to the 6-cM cluster of Bx1 to Bx5, Bx6, and Bx8 genes, however, is loose. Two other OMTs, Zrp4 and the maize COMT1 Brown midrip3 (Bm3), were located in the same chromosomal region.

DISCUSSION

All Genes of Benzoxazinone Biosynthesis in Maize Are Isolated

The isolation of Bx6 and Bx7 completes the characterization of benzoxazinoid biosynthesis. The pathway displays the typical features of plant secondary metabolic biosynthesis: The branch point from the primary metabolism is created by gene duplication, in this case of the T5A (Trp synthase α) gene, and subsequent modification of the duplicated gene to Bx1 (Frey et al., 1997). The same sequence of events is reported for the branch point reaction of pyrrolizidine alkaloids and saponins (Ober and Hartmann, 1999; Qi et al., 2004). A set of enzymatic functions commonly found in secondary metabolic pathways, namely, cytochrome P450s (BX2–BX5), a 2ODD (BX6), and an OMT (BX7), are recruited for functionalization of the primary product. The enzymes employed in the pathway are quite specific: It has been shown that the P450 enzymes are substrate specific and produce only one oxygenation product (Glawischnig et al., 1999). Substrate specificity was also observed for the last enzymes in the pathway, the 2ODD BX6 and the OMT BX7. Both enzymes accept exclusively the glcs as substrates. BX6 and BX7 do not have a close relative among the characterized members of the respective gene family (Fig. 5; Frey et al., 2003).

The 2-Oxoglutarate-Dependent Dioxygenase BX6 and OMT BX7 Require a Glucosylated Substrate

2ODDs are well characterized in flavonoid biosynthesis (flavanon 3β-hydroxylase, flavonol synthase, anthocyanidin synthase); the biosynthesis of GAs (e.g. GA 7- and GA 20-oxidase), ethylene (1-aminoacyclopropane-1-carboxylate oxidase), and abscisic acid (9-cis-epoxy-carotenoid dioxygenase); and in alkaloid biosynthesis. The pathway-specific 2ODDs can have overlapping
substrate and product spectra (e.g. flavonoid biosynthesis; Turnbull et al., 2004) and may catalyze successive reactions of a pathway (e.g. hydroxylation and epoxidation of hyoscyamine to scopolamine by H6H in Anisodus tanguticus; Liu et al., 2005). Other ODDs use only defined substrates such as the flavanol-6-hydroxylase of Chrysosplenium americanum (Anzellotti and Ibrahim, 2000, 2004) that requires a defined methylation pattern of the substrate. The 2ODD BX6 of benzoxazinone biosynthesis, originally defined by the analysis of insertion mutants (Frey et al., 2003), similarly exhibits substrate specificity: The only benzoxazinoid that is accepted by the enzyme is DIBOA-glc. The reaction is characterized by a $V_{\text{max}}$ of 59 $\mu$kat g$^{-1}$ that is in the higher range determined for 2ODDs (e.g. petunia [Petunia hybrida] flavan 3β-hydroxylase, substrate naringenin, 31.8 $\mu$kat g$^{-1}$; Wellmann et al., 2004) and a relatively high $K_{m}$ of 373 $\mu$M for DIBOA-glc. Since benzoxazinoids in maize seedlings reach concentrations of 40 mM (data not shown), the natural milieu contains probably sufficient substrate concentrations for BX6.

OMTs have been originally categorized as promiscuous enzymes that methylate phenylpropanoid and alkaloid compounds (Frick and Kutchan, 1999). Progress in cloning of OMTs and the advent of first crystal structures (Zubieta et al., 2001, 2002; Gang et al., 2002) revealed that OMTs may have subtle structure-function relationships and are substrate specific. Recently, the evolution of defined substrate requirements was demonstrated for compounds in the scent of V. planifolia (Li et al., 2006). BX7 may represent an OMT with a narrow substrate spectrum. TRIBOA-glc is the only known substrate that is metabolized, the aglucone is not accepted. Several common substrates of OMTs were assayed but no methylation was observed. Especially, apigenin, the substrate of the flavonoid-7-methyltransferase isolated from barley (Christensen et al., 1998), is not metabolized; flavonoid-7-methyltransferase is the closest known relative to BX7 with a defined substrate requirement.

In conclusion, BX7 and BX6 represent enzymes that perform precisely one catalytic step each in the benzoxazinoid biosynthetic pathway. Both enzymes have $K_{m}$ values at the upper level found for the enzyme class combined with a sound $V_{\text{max}}$.

Secondary metabolites are often stored in the vacuole. Glycosylation is discussed as an essential feature for transport across the tonoplast. In maize and petunia, cyanidine 3-glc is transported to the tonoplast by a carrier protein (BZ2 and AN9, respectively) and delivered to a multidrug resistance-like protein in the vacuolar membrane (Goodman et al., 2004). However, glycosylation is not necessarily the final step of the

Figure 5. Phylogenetic net of plant OMTs. Enzymes with defined substrates were subjected to the analysis using the program TCOFFEE for the alignment (Poirot et al., 2003), and the evolutionary network was computed with SplitsTree (Huson and Bryant, 2006). COMTs from different species form a distinct relation group; a second group comprises enzymes (SAGAMT, JAGAMT, IAMT, and BAMT) responsible for methylation of plant hormones (salicylic acid, jasmonic acid). The third cluster includes loosely related OMTs involved in methylation of intermediates of different secondary metabolite pathways. BX7 is linked to this group.
biosynthetic pathway. Examples for glucosylated intermediates are given in the glucosinolate biosynthesis of Arabidopsis (Arabidopsis thaliana; Grubb et al., 2004), in loganin biosynthesis of Lonicera japonica (Katano et al., 2001), and in aurone biosynthesis of Antirrhinum majus (Nakayama et al., 2000). These pathways include a sulfotransferase, a cytochrome P450 enzyme, and a polyphenol oxidase, respectively, that modifies the glucosylated substrate. To our knowledge there are no reports of plant 2ODDs and class II OMTs that are specific for a glucosylated substrate. At first glance, one would expect the biosynthetic pathway of benzoxazinoid biosynthesis to proceed through the final modification step to DIMBOA and to conclude with glucosylation. However, DIBOA is the first toxic intermediate of the pathway (see below). Reduction of its reactivity by glucosylation might be required to reduce autotoxicity and to provide a stable metabolite for further modifications.

Modification of glcs may be catalyzed by enzymes with vacuolar location, e.g. the aureusidin synthase AmAS1 of A. majus is located in the vacuole (Ono et al., 2006). However, cytoplasmic localization of BX6 was substantiated by the BX6-GFP fusion analysis. According to our findings, both enzymes, BX6 and BX7, display no characteristics of vacuolar proteins: No signal peptides are detectable and the pH optima are not shifted to the acidic range of vacuoles. Hence, it is inferred that DIBOA-glc is converted to DIMBOA-glc prior to translocation to the vacuole.

Evolution of DIMBOA-glc Biosynthesis

Upon cell damage, the benzoxazinone glc is released from the vacuole that is used as a storage compartment.

The toxic aglucone is produced by a specific glucosidase (Esen, 1992). The toxicity depends largely on the reactivity of both the N-OH function and the presence of a cyclic hemiacetal unit. The hemiacetal undergoes an oxocyclo tautomerization. It has been shown that the aldehyde group of the oxo form reacts with the α-NH2 group of N-α-acetyl-Lys, a model substrate for Lys residues in proteins (Perez and Niemeyer, 1989).

DIBOA and DIMBOA function as enzyme inhibitor of, for example, a-chymotrypsin (Cuevas et al., 1990), aphid cholinesterase (Cuevas and Niemeyer, 1993), and plasma membrane H+-ATPase (Friebe et al., 1997). DIMBOA biosynthesis appears as the core pathway for benzoxazinoids. In some species (e.g. maize and wheat), DIBOA is further modified to yield DIMBOA. DIMBOA provides the plant with additional functions. It is a distinctively more reactive compound than DIBOA. As a donor, the 7-MeO group facilitates N-O bond heterolysis (Hashimoto and Shudo, 1996) and the dehydration of DIMBOA (Hofmann and Sicker, 1999). The first process leads to the formation of a reactive, multicentered cationic electrophile; the latter results in the generation of a reactive formyl donor toward -NH2, -OH, and -SH groups. Hence, biosynthesis of DIMBOA instead of DIBOA seems to be of evolutionary advantage for the plant by producing a more reactive chemical defense.

In plants, genes associated with common metabolic pathways are generally unlinked. Remarkably, all six genes of the core biosynthesis in maize, including a...
lyase, P450s, and a glucosyltransferase, and the ODD Bx6 are linked within 6 cM on the short arm of chromosome 4 (Fig. 7). The OMT Bx7 is more loosely associated but also located on the short arm of chromosome 4. In wheat, the genes of the core biosynthesis are found on two chromosomes and it was proposed that clustering was similar to maize in the original state of a putative a wheat progenitor (Nomura et al., 2003). One other example for genetic linkage of defense pathway-related genes is reported in oats (Avena sativa). Five genes of saponin biosynthesis, including a gene for a glycosylating enzyme, map within 3.6 cM in Avena (Qi et al., 2004). It has been suggested that clustering has the potential to facilitate coordinate regulation of expression at the chromatin level (Qi et al., 2004). Additionally, we suggest that clustering of the genes is of selective advantage in a population once beneficial allelic combinations are established in coupling phase.

There is ample evidence that the core benzoxazinoid biosynthesis is of monophyletic origin in grasses. It has been shown for wheat that a (not-yet cloned) 2ODD is responsible for C-7 oxygenation (Frey et al., 2003), hence, the same enzyme class is used for modification of DIBOA in wheat and maize. However, it remains to be shown if the C-7 hydroxylation and methylation are carried out by orthologous functions in the Poaceae.

**MATERIALS AND METHODS**

All chemicals were used for pro analysis or HPLC grade. The substrates apigenin (5,7,4'-trihydroxylavon), caffeic acid (3,4-di-hydroxy-cinnamic acid), ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid), and quercetin were purchased from Sigma-Aldrich. Plasmid Bluescript KS+ (Stratagene) was used as a cloning vector. Sequences were isolated from libraries described by Frey et al. (1997).

**Plant materials**

Protein purification was performed with the hybrid maize (Zea mays) line LG22.44 (Limagrain). Whole seedlings were used for protein preparation. Expression data were collected on inbred line B73. Rye (Secale cereale) inbred Halo (Lochow-Petkus GmbH) and Lamiun galeobdolon grown in Staedtengarten, Freising, were used for isolation of DIBOA-glc.

**Synthesis of TRIBOA**

Two alternative syntheses for TRIBOA have been already reported (Kluge et al., 1995). In the former procedure improvements have been introduced consisting in the careful extractive removal of tiny amounts of lactam that hitherto accompanied the hydroxamic acid precursor 7-benzyloxy-4-hydroxy-2-methoxy-2/(1,4-benzoxazin-3(4)/H)-one. A procedure has been developed to extract the cyclic hydroxamic acid from the crude cyclization product with NaOH followed by acidification. The material thus obtained was hydrogenated at ambient pressure over Pd-C in methanol to yield 4,7-dihydroxy-2-methoxy-2H-1,4-benzoazin-3(4)H-one. Eventually, this methyl acetal of TRIBOA was cleaved with boron trichloride in methylene chloride to yield crude TRIBOA. TRIBOA was purified by a column chromatography over Lichroprep RP-18 silica gel and isolated from the aqueous eluent fraction carefully by lyophilization. TRIBOA thus obtained was free of other heterocycles (according to thin-layer chromatography and to NMR analysis) and showed the correct mp 172°C to 173°C (off-white microcrystalline powder).

**Isolation of Substrates and Standards**

DIBOA-glc, DIMBOA-glc, and DIBOA were isolated as described by von Rad et al. (2001). TRIBOA-glc was generated by two alternative approaches. For the generation of the reference substance chemically synthesized TRIBOA was glucosylated in vitro using the heterologously expressed UDP-glucosyltransferase Bx6 (von Rad et al., 2001). Routine large-scale production of TRIBOA-glc was carried out by incubation of DIBOA-glc with raw extracts of isopropylthio-β-galactoside-induced Escherichia coli BL21(DE3) expressing Bx6 from the plasmid pET3a (Studier and Moffatt, 1986). Three hours after induction with 1 mM isopropylthio-β-galactoside, the cells were resuspended in 100 mM Tris-HCl (pH 7.5), 20 mM NaCl, 5 mM dithiothreitol (DTT), 1 mM phenylmethyl-sulfonyl fluoride, 20% glycerol, and 1 mM/liter lysosome (1 g of bacteria pellet per 3 mL of buffer). Five cycles of freeze (liquid N2) and thaw (ice) were applied. RNase and DNase (10 μg/mL each) were added, and solution was cleared by centrifugation. Aliquots were stored at −70°C. For TRIBOA-glc synthesis, 100 μL of the protein solution were used for 1 mL assay volume. The reaction buffer contained 100 mM MES-NaOH (pH 6.0), 5 mM DTT, 10 mM ascorbate, 10 mM 2-oxoglutarate, 4 mM FeSO4, and 3 mM DIBOA-glc. The reaction was incubated at 30°C with gentle agitation and after 45 min stopped by the addition of 1 volume of methanol. Precipitated protein was removed by centrifugation and the supernatant was applied to HPLC. TRIBOA-glc was eluted with 9% acetonitrile and 91% of 0.3% formic acid using Merck LiChroCART RP-18e.

**HPLC Analysis**

All samples were analyzed on a Beckman HPLC System Gold, with Programmable Solvent Module 126, Diode Array Detector Module 168, and Autosampler 508. Data were collected and processed by using 32 Kарат software. Instrumental conditions for analysis were: Merck LiChroCART RP-18e (250 × 4 mm, 5 μm analytical, and 250 × 10 mm, 10 μm preparative) reversed-phase column at 25°C. Mobile phases were: 0.3% formic acid (A) and acetonitrile (B). Injection volume was 50 μL or 2 mL, and the flow rate was 1 or 5 mL/min in analytical and preparative analysis, respectively. The following gradient was used for separation for the analytical analysis: at 0 min, 7.5% B; 1 min, 7.5% B; 2 min, 10.5% B; 9 min, 10.5% B; 10 min, 13% B; 11 min, 13% B; 12 min, 12% B; 17 min, 22% B; and 18 min, 100% B. Under these conditions, the following retention times were obtained for each compound: DIBOA-glc, 12 min; TRIBOA-glc, 6.4 min; DIMBOA-glc, 16.1 min. The detection was carried out at: 254 nm for DIBOA-glc and 266 nm for DIMBOA-glc and TRIBOA-glc.

**MS**

The HPLC-MS investigations were performed with a HP 1100 liquid chromatograph equipped with a diode array detector (280 nm) and a triple quadrupole mass spectrometer (API 2000, Applied Biosystems). A Nucleosil C18 (5 μm) column was used, 3 mm × 250 mm × 5 μm. The solvent system consisted of pure water (solvent A) and methanol/isopropanol (95/5), containing 0.025% acetic acid (solvent B), respectively. The mobile phase was used as a three-step gradient: The first part was isocratic (8% B for 2 min), subsequently followed by a linear gradient (from 8% to 50% B in 9 min) and followed by a third, again isocratic part (50% B). The flow was 150 μL/min; mass spectra were recorded in the negative mode.

**Purification of BX7**

Maize seedlings were grown on wet paper for 4 d in the dark at 28°C. All purification procedures were carried out at 4°C. Protein was extracted from 120 g seedling shoots with 4 volumes (w/v) extraction buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, and 10% glycerol) and 0.3 volumes (w/v) Polyclar (Serva). After filtration through miracloth and centrifugation for 1 h at 10,000g, the supernatant was sterile filtered (0.2 μm) and aliquoted. Aliquots of the protein extract (about 50 μg each) were applied to the affinity column. The affinity column was prepared according to the manufacturer’s instructions and as described by Attieh et al. (1995). Briefly, 20 mL of 20% ethanol was added to...
5 mL of adenosine 5-monophosphate agaroase (Sigma). After 1 h of swelling, the supernatant was decanted, and the slurry was suspended first in water and subsequently in 50 mM Tris-HCl, pH 8.5. The agarose was incubated with 1,000 units of alkaline phosphatase type VII (Sigma-Aldrich) at 37°C overnight in a continuously rotating reaction vial. The material was washed once with 20% ethanol and twice with water, the slurry transferred to a column, and equilibrated in extraction buffer. After application of the protein extract, the column was washed with a flow of 0.2 mL/min with extraction buffer for 1 h. After washing with buffer B (extraction buffer with 0.2 mM KCl, flow 0.2 mL/min), proteins were eluted with a gradient of SAM in extraction buffer (0–4 mM in 100 min, flow 0.2 mL/min). Active fractions of four runs of the affinity chromatography were pooled, concentrated with VivAspin columns (molecular weight cutoff 10,000; Sartorius), and a total of about 50 μg protein applied to a MonoQ column (Amersham Pharmacia Biotech, flow rate 40 mL/h) equilibrated in buffer B. Elution was with 20 column volumes 0.2 to 2.0 mM KCl in buffer B. Fractions were assayed for methyltransferase activity and subjected to PAGE analysis. Candidate bands were cut from the gel (see Supplemental Fig. S3) and digested following the protocol of Schäfer et al. (2001). Sample preparation for MALDI-MS was performed using ZipTips (Qiagen) following the manufacturer’s protocol. For MALDI-MS and MALDI/MS/MS analysis, an Ultрафlex I ToF/ToF mass spectrometer (Bruker Daltonik) was used. Data analysis was performed using the BioTools, RapideNovo (Bruker Daltonik), and Mascot (Matrix Science) software packages. For determination de novo amino acid sequences, no modifications were allowed and the b and y ion series were weighted 5 times higher than other ion series. The amino acids Ile and Gin were excluded. The most sequence parts determined with highest significance were searched against the National Center for Biotechnology Information and OMT sequences selected from the Pioneer EST collection. The selected sequences were in silico digested with trypsin and directly compared to the observed MS spectra of the tryptic digests. The sequence with the highest peptide overlap was selected.

**Heterologous Protein Expression and Purification of His-Tag Proteins**

For heterologous expression Bx6 cDNA was fused to the C-terminal His-tag of pET28a-His and Bx7 cDNA was joined 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 nickel-nitriilotriacetic acid agaroase (Qiagen). Eluted proteins were dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM DTT, frozen in liquid N₂, and stored in aliquots in −70°C. Protein concentrations were determined using the Bicinchonin protein assay kit with bovine serum albumin as standard.

**Enzyme Assays**

Heterologous Bx6 expression in *E. coli* was as a C-terminal His-tagged protein and without an His-tag. Purified His-tagged protein and untagged protein in crude extract were assayed to determine the influence of the tag on Bx6 activity. Both enzymes had very similar activity, indicating that the His-tag does not interfere with catalysis. Purified His-tagged protein was used for determination of kinetic parameters; crude extract was used for determination of pH dependency.

For determination pH optimum of Bx6 activity the assay (1.5 mg/mL) contained 100 mM buffer (acetic, 4.5–5.0; citrate, pH 5.0–6.4; MES-NaOH, 5.0–6.5; Tris-HCl, 6.5–9.5; AMP-HCl, 9.0–10.5; CAPS-NaOH, 10.0–11.0, 2 mM DTT, 50 μM SAM, 0.1 mM TRIBOA-glc, and 0.1 mg/mL His-tagged Bx7 protein.

**Subcellular Localization**

The pH dependency of Bx7 was monitored as described above, using 50 mM buffer (acetic, 4.5–5.0; citrate; MES-NaOH, 5.0–6.5; Tris-HCl, 6.5–9.5; AMP-HCl, 9.0–10.5; CAPS-NaOH, 10.0–11.0, 2 mM DTT, 50 μM SAM, 0.1 mM TRIBOA-glc, and 0.1 mg/mL His-tagged Bx7 protein.

For transient expression of the GFP-reporter constructs, protoplasts were isolated from aseptically grown plants, 12 d after imbibition. Preparation and electroporation was as described by J. Sheen (http://genetics.mgh.harvard.edu/sheenweb). Thirty micrograms of the pUC19-based plasmid were used for each electroporation of 1 to 2 × 10⁷ protoplasts. Analysis was 24 h after electroporation with Zeiss Axiohot equipped with HQ-Filterset for enhanced GFP (AHF Analysentechnik AG). Photographs were taken with DCS 670 Digital Nikon F5 SLR camera and analyzed with the software Kodak DCS Photodok. Protoplasts from transgenic plants were isolated and analyzed accordingly.

**Determination of Transcription Levels by Quantitative Reverse-Transcription-PCR**

RNA isolation, cDNA synthesis, and real-time reverse-transcription-PCR was as described (von Rad et al., 2001). The gene-specific primer pair Bx6F: 5′-CTCTAGAGCTGCCTGACC-3′ and Bx6R: 5′-TCGGTATAGCCCGCTCTGACCC-3′ was used for amplification of 250-bp fragment specific for Bx6. A Bx7-specific fragment of 440 bp was amplified with the primer pair Bx7F: 5′-TCAGAGCGCTGCGGGGAGGACG-3′ and Bx7R: 5′-ATGCTCTCTGAAGAGCTGCGCC-3′. Two biological replicates were used for RNA isolation and the analysis of every RNA sample was done in replicate.

**Mapping**

Bx7 was mapped by PCR amplification of allele-specific fragments. The primer pair CM37F: 5′-CAACACACACACTATCCGG-3′ and CM37R: 5′-GAGCTGCTGCGGAGTAGTACC-3′ was amplified a band of 204 bp on CM3 and no band on T232 genomic DNA. The primer pair T232F: 5′-CAACACACACTCCGCGCA-3′ and T232R: 5′-GAGCTGCTGCGGAGTAGTACC-3′ amplified a band of 218 bp on T232 genomic DNA and no band with CM3. Annealing was at 65°C for 30 s and elongation was for 30 s. A final concentration of 3% dimethyl sulfoxide was included in the reaction buffer.

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Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: ARAHTOM, NP_200227; BAMI, AAF82824; B7X, EU192149; CATROCOMT, AY028439; CLABR, O23760; CHRAETOM, Q42653; COFCACOMT, AAN93727; COPJACOMT, Q86486; COPJACOMT, Q98622; COPJACOMT, Q9EL16; COPIAOJET, Q71E5; GRAMINIVUN, AAC18643; HYFlavonoid-7OMT, CA54616; IAMT, NP_200336; JAGMT, NP_200441; MEDSAOMT, O24529; MESCRIPTM, P45986; OCIBACVMT, Q93WU3; OCBMAEOMT, Q93WU2; ORYSAOMT, Q6ZD89; POPKICOMT, Q34047; PRUDUCOMT, Q43609; SAGAMT, NP_194372; ZINELCOMT, ZEU19911; ZMAISCOMT, Q06509; ZMAISZRFR4, P47917.

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure S1. Purification of BX7.
Supplemental Figure S2. Comparison of BX7 sequence with identified peptide sequences of the purified enzyme.

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