



Zea mays Glucosyltransferase BX9- An Essential Enzyme for Benzoxazolinone Detoxification

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ABSTRACT

Purification of the benzoxazolinone (BOA)-glucosylating activity from maize roots by chromatographic methods and LC/MS/MS peptide analysis after 2d electrophoresis identified glucosyltransferase BX9 as the enzyme responsible for BOA-6-O-glucosylation. Immunoprecipitation and experiments with transgenic *Arabidopsis thaliana* lines corroborate the function of BX9 in detoxification of the intermediate BOA-6-OH. The BOA-6-O-glucosylation pathway is a “ready to go” mechanism, important to detoxify benzoxazolinone (BOA), before the detoxification pathway leading to glucoside carbamate synthesis is established. Oxidative modifications of BX9 after exposure to BOA seem to trigger the enzyme for acceptance of BOA-6-OH as a substrate. Consequently, reducing sulphur compounds inactivated BOA-6-OH glucosylation. BX9 has a higher affinity to BOA-6-OH than to DIMBOA when assayed at pH 8.0. For the substrate BOA-6-OH, K_m -values were determined to be 120.7 μ M, the K_m value for UDPG were 360 μ M. The intermediate BOA-6-OH is a linker between BOA and MBOA detoxification, when MBOA is demethylated.

Glucoside carbamate, a major BOA detoxification product later in the process, is a potent inhibitor of BOA-6-O-glucosylation (K_i 84 μ M). BOA detoxification pathways are competitive to the formation of benzoxazolinone derived phenoxazinone, a highly toxic compound for many organisms including plants. Benzoxazolinone detoxification is therefore of great importance for the survival of plant seedlings.

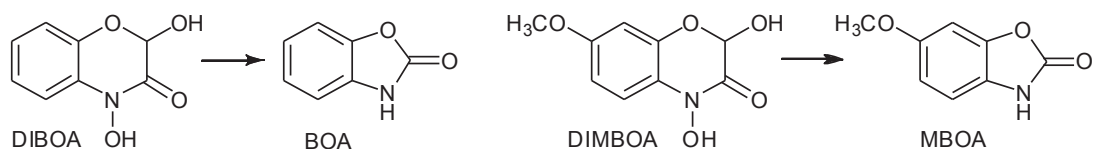
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Introduction

Benzoxazinone- β -D-glucosides are characteristic secondary compounds of several Poaceae including maize, wheat, and rye and some dicotyledonous species (Dick *et al.* 2012; Sicker and Schulz 2002). The aglycons possess antimicrobial, insecticidal and herbicidal activity and have therefore received attention. The biosynthesis of the compounds has been thoroughly investigated in maize (Dick *et al.* 2012). Following the synthesis of benzoxazinone DIBOA, the molecule is subsequently glucosylated at the 2-position by the glucosyltransferases BX8 and BX9. Glucosylated DIBOA is the precursor of DIMBOA-glucoside, the major benzoxazinone in maize (Frey *et al.* 1997; Glawischnig *et al.* 1999; von Rad *et al.* 2001).

The allelopathic potential of benzoxazinoids was demonstrated in many studies with rye and meanwhile, it is an important tool to suppress

certain weeds in environmentally friendly farming systems (Schulz *et al.* 2013). The allelopathic potential of maize is less studied. In solution, the aglycons DIBOA and DIMBOA are unstable. They undergo heterocyclic ring contraction and the release of formic acid lead to the benzoxazolinones BOA or MBOA (Niemeyer 2009). Benzoxazinoids are exuded by young roots or are released into the environment by plant rotting. The compounds are converted into further degradation products by microorganisms, the phenoxazinones, which are present in the soil for several months. The known degradation sequence starts with microbial opening of the heterocycle, decarboxylation and release of 2-aminophenol. Phytotoxic 2-aminophenoxazinones, bio-inactive acetamides and malonamic acids are subsequently occurring products (Macias *et al.* 2005). Specialized microorganisms, such as certain fungi are able to eliminate phenoxazinones by H_2O_2 release and Fenton reactions (Schulz *et al.* 2012).



Known Plant Detoxification Products of BOA and MBOA

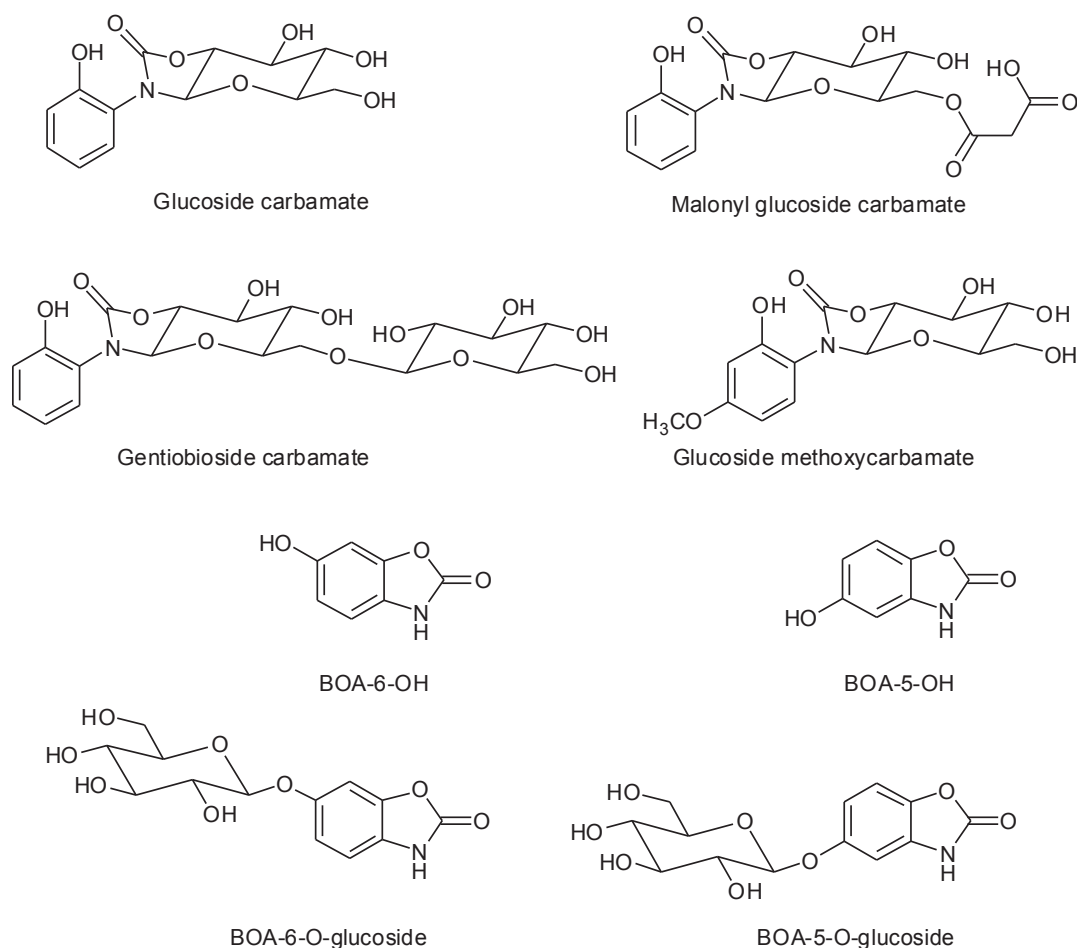


Figure 1. Benzoxazolinone detoxification products found in higher plants.

Although they are less toxic than the precursor molecules, benzoxazolinones can be deleterious to other plants, when they are absorbed in sufficient amounts. The generation of more toxic hydroxylated BOA molecules should be pivotal (Schulz *et al.* 2013) as is the generation of phenoxazinones by root associated microorganisms. ROS production (Reactive Oxygen Species) and oxidative stress are key events in the mode of action. Membrane damage, lipid peroxidation, protein oxidation, enzyme inactivation and other fatal events take place (Friebe *et al.*, 1997; Sanchez-Moreiras *et al.*, 2005; 2011; Burgos *et al.*, 2004; Singh *et al.*, 2005;

Batish *et al.*, 2006; Hussain *et al.*, 2008; Kato-Noguchi *et al.*, 2010; Hussain and Reigosa, 2011; Schulz *et al.*, 2012a, b, 2013). Senescence and death occurs, unless plants start rescue and defense programs (Baerson *et al.* 2005). When exposed to BOA, maize roots react immediately with an increase of malondialdehyde (MDA), a marker for oxidative stress and lipid peroxidation. The return to MDA control levels after 4-6 hours is a clue for the successful activation of defense mechanisms in the maize and the switch to highly effective detoxification by glucoside carbamate synthesis (Schulz *et al.* 2012a; Schulz *et al.* in press).

When confronted with BOA, plants react in a species- and dose-dependent manner. Generally, members of the Poaceae were found to be less sensitive to the compounds than most of the dicotyledonous species. One important reason for the different sensitivity is the better developed ability of grasses to reduce the toxicity of benzoxazolinone(s), in comparison to many dicot species. Roots are the predominant and primary organs for detoxification processes. Almost all investigated weed and crop seedlings detoxify benzoxazolinone (BOA) via 6- or 5- hydroxylation and subsequent O-glycosylation (Hofmann *et al.* 2006). In most cereals and wild grasses, glucoside carbamate is the major product (Fig. 1), (Wieland *et al.* 1998; Sicker *et al.* 2004; Schulz and Wieland 1999; Schulz *et al.* 2006). The highly complex, compartment spanning and redox-regulated glucoside carbamate synthesis was elucidated in maize (Schulz *et al.*, in press). However, the accumulation of glucoside carbamate starts relatively late, about 6h after exposure to BOA, whereas during the first hours BOA-6-O-glucoside is synthesized. The detoxification by BOA-6-O-glucoside formation in maize is therefore regarded as a constitutive mechanism, valuable to bridge over the time until glucoside carbamate synthesis is possible. The high importance of benzoxazinoid detoxification prior to the possible formation of phenoxazinones in larger amounts was underlined by the work of Venturelli *et al.* (2015), who described the inhibition of histone deacetylases by APO/AMPO and enhancement of the effect by locus-specific alterations of histone acetylation and gene expression. These actions are discussed as major reasons for phenoxazinone-caused plant growth reduction.

In this study, we focus on the identification of a maize glucosyltransferase and its biochemical properties enabling the enzyme for the BOA-6-OH glucosylation step during the early process of BOA detoxification until the switch to glucoside carbamate, requiring a massive shifting of enzymes into the extraplasmic space, is possible.

Material and Methods

Plant Material, incubation conditions and analyses of extracts

Seven-days-old, hydroponically grown maize seedlings (cultivars *Zea mays* L. "Nicco", "Pedro" and "Cassila" with similar BOA detoxification behavior, all gifts from KWS, Germany) were incubated for 24 hours in 25mM MES-KOH pH 5.8; 0.5mM KCl; 0.25mM CaSO₄; 0.1% (w/v) Na-ascorbate, containing 500µM BOA (Fluka) or in tap water containing 0.5 mM BOA. Controls contained no benzoxazolines. Maize seedlings were also incubated with 2 mM MBOA and with 0.5 mM MBOA without and in presence of

1 and 5 mM methionine. Growth conditions were as described in Sicker *et al.* (2001).

Arabidopsis thaliana Col-0 and the transgenes 35S::Bx8, 35S::Bx9 and 35S::Co-Bx8 were grown from seeds in a phytotron at 20°C (with 150 µE light intensity, a 16 h light period and 55% relative humidity) for four weeks in pots with a Vermiculite/soil mixture (1:3). Plants were harvested and soil particles were removed from the root system by repeated water baths. 12 plants were incubated with 250 ml incubation medium containing 0, 0.5, 1.0 and 2.0 mM BOA for 24 h. Plants were washed and separated in shoots and roots, which were either used for protein extraction or for methanolic extract preparation. Methanolic extracts of maize and *Arabidopsis* roots and shoots were prepared as described in Schulz *et al.* (2012b) with 50% methanol and analyzed by HPLC.

Maize UGT Purification

After incubation, the maize roots were harvested and powdered on ice with quartz in ice-cold extraction buffer (w/v:1/5) containing 100mM MES-KOH pH 6.0; 10% (v/v) glycerol; 2% (w/v) Na-ascorbate; 1mM DTT, 100µM Pefabloc / g roots and 0.1 g Polyclar / g roots). The homogenate was filtrated through Miracloth (Calbiochem) and centrifuged (20,000g, 20min, 4°C). The crude extract was precipitated with 30-60% ammonium sulfate and subsequently centrifuged (40.000 g, 1h, and 4°C). The pellet was resuspended in extraction buffer and applied to a Sephadex G-100 (Sigma) column, pre-equilibrated with extraction buffer. The elution was performed with the same buffer (flow rate 0.3 mL min⁻¹). Fractions with enzyme activity were pooled and concentrated by ultrafiltration (PBGC membrane, Millipore). Protein concentrations were determined with the Bradford method. The concentrate was further purified by FPLC (Biosys 2000, Beckman Coulter). For each run, 1mL of the concentrate was loaded onto a QHyperD 10 column (4.6x100mm, Beckman ProScale; flow rate 1mL min⁻¹) pre-equilibrated with buffer B (100mM MES-KOH pH 6.0; 10% (v/v) glycerol; 1mM DTT). After washing with buffer B (1 ml / min), the enzyme was eluted with a linear gradient of 0 – 0.5 M KCl in buffer B. Active fractions, eluting at 0.23 M KCl, were pooled and concentrated by ultra-filtration. The concentrate, supplemented with 5mM UDPG, was subjected to affinity chromatography on a HiTrap Blue column (0.7 x 2.5cm, Pharmacia) pre-equilibrated with buffer B. After washing with 5mL buffer B (0.5mL per run; flow rate 0.5mL min⁻¹), the enzyme was eluted with a linear gradient of 0 – 500 µM BOA-6-OH in 100 mM TRIS pH 8.4. The enzyme elutes at

pH 7.8 and a BOA-6-OH concentration of 400 - 420 μ M. Alternatively, the enzyme was eluted with 40 mM UDPG in 50 mM HEPES-KOH, pH 7.5 containing 5 mM DTT. Pre-runs with 5 mM ascorbate, malate or pyruvate were performed to reduce the amount of proteins co-purified by the previous steps. After elution with BOA-6-OH active fractions were pooled, concentrated and either used for 2d electrophoresis. For further enzyme characterizations elution with UDPG was preferred (see below).

UGT Assays

The standard reaction mixture contained 25 μ L 200 mM HEPES for adjustment to pH 8, 2.5 mM UDPG in extraction buffer, 200 μ M BOA-6-OH in ethanol and 68 μ L enzyme solution in a total volume of 100 μ L. The mixture was incubated at 30°C for 10 min, the reaction was stopped by boiling and centrifuged (10000g, 15min, 4°C). Other substrates (sugar acceptors) were 200 μ M DIMBOA, 6-hydroxyindolon, BOA-5-OH, DIBOA or 2,4 D. *O*-glucosylation was also assayed at pH 6.5 with all sugar acceptor substrates. Adjustment of the pH was done by addition of 5 μ L 200mM HEPES and 20 μ L extraction buffer. For K_m determinations the UDPG concentration ranged from 0.5 to 15 mM, the BOA-6-OH concentration from 20 to 500 μ M. Michaelis-Menten constants (K_m) were determined from Lineweaver-Burk plots. For K_i (glucoside carbamate) determination 0, 10, 25 and 50 μ M glucoside carbamate were used with the standard reaction mixture. The K_i value was determined from Hanes plots.

Assays were analyzed by HPLC (Schulz *et al.*, 2012), concentrations of produced BOA-6-*O*-glucoside were calculated on the basis of external standards (Wieland *et al.*, 1998; Sicker *et al.*, 2001). Concentrations of produced BOA-6-*O*-glucoside or -*N*-glucoside / carbamate were calculated on the basis of synthetic BOA-6-OH and BOA-*N*-glucoside / glucoside carbamate used as external standards (Wieland *et al.* 1998; Sicker *et al.* 2001). BOA-5-OH was synthesized as previously described (Hofmann *et al.* 2006), 6-hydroxyindolone was synthesized by Aurora, Wien. 2,4 D was from Sigma.

UGT activity determination in transgenic *Arabidopsis* lines

Arabidopsis roots and shoots were homogenized by mortaring on ice with sea sand, PVPP (20% of fresh weight), 5 μ L protease inhibitor cocktail (Sigma)/ 100 mg FW, and the extraction buffer used for maize UGT purification (1:3, FW:buffer volume). The homogenate was centrifuged for 20 min at 4°C and 20.000g. The supernatant was immediately used for UGT activity

determinations, performed as described with maize protein. Assays contained 50 μ g protein in average, according to the Bradford method. Analyses of the assays were done by HPLC.

Two-dimensional Electrophoresis

The pooled active fractions from the HiTrap Blue column were concentrated by ultra-filtration. The concentrated proteins were precipitated with ice-cold methanol (3:1). After centrifugation, the pellet was dissolved in 9.5M urea; 4% Chaps; 1.5% DTT; 2% Pharmalyte 3-10 for IEF (Pharmacia); 0.8% *n*-hexyl- β -D-glucopyranoside; 0.004% *n*-decyl- β -D-maltopyranoside and 0.002% SDS.

For isoelectric focusing (IEF) a Bio-Rad Protean IEF Cell and Ready Strip IPG strips pH 4-7 (Bio-Rad) were used. Focusing with 50 μ Am / gel was completed after 20.000Vh. The second dimension was performed on a 15% gel. Coomassie stained protein spots were excised and subjected to LC/MS/MS analysis.

Western blots were performed as described by von Rad *et al.* (2001) using polyclonal antibodies raised against recombinant BX9. Alkaline phosphatase coupled anti-rabbit antibodies (Sigma) were used for visualization.

Identification of the UGT by LC/MS/MS

Gel spots were repeatedly dehydrated and rehydrated prior to digestion by soaking them in dehydration (2:1 acetonitrile/25mM ammonium bicarbonate) and rehydration solutions (25mM ammonium bicarbonate) in turn. Spots were dehydrated four times. Following the fourth dehydration, the supernatant was removed and spots were dried in a vacuum centrifuge.

Spots were then resuspended in ~10ng/microliter solution of trypsin in 25mM ammonium bicarbonate with ~5mM calcium chloride. Small amounts of trypsin solution were added as the spots rehydrated. Once rehydrated, the spots were covered with a minimal volume of ammonium bicarbonate solution and incubated 4.5 hours at 37°C, and generated peptides were extracted by adding roughly twice the digest buffer volume of acetonitrile with 1%-2% formic acid. Samples were vortexed and then the peptide-containing supernatant was transferred to new tubes, which underwent vacuum centrifugation to remove the organic solvent.

MS - Methods

LC MS/MS data were collected using a Q-ToF II tandem mass spectrometer coupled to a CapLC

system fitted with a 75µm NanoEase Atlantis dC18 analytical column and a NanoEase Symmetry300 C18 Trap column in a forward flushing configuration (Waters Corporation). All chromatographic solutions contained 0.1% formic acid. Peptide mixtures were separated with a gradient of 5-50% acetonitrile in water (Aldrich HPLC grade) over a 15 minute period, followed by column cleaning and re-equilibration. Data were collected in survey mode using argon as a collision gas at a pressure of $4\text{--}5 \times 10^{-5}$ bar. Capillary voltage was 1.6 kV and the cone voltage was 45 V. The collision voltage was kept at 10V during MS-ToF mode collection.

Multiply-charged precursor ions of sufficient intensity were fragmented in MSMS mode with collision voltage between 20 and 70 volts, depending on precursor charge and mass. Multiple runs were performed with each sample. Peak files were generated by Masslynx3.5 (Waters Corp.) and de novo sequencing was performed both with LutfiskXP version 1.0 (Richard S. Johnson) and ProteinLynx/Global Server2.1 (Waters Corp). Resulting de novo sequences were visually checked and the highest scoring sequences were used to form queries for MSBLAST searches (Shevchenko *et al.*, 2001).

Modulation of glucose transferase activities

The active protein fraction from anion exchange chromatography (2ml) was incubated with 25 mM glutathione disulfide at 30°C (Barrett *et al.*, 1999). Aliquots (68µl) of the mixture were taken after 20, 40, 60, 80, and 90 min and assayed for glucosyltransferase activity using DIMBOA or BOA-6-OH as acceptor molecule. In a parallel analysis the incubation of

the protein with 25 mM GSSG for 90 min was followed by buffer exchange to extraction buffer without DTT by centrifugation (Centricon tubes). For reconstitution, the resulting solution was incubated in presence of 20 mM DTT at 30°C. Aliquots (68 µl) were taken every 10 min over a period of 120 min and assayed for DIMBOA or BOA-6-OH glucosylation. In another series of experiments, the NO-donor GSNO (S-nitrosoglutathione) was used (Lindermayer *et al.* 2005). The protein was preincubated for 15 min at 20°C before incubation with 250 µM GSNO and analysis. In a third series, the protein was assayed in presence of 200 µM H_2O_2 , and 10 or 20 mM DTT.

Immunoprecipitation of BX9 from maize roots

Maize root tips (3 cm, 2.8 g FW) were harvested and proteins extracted as described above. The crude extracts were used for immunoprecipitation with BX9 antibodies. Immunoprecipitation was done with the Pierce Direct IP Kit (Fisher Scientific) according to the instructions of the manufacturer. Elution of the bound protein was with Pierce elution buffer, pH 2.8 in a total volume of 100 µl. Crude protein extracts, flow throughs and eluates were assayed for UGT activity as described and aliquots of all fractions were subjected to western analysis after SDS-PAGE.

Results

Protein crude extracts from maize roots of control plants contain a constitutive glucosyltransferase activity able to glucosylate BOA-6-OH, but BOA-N-glucosylation for glucoside carbamate production could not be reproducibly measured in extracts prepared for BOA-OH glucosyltransferase measure-

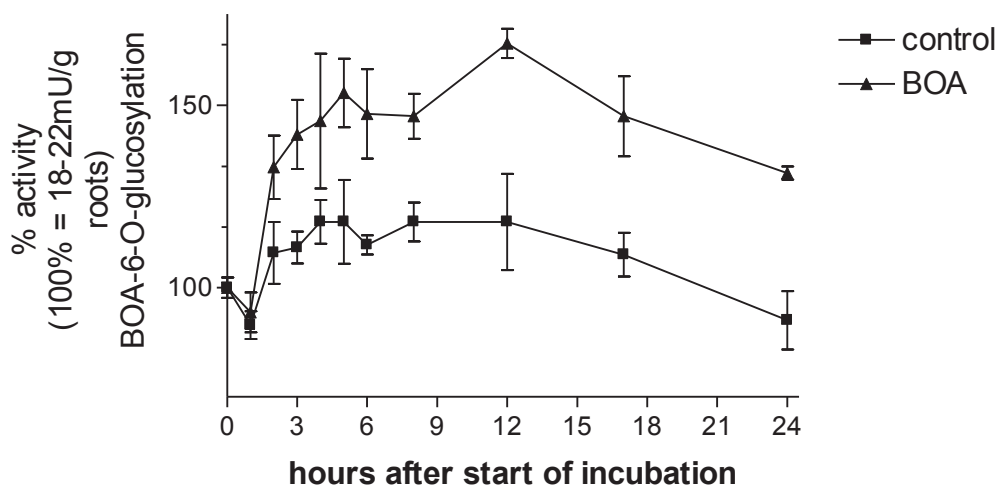


Figure 2. BOA-6-OH glucosylating activity during the course of BOA incubation in comparison to the control plants, which incubated with water. Values present the means $\pm$ standard deviations.

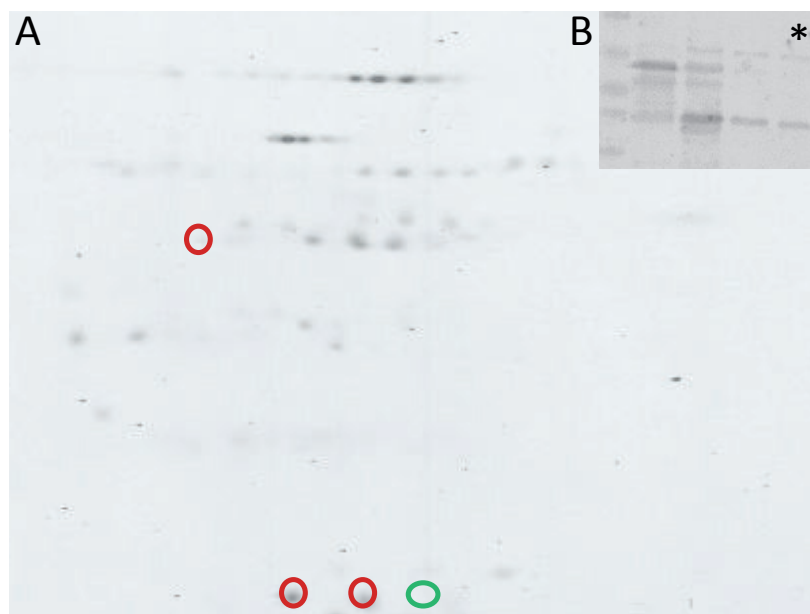


Figure 3. Coomassie stained gel after 2d-electrophoresis of the Hi Trap Blue fraction indicated the co-purification of different other proteins. The red markers encircle BX9 and its fragments, the green marker a BX8 fragment. B: The insert shows a Western blot of the different purification steps, indicating the loss of intact BX9. B-lines: marker proteins, crude extract, gel filtration, anion exchange chromatography, affinity chromatography (*).

ments. The BOA-6-OH glucosylating activity was enhanced after incubation with BOA. After 1 h, the activity increased up to 35% during the next hour and stayed about 45 % in average above the control levels during the whole course of monitoring with a maximum of activity after 6-12 hours of incubation, followed by a plateau and a slight decrease to the end of the incubation period after 24 h (Fig. 2).

Although BOA-6-O-glucoside does not accumulate anymore, the BOA-6-OH glucosylating activity was still above the control level after a 24h BOA exposure.

Addition of more than 1 mM DTT to the extraction buffer resulted in a 30-25% loss of the BOA-6-O-glucosylating activity. Following ammonium sulfate fractionation, the activity was enriched by chromatography on Sephadex G 100, anion exchange chromatography using a Q-Hyper D 10 column and chromatography on Hi Trap Blue. From all columns, the activity eluted in a single peak, but some activity was found in the wash fraction of the Q-Hyper D 10 column. The overall enrichment was 395 – fold (table 1), but the final yield was low (0.5 %). Elution from the Hi Trap Blue column was not only possible with BOA-6-OH and 40 mM UDPG but also with 20 mM homocysteine or 2 mM NADH. The use of homocysteine and NADH for elution led to a rapid loss of activity. With desalted $(\text{NH}_4)_2\text{SO}_4$ fractions, low BOA-N-glucosylation activity was measurable. However, during the further steps of the purification procedure, this activity was lost.

Identification of BX9 as a glucosyltransferase responsible for BOA-6-OH glucosylation

Two-d-electrophoresis and LC/MS/MS analysis of the Hi Trap Blue fraction revealed large amounts of a BX9 fragment in the range of 21 kDa whereas only traces of the complete protein were found (Table 2, Fig. 3). Several proteins were co-purified, mainly methionine synthase, malate dehydrogenase, mono-hydroascorbate reductase, enolase, four glutathione-S-transferases and traces of a BX8 but no other UGTs or their fragments. The BX9 fragment, which was already abundant in SDS-extracted proteins from roots, represents the C-terminal domain of the enzyme. Although only this fragment was mainly enriched, BX9 might be responsible for BOA-6-OH detoxification.

Further evidence for BX9 as a BOA-6-OH glucosylating enzyme was obtained by pull down experiments using maize crude protein extracts and BX9 antibodies. Incubation of maize crude extracts with the antibody led to a complete loss of activity and when the immuno-bound enzyme was eluted the BOA-6-OH-glucosylating activity was recovered (Fig. 4). The BX9 fragments are enriched in the flow through but no BOA-6-OH glucosylating activity was detected for this fraction. Hence the native BX9 is responsible for BOA-6-OH detoxification.

Table 1. Enrichment of BOA-6-*O*-glucosylating activity.

Purification step	Total activity (nkat)	Specific activity (nkat mg ⁻¹ protein)	Yield (%)	Purification factor
Crude extract	13.75	0.2	100	1
Ammonium sulphate precipitation	2,78	0.34	20.3	1.72
Gel filtration	1.84	1.41	13.4	7.16
Anion exchange chromatography	0.03	12.61	0.22	64.17
Affinity chromatography	0.07	78	0.50	395.3

BOA incubation accelerate BX9 cleavage

The increase of the 23/21 kD fragments could be caused by the allelochemical already during the incubation. Western blots with BX9 antibodies and SDS-extracted maize proteins clearly showed that BX9 undergoes fragmentation in presence of BOA. The fragments occur mainly in the root hair and maturation zones (Fig.5).

*Biochemical characteristics of BOA-*O*- and *N*- glucosylation*

To get more insights in biochemical characteristics that lead to the different BOA glucosylations, the Q-Hyper D10 column fraction was used for a characterization of the BOA-6-OH glucosylation and the desalted ammonium sulfate fraction for *N*-glucosylation (Tab. 3). The pH optimum of BOA-6-*O*-glucosylation was at pH 8 with half maxima at pH 7 and 9.3, the optimum of BOA-*N*-glucosylation was found between pH 6.5 and 7 with half maxima at pH 6 and 7.5. Thus, *O*-glucosylation is shifted to the more alkaline range in comparison to BOA-*N*-glucosylation. Substrate specificities differed

depending on pH. At pH 8, the highest activity was found with BOA-6-OH (150%), followed by the natural substrate DIMBOA (100%) and DIBOA (53.3%). A low activity was determined with BOA-5-OH and 2,4D, whereas BOA and 6-hydroxyindolone were not accepted. At pH 6.5 only 30% of the activity was found with BOA-6-OH but still 61.5% with DIMBOA. Glucosylation of DIBOA dropped to 8.6 %, but 6-hydroxyindolone was accepted (21%). BOA-5-OH and 2,4-D were not accepted as substrates at pH 6.5. BOA was *N*-glucosylated, but with very low efficiency (less than 5%). The low activity was not in agreement with the fact that BOA-*N*-glucosylation is the major detoxification reaction in the roots and presents a residual activity as BOA-*N*-glucosylation takes place in the extraplasmic space (Schulz *et al.* in press).

For the substrate BOA-6-OH, K_m -values were determined to be 120.7 μ M for BOA-6-OH. Lower BOA-6-OH concentrations led to sigmoid graphs, higher ones than 400 μ M to substrate inhibition. The K_m values for UDPG were 0.36 mM. Glucoside carbamate was an effective inhibitor of BOA-6-OH glucosylation (K_i 89.7 μ M) (Fig. 6).

Table 2. Sequences of identified BX9 Peptides and their position within the BX9 sequence.

AA Position	Peptide
15-31	RVVVFPPFQGHFNPVM
49-61	RGLAITVFHSGALDPADYPADY
64-73	VPVTVEADPK
194-207	RVDTSDLEEF AELLA
238-253	ALSVPVFAVAPLNK
323-338	RGFESGALPDGVEDEV
395-401	RYVCDVWK
428-436	EGEEIKER
446-459	GIGIGVDVDETTSP
460-471	RTDLTDLVDLIK

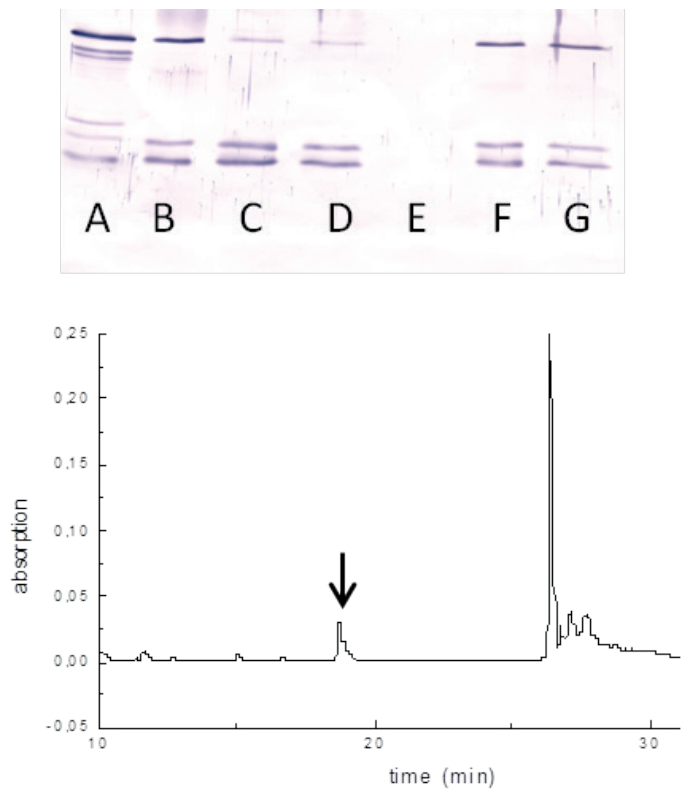


Figure 4. Western blot of the immunoprecipitation steps with BX9 antibodies. I: crude protein extract B: antibody treated crude extract, C: column flow through; D, E: 1. and 2. wash; F and G: 1st and 2nd elution. A: crude extract (aliquot of 500µl containing 4-6 mU enzyme activity); B: no activity; C: 0.04 nkat; D: 0.027 nkat; E no activity; F,G: 0.01-0.013 nkat The fractions obtained during the immunoprecipitation procedure contained co-eluting antibody, which hampered correct determination of the enzyme activity and BX9 protein concentration. Activity found in F and G could therefore considerably higher than the determined 10-20% of the one found in the crude extract. II: HPLC chromatogram using 50µl of eluate assay with BOA-6-OH and UDPG as substrates. Arrow points to product BOA-6-O-glucoside. 100µl Assays with 20 µl protein solution.

Modulation of BX 9 Activities by sulfur containing effector molecules

Several substances known to be involved in stress signal transduction and redox-regulations were tested for their influence on BOA-6-OH and DIMBOA-glucosylation (West *et al.* 2006; Lindermayer *et al.* 2005; Klatt and Lamas 2000), (Tab. 3). Short term treatments with S-nitrosoglutathione, a compound used for S-nitrosylation and NO-mediated protein

S-glutathionylation reduced the BOA-6-O-glucosylation to 20% and inhibited DIMBOA glucosylation up to 50%. The addition of 200 µM H₂O₂ had no effect on BOA-6-O-glucosid production, but decreased DIMBOA glucosylation to 60% and assaying in presence of 20 mM DTT inhibited BOA-6-OH glucosylation to 25 %. A strong inactivation by GSSG toward all sugar acceptors was observed. For DIMBOA, the activity could be reconstituted by DTT up to 60% within 2h, reconstitution of BOA-6-OH glucosylation with DTT failed.

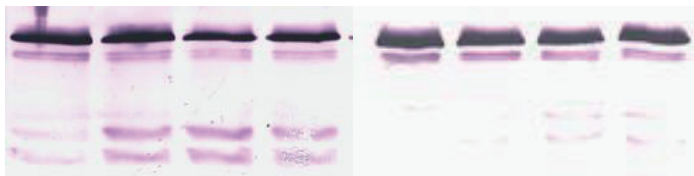


Figure 5. Western blot of proteins extracted from 1cm root segments (left to right: tip-1cm, 1-2cm, 2-3cm, 3-4cm) with BX9 antibodies. A: extracts from BOA-incubated (24h) B: extracts from control plants, which were incubated only with water. BX9 fragments are mainly in the 2-4 cm part of the root.

Table 3. Biochemical characteristics of the glucosylating activity. Substrate concentrations: 2.5 mM UDPG, glucose acceptor substrates: 200µM. 100% = 8.5 nkat/mg protein.

Activity Properties			
pH optimum	8.0, BOA-O-glucosylation		
Substrate specificity	at pH 8.0		at pH 6.5
	DIMBOA	100%	DIMBOA 61.5 %
	DIBOA	53%	DIBOA 53 %
	BOA-6-OH	150%	BOA-6-OH 30%
	BOA	0	BOA < 5%
	6-hydroxindolone	0	6-hydroxindolone 21%
Km values	BOA-6-OH 120.7 µM		
	UDPG 360 µM		
K _i value	glucoside carbamate 89.7 µM		

From this characterization we conclude, that the redox-status of the enzyme is important. Most likely, reduced or oxidized strategically important –SH groups of BX9 could be crucial for BOA-6-OH glucosylation. An oxidized form of the enzyme could be necessary for full activity when BOA-6-OH is the sugar acceptor molecule. This oxidized form seems to be unstable and undergoes cleavage during the course of the BOA incubation. The results agree with the inactivation of the BOA-6-OH competent enzyme by homocysteine and NADH.

BOA-detoxification in transgenic *Arabidopsis* lines

We used transgenic BX9, BX8 and Co-BX8 (which expresses the *Consolida orientalis* BX8 gene, Frey *et al.*) *Arabidopsis* lines to substantiate a role of BX9 in BOA-6-OH glucosylation (Fig. 7). BOA-6-O-glucoside accumulation was significantly higher in the BX9 line when the incubation was done with 0.5 mM

and 1mM BOA, whereas 2 mM led to a similar accumulation as found in WT and BX8 plants. The Co-BX line accumulated the highest amount of BOA-6-O-glucoside. With 2 mM BOA 100 to 200 % more of this compound compared to the BX9 line was found.

BOA-6-OH glucosylating UGT activity was measured with extracts of soluble proteins prepared from the transgenic *Arabidopsis* lines after 24h incubations with 0, 0.5, 1 and 2 mM BOA (Fig. 8). In roots of WT plants not exposed to BOA, the BOA-6-OH glucosylating activity was in the range of 10 mU / g FW (0.17nkat). The activity was 4-5 fold increased after 0.5 mM BOA incubation, whereas higher BOA concentration had no additional effect. It is known that several genes encoding UGTs are induced by BOA (Baerson *et al.* 2005). Their activities certainly interfere with the maize UGTs in the transgenic lines. The BX8 and the Co-BX8 lines exhibited both a higher activity in the control roots (2-3 fold:Co-BX8; 2-2.5 fold: BX8, compared to the WT.

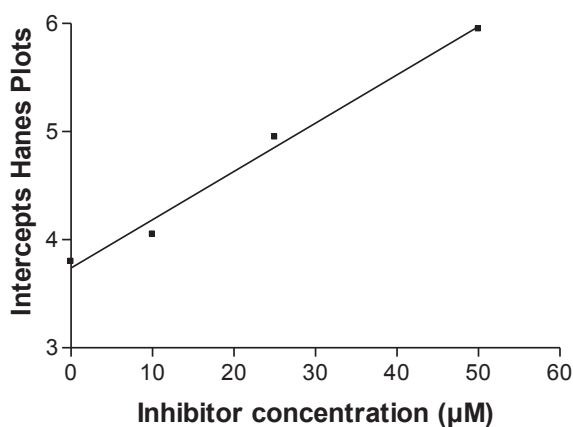
**Figure 6.** Glucoside carbamate is an inhibitor for BOA-6-OH glucosylation.

Table 4. Influence of effectors on the glucosylating activity at pH 8.0, substrate concentrations 200 µM, 100%=8.5 nkat/mg protein.

Effectors	% Activity O-glucosylation	
	DIMBOA	BOA-6-OH
20mM S-nitrosoglutathione	50	20
200 µM H ₂ O ₂	40	100
20 mM DTT	100	25
25 mM GSSG	0	0
Reconstitution with DTT	60 (2h)	no reconstitution

Incubation with 0.5 mM BOA led to an increase in the lines (Co-BX8: 50%, BX8: 25%), 1 mM BOA increased the activity in BX8 line 30% compared to the WT, the Co-BX8 line activity was similar to the WT. 2 mM BOA incubations decreased the activity in the roots of both lines, probably due to interferences with the induced endogenous detoxifying glucosyltransferase.

The BX9 line showed a different activity profile. The highest BOA-6-OH glucosylation was found in the control (5 fold compared to the WT), increasing BOA concentrations led to a loss of activity that could dropped below the average WT value when 1 or 2 mM BOA were used for the incubation. The UGT activity profiles coincide with the BOA-6-O-glucoside accumulation pattern in the roots. The higher accumulation of this detoxification product in BX9 plants has to be assigned to the high enzyme activity already before the start of the incubation. The decrease of BOA-6-OH glucosylation with higher BOA concentrations can be explained again by interference with the *Arabidopsis* UGT or the BOA-6-OH competent form of BX9 is no longer generated. BX9 cleavage, occurring also in *Arabidopsis*, cannot be the only reason, since the enzyme is constantly synthesized. The BX9 cleaving product can be demonstrated by Western blotting (Fig. 9). The Western blot also shows

that BX8 is heavily degraded under BOA influence.

Possible crosslinks of BOA and MBOA detoxification

Methylation and demethylation or transmethylation reactions may interlink the BOA/MBOA detoxification pathways. Transmethylation reaction could also be involved in cell wall incorporation of detoxification products. Therefore, the influence of methionine on the accumulation of detoxification products was tested. Methionine is, for instance, an essential methyl-group donor in pathways leading to the methylation of lignin precursors (van der Mijnsbrugge *et al.*, 2000; Ravel *et al.* 2004; Barrière *et al.* 2009).

Incubations with MBOA resulted in the accumulation of BOA-6-O-glucoside, but the presence of methionine shut down BOA-6-O-glucoside accumulation (Fig. 10). Thus, the accumulation of BOA-O-glucoside as a detoxification product of MBOA is influenced by the methyl-donor methionine and indicates trans-methylation reactions. This needs, however, further proof. The experiment demonstrates that BX9 could be important for detoxifying MBOA derived BOA-6-OH. The intermediate BOA-6-OH is therefore a linker between BOA and MBOA detoxification.

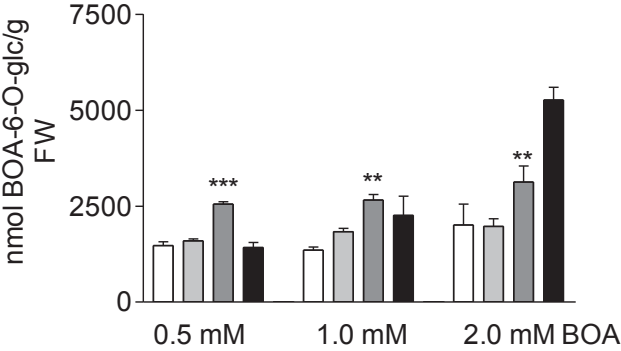


Figure 7. Accumulation of the two BOA-6-O-glucoside in transgenic *Arabidopsis* lines and the wild type Col-0, (Mean ± ES; N = 6). Asterisks indicate significant differences between BX9 line and the other lines/WT (P < 0.005).

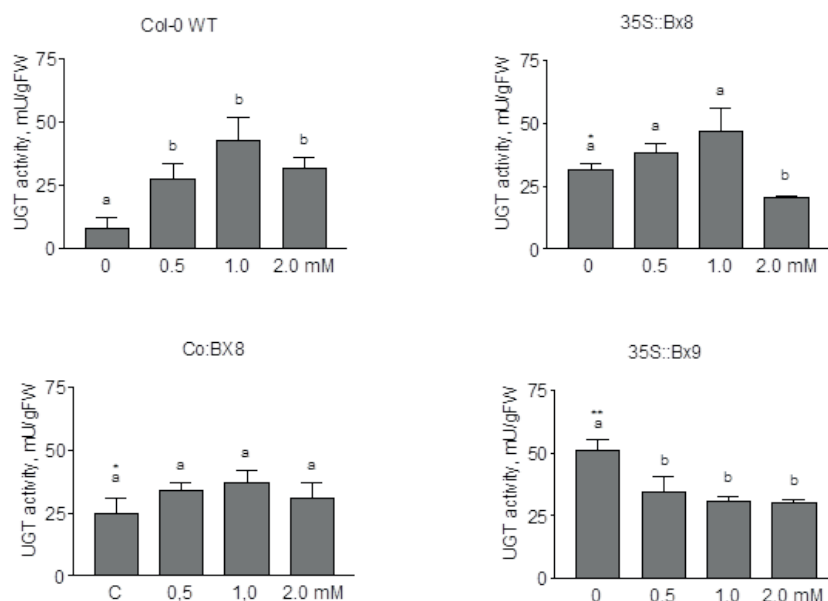


Figure 8. Root UGT activity profiles for BOA-6-O-glucosylation in the three transgenic lines and the WT after incubations no BOA and with different BOA concentrations. The BX9-line shows a different activity profile, compared to the wild type and the other transgenic lines (mean $\pm$ standard error; N = 6). Asterisks indicate significant differences between the WT and the different lines (P < 0.005). Means $\pm$ SE with different letters are significantly different within one line. Data sets of the different lines were analyzed by one-way ANOVA.

Discussion

The BOA-6-O-glucosylation pathway is a “ready to go” mechanism, important to detoxify BOA during the first hours of BOA exposure. It seems to be an important mechanism to avoid autotoxification when high amounts of MBOA are present in the root tip. Possibly the latter event is the major function of BX9 but not of BX8. BX9 was identified as a protein which can be nonconventional exuded and tightly bound to cell wall polymers (Schulz *et al.* in press). The latter behavior was not yet known when the purification of the BOA-6-OH glucosylating activity was started. Regarding the actual knowledge, the shifting from the cytosol to the extraplasmic space and concurrent breakdown of the cytosolic protein in two major fragments would explain first, the increasing occurrence of the cytosolic fragments during BOA incubations and secondly, the low enrichment of the enzyme during purification.

Since the addition of H₂O₂ had no influence on BOA-6-O-glucosylation, BOA-6-O-glucoside synthesis may be catalyzed by an already oxidized but unstable form of cytosolic BX9. Consequently, reducing sulfur compounds inactivated BOA-6-OH glucosylation as well as glutathionylation by GSSG. It is assumed that oxidation changes the enzyme

conformation and the sugar acceptor specificity, but finally destabilizes the enzyme and proteolytic fragmentation is easier possible.

BX9 is sensitive to oxidations eliciting substrate specificity alterations, and to nitrosylation and glutathionylation at -SH groups. As concluded from the 60% deactivation in presence of H₂O₂, the redox status is essential for acceptance of the substrate DIMBOA, which is corroborated by the experiments with GSNO and GSSG. At present, no redox-modulated glucosyltransferase with affinity to small sugar acceptor molecules is described. As a consequence of redox modulations the conformation of the enzyme might be changed and a number of structurally related molecules hitherto not accepted for substrates can be specifically used for glucosylation. Redox modulations of proteins are growingly recognized and are also assumed for apoplastic proteins (Go and Jones 2013; Cohen *et al.* 2014). Reversible glutathionylation was found to regulate actin polymerization and many other proteins (Wang *et al.* 2001). The modification could explain why recombinant BX9 failed in BOA-6-OH glucosylation. Another explanation for the failure could be a necessity of a tight interaction with a mono-oxygenase that introduces the hydroxyl group in position 6 of the BOA molecule. This would avoid the accumulation of toxic BOA-6-OH.

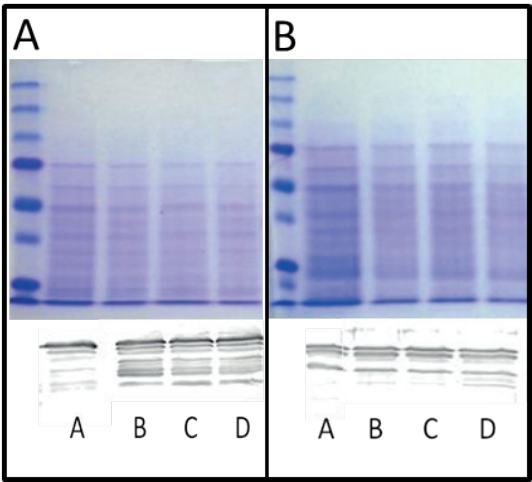


Figure 9. SDS-PAGE of root proteins from the transgenic BX8 (A) and BX9 (B) lines after 24h incubation with 0 (A), 0.5 (B), 1.0 (C) and 2.0 (D) mM BOA. The 0-control is next to the marker proteins. Lower line: Western blots of the BX8 (A) and BX9 (B) lines with polyclonal BX8- and BX9-antibodies. The latter react with the complete protein and degradation products of the proteins. Particularly BX8 shows massive degradation in BOA incubated roots.

Cleavage of cytosolic BX9 could be a key event in the switch to glucoside carbamate synthesis. Although the fragments have no catalytic function, the stability of the C-terminal fragment is surprising. This fragment contains the UDPG binding domain with the unusual feature of two neighbored cysteines. It can be speculated that the cysteines can serve as a redox sensor and the fragments have a redox reporter function during the detoxification process and are therefore not quickly degraded. Also reversible interactions with –SH groups of other proteins, including those of BX9, cannot be excluded. Moreover, vicinal thioles are thought to be involved in signal transduction (Cohen *et al.* 2014).

Concomitant glycosylation for detoxification of BOA-6-OH and performance of glycosylation of

DIBOA during the biosynthesis of DIMBOA-glc is clearly an advantage which was evolved by gene duplication in maize. In contrast, *Consolida regalis* has only one enzyme that has to fulfill both functions. It is postulated that during detoxification the biosynthesis of benzoxazinones is almost disrupted, but this needs further investigations.

The ability to glucosylate BOA-6-OH by induced or constitutive enzymes is rather wide-spread in plants. Corresponding glucosyltransferase activities were measured, for example, not only in protein extracts from *Arabidopsis thaliana*, but also with other species such as *Abutilon theophrasti* or *Avena sativa*. An increase of BOA-6-O- glucosylation activity after BOA incubation is also a common effect (Schulz *et al.* 2006; Haghi Kia *et al.* 2014). None of the above mentioned

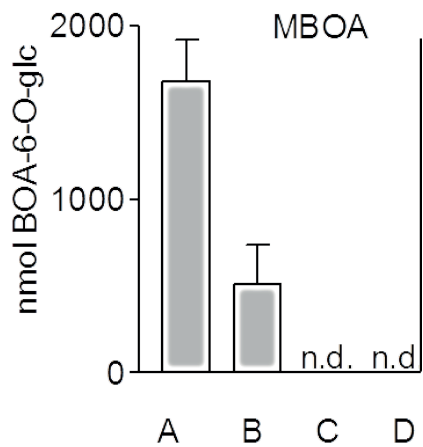


Figure 10. BOA-6-O-glucoside, one of the detoxification products of MBOA, is not produced in presence of methionine. The MBOA Incubations with 2.0 (A) and 0.5 mM MBOA (B,C,D); C + 1 mM methionine ; D +5 mM methionine.

species produces benzoxazinones, but all of them are able to perform BOA detoxification by BOA hydroxylation in position 6 and glucosylation of this intermediate, whereby *Hordeum vulgare*, *Lolium perenne* and *Avena sativa* switch later to glucoside carbamate production during the detoxification process.

Formation of N- and O-glucosides of xenobiotics is well known. For example, 3,4-dichloraniline detoxification via N- and O-glucosylation were found with glucosyltransferase GT72B1 from *Arabidopsis thaliana* suspension cultures and root cultures (Loutre *et al.* 2003). Sandermann *et al.* (1991) reported on N- and O-glucosylation of chlorinated anilines and phenols in soybeans due to distinct isozymes. Therefore, redox regulated substrate specificity might be not generally valid. On the other hand, in many studies, the natural substrates of the recombinant enzymes remain obscure. As stated by Bowles *et al.* (2005), the natural function of most of the enzymes studied by *in vitro* characterizations, often performed with recombinant proteins, remained extensively unknown. Redox modulations with pronounced effects on the substrate specificity and the mode of glucosylation may be one reason for the difficulties to pinpoint the natural function of recombinant glucosyltransferases. To our opinion, cytosolic UGTs are important when xenobiotics, including allelochemicals, have already passed the plasma membrane and entered the protoplast. An advanced detoxification strategy is to transfer enzymes needed for detoxification reactions into the extraplasmic space.

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References

- Baerson SR, Sanchez-Moreiras A, Pedrol-Bonjoch N, Schulz M, Kagan IA, Agarwal AK, Reigosa MJ, Duke SO. 2005. Detoxification and transcriptome response in *Arabidopsis* seedlings exposed to the allelochemical benzoxazolin-2(3H)-one. *J. Biol. Chem.* 280: 21867-21881
- Barrière Y, Méchin V, Lafarguette F, Manicacci D, Guillon F, Wang H, Lauresergues D, Pichon M, Bosio M, Tatout C. 2009. Toward the discovery of maize cell wall genes involved in silage maize quality and capacity to biofuel production. *Maydica* 54: 161-198
- Barrett WC, DeGnore JP, König S, Fales HM, Keng Y-F, Zhang Z-Y, Yim MB, Chook PB. 1999. Regulation of PTP1B via Glutathionylation of the active site cysteine 215. *Biochemistry* 38: 6699-6705
- Batish DR, Singh HP, Setia N, Kaur S, Kohli RK. 2006. 2-Benzoxazolinone (BOA) induced oxidative stress, lipid peroxidation and changes in some antioxidant enzyme activities in mung bean (*Phaseolus aureus*). *Plant Physiol. Biochem.* 44: 819-827
- Bowles D, Isayenkova J, Lim E-K, Poppenberger B. 2005. Glucosyltransferases: managers of small molecules. *Curr. Opin. Plant Biol.* 8: 254-263
- Burgos N R, Talbert R E, Kim K.S, Kuk YI. 2004. Growth inhibition and root ultrastructure of cucumber seedlings exposed to allelochemicals from rye (*Secale cereale*). *J. Chem. Ecol.* 30:671-689
- Cohen MF, Gurung S, Fukuto JM, Yamasaki H. 2014. Controlled free radical attack in the apoplast: A hypothesis for roles of O, N and S species in regulatory and polysaccharide cleavage events during rapid abscission by *Azolla*. *Plant Sci* 217: 120-126
- Dick R, Rattei T, Haslbeck M, Schwab W, Gierl A, Frey M. 2012. Comparative analysis of benzoxazinoid biosynthesis in monocots and dicots: independent recruitment of stabilization and activation functions. *Plant Cell* 24:915-28
- Frey M, Chomet P, Glawischnig E, Stettner G, Gruen S, Winklmaier A, Eisenreich W, Bacher A, Meeley RB, Briggs SP, Simcox K, Gierl A. 1997. Analyses of a chemical plant defense mechanism in grasses. *Science* 277: 696-699
- Friebe A, Roth U, Kück P, Schnabl H, Schulz M. 1997. Effects of 2,4-dihydroxy-1,4-benzoxazin-3-ones on the activity of plasma membrane H⁺-ATPase. *Phytochemistry* 44:979-983
- Glawischnig E, Gruen S, Frey M, Gierl A. 1999. Cytochrome P 450 monogenases of DIBOA biosynthesis: Specificity and conservation among grasses. *Phytochemistry* 50: 925-930
- Go Y-M and Jones D P. 2013. The Redox Proteome. *J Biol Chem* 288: 26512-26520
- Haghi Kia S, Schulz M, Ayah E, Schouten A, Müllenborn C, Paetz C, Schneider B, Hofmann D, Disko U, Tabaglio V, Marocco A. 2014. *Abutilon theophrasti*'s defense against the allelochemical benzoxazolin-2(3H)-one: Support by *Actinomyces elegans*. *J Chem Ecol* 40: 1286-1298
- Hofmann D, Knop M, Hao H, Hennig L, Sicker D, Schulz M. 2006. Glucosides from MBOA and BOA detoxification by *Zea mays* and *Portulaca oleracea*. *J Nat Prod* 69:34-37
- Hussain MI, Gonzalez L, Reigosa M.J. 2008. Germination and growth response of four plant species towards different allelochemicals and herbicides. *Allelopathy J.* 22:101-110
- Hussain MI, Reigosa M.J. 2011. Allelochemical stress inhibits growth, leaf water relations, PSII photochemistry, non-photochemical fluorescence quenching, and heat energy dissipation in three C₃ perennial species. *J Exp Bot* 62:4533-4545
- Kato-Noguchi H, Macias FA, Molinillo JM G. 2010. Structure-activity relationship of benzoxazinones and related compounds with respect to the growth inhibition and α -amylase activity in cress seedlings. *J Plant Physiol* 167:1221-1225
- Klatt P, Lamas S. 2000. Regulation of protein function by

- S-glutathiolation to oxidative and nitrosative stress. *Eur J Biochem* 264: 4928-4944
- Lindermayer C, Saalbach G, Durner J. (2005) Proteomic identification of S-nitrosylated proteins in *Arabidopsis*. *Plant Physiol* 137: 921-930
- Loutre C, Dixon DP, Brazier M, Slater M, Cole DJ, Edwards R, 2003. Isolation of a glucosyltransferase from *Arabidopsis thaliana* active in the metabolism of the persistent pollutant 3,4-dichloroaniline. *Plant J* 34: 485-493
- Macías FA, Oliveros-Bastidas A, Marín D, Castellano D, Simonet AM, Molinillo JMG. 2005. Degradation studies on benzoxazinoids. Soil degradation dynamics of (2R)-2-O-beta-D-glucopyranosyl-4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA-Glc) and its degradation products, phytotoxic allelochemicals from Gramineae. *J Agric Food Chem* 53: 554-561
- Niemeyer H M. 2009. Hydroxamic acids derived from 2-hydroxy-2h-1,4-benzoxazin-3(4H) one: key defense chemicals of cereals. *J. Agric. Food. Chem.* 57:1677-1696
- Ravanel S, Block MA, Rippert P, Jabrin S, Curien G, Rébeillé F, and Douce R 2004 Methionine metabolism in plants. Chloroplasts are autonomous for de novo methionine synthesis and can import S-adenosylmethionine from the cytosol. *J Biol Chem* 279: 22548-22557
- Sanchez-Moreiras AM, Reigosa MJ. 2005. Whole plant response of lettuce after root exposure to BOA (2(3H)-benzoxazolinone). *J Chem Ecol* 31: 2689-2703
- Sandermann H, Schmitt R, Eckey H, Bauknecht T. 1991. Plant biochemistry of xenobiotics – isolation and properties of soybean O-glucosyl and N-glucosyl and O-malonyltransferase and N-malonyltransferase for chlorinated phenols and anilines. *Arch Biochem Biophys* 287: 341-350
- Schulz M, Wieland I. 1999. Variations in metabolism of BOA among species in various field communities - biochemical evidence for co-evolutionary processes in plant communities? *Chemoecology* 9:133-141
- Schulz M, Knop M, Kant S, Sicker D, Voloshchuk N, Gryganski A. 2006. Detoxification of allelochemicals - the case of benzoxazolin-2(3H)-one (BOA). In: *Allelopathy: a physiological Process with ecological implications*. (Reigosa, M.J., Pedrol, N., Gonzales, L. eds) Springer Dordrecht, The Netherlands. Pp. 157-170
- Schulz M, Sicker D, Baluska F, Sablofski T, Scherer HW, Ritter FM. Benzoxazolinone detoxification and degradation – A molecule's journey. In: *Abd El-Ghany Hasaneen MN (ed) Herbicides - Properties, Synthesis and Control of Weeds*. InTech, Rijeka, Croatia, 2012a. P 17-42
- Schulz M, Tagaglio V, Marocco A, Macias FA, Molinillo JMG. 2013. Benzoxazinoids in rye allelopathy – from discovery to application in sustainable weed control and organic farming. *J Chem Ecol* 39: 154-174
- Schulz M, Marocco A, Tagaglio V. 2012b. BOA detoxification of four summer weeds during germination and seedling growth. *J Chem Ecol* 38:933-946
- Schulz M, Filary B, Kühn S, Colby T, Harzen A, Schmidt J, Sicker D, Hennig L, Hofmann D, Disko U, Anders N. Benzoxazolinone detoxification by N-Glucosylation: The multi-compartment- network of *Zea mays* L. 2015, accepted
- Schulz M, Filary B, Kühn S, Colby T, Harzen A, Schmidt J, Sicker D, Hennig L, Hofmann D, Disko U, Anders N. 2016. Benzoxazolinone detoxification by N-Glucosylation: The multi-compartment- network of *Zea mays* L. *Plant Sign.& Behav.* <http://dx.doi.org/10.1080/15592324.2015.1119962>
- Sicker D, Schneider B, Hennig L, Knop M, Schulz M. 2001. Glucoside carbamate from benzoxazolin-2(3H)-one detoxification in extracts and exudates of corn roots. *Phytochemistry* 58: 819-825
- Sicker D, Schulz M. 2002. Benzoxazinones in plants: Occurrence, synthetic access, and biological activity. *Studies in Natural Products Chemistry* 27, 185-232
- Sicker D, Hao H, Schulz M. 2004. Benzoxazolin-2-(3H)-ones. Generation, Effects and detoxification in the competition among plants, In: *Recent Advances on Allelopathy Vol. II*. (F.A. Macias, Galindo, J.C.G., Molinillo, J.M.G., Cutler, H.G., eds.), CRC Press, Boca Raton, Florida USA, pp 77-102
- Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Mann M. 1996. Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proc Natl Acad Sci USA* 93: 14440-14445
- Van Der Mijsbrugghe K, Meyermans H, Van Montagu M, Bauw G. Boerjan W. 2000 Wood formation in poplar: identification, characterization, and seasonal variation of xylem proteins. *Planta* 210: 589-598
- Venturelli S, Belz RG, Kämper A, Alexander Berger, von Horn K, Wegner A, Böcker A, Zabulon G, Langenecker T, Kohlbacher O, Barneche F, Weigel D, Lauer UM, Bitzer M, Becker C. 2015. Plants release precursors of histone deacetylase inhibitors to suppress growth of competitors. www.plantcell.org/cgi/doi/10.1105/tpc.15.00585
- Von Rad U, Hüttel R, Lottspeich F, Gierl A, Frey M. 2001. Two glucosyltransferases involved in detoxification of benzoxazinoids in maize. *The Plant J.* 28: 633-642
- Wieland I, Kluge M, Schneider B, Schmidt J, Sicker D, Schulz M. 1998. 3-β-D-Glucopyranosyl-benzoxazolin-2(3H)-one- A detoxification product of benzoxazolin-2(3H)-one in oat roots. *Phytochemistry* 48:719-722
- Wang J, Boja ES, Tan W, Tekle E, Fales HM, English S, Mieyal JJ, Chock PB. 2001. Reversible glutathionylation regulates actin polymerization in A431 cells. *J Biol.Chem* 276: 47763-47766
- West MB, Hil I BG, Xuan YT, Bhatnagar A. 2006. Protein glutathiolation by nitric oxide: an intracellular mechanism regulating redox protein modification. *FASEB J* 20: 1715-1717

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