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RESEARCH PAPER

Benzoxazolinone detoxification by *N*-Glucosylation: The multi-compartment-network of *Zea mays* L.

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ABSTRACT

The major detoxification product in maize roots after 24 h benzoxazolin-2(3*H*)-one (BOA) exposure was identified as glucoside carbamate resulting from rearrangement of BOA-*N*-glucoside, but the pathway of *N*-glucosylation, enzymes involved and the site of synthesis were previously unknown. Assaying whole cell proteins revealed the necessity of H_2O_2 and Fe^{2+} ions for glucoside carbamate production. Peroxidase produced BOA radicals are apparently formed within the extraplastic space of the young maize root. Radicals seem to be the preferred substrate for *N*-glucosylation, either by direct reaction with glucose or, more likely, the *N*-glucoside is released by glucanase/glucosidase catalyzed hydrolysis from cell wall components harboring fixed BOA. The processes are accompanied by alterations of cell wall polymers. Glucoside carbamate accumulation could be suppressed by the oxireductase inhibitor 2-bromo-4′-nitroacetophenone and by peroxidase inhibitor 2,3-butanedione. Alternatively, activated BOA molecules with an open heterocycle may be produced by microorganisms (e.g., endophyte *Fusarium verticillioides*) and channeled for enzymatic *N*-glucosylation. Experiments with transgenic *Arabidopsis* lines indicate a role of maize glucosyltransferase BX9 in BOA-*N*-glycosylation. Western blots with BX9 antibody demonstrate the presence of BX9 in the extraplastic space. Proteomic analyses verified a high BOA responsiveness of multiple peroxidases in the apoplast/cell wall. BOA incubations led to shifting, altered abundances and identities of the apoplast and cell wall located peroxidases, glucanases, glucosidases and glutathione transferases (GSTs). GSTs could function as glucoside carbamate transporters. The highly complex, compartment spanning and redox-regulated glucoside carbamate pathway seems to be mainly realized in Poaceae. In maize, carbamate production is independent from benzoxazinone synthesis.

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Introduction

Benzoxazinoids (benzoxazinones and benzoxazolines) are characteristic secondary products of several *Poaceae*, including the cereals rye, wheat and maize, as well of a few dicots.[1-7] The compounds possess a broad bioactivity, whereby the insecticidal properties are well described,[8] e.g., DIMBOA was found to be associated with maize resistance against the European corn borer, *Ostrinia nubilalis*.[9-11] Benzoxazinoids and their derivatives, the phenoxazinones, are also allelochemicals that can be used in sustainable agricultural systems for weed suppressing.[12] However, the successful application depends on the weed species, and on geno- and ecotypes of some species. Some weeds, as well as many crops, have developed strategies to overcome growth inhibition caused by benzoxazinoids present in mulches, for instance of rye.[13,14]

In maize numerous benzoxazinoids have been identified. Among them, DIMBOA and HMBOA are the most abundant benzoxazinones, whereas MBOA is the most important benzoxazolinone. Others, such as DIBOA, are found in low concentrations.

Benzoxazinone biosynthesis has been thoroughly elucidated.[7,15,16] The biosynthesis includes the glucosylation of DIBOA to DIBOA glucoside, the precursor of DIMBOA glucoside. The glucosylation is catalyzed by 2 glucosyltransferases, BX8 and BX9, whereby BX9 is a duplicate of BX8.[17] The compounds are stored in the vacuole as glucosides, until they are exuded or they are hydrolyzed upon damage of the tissue by herbivores. The release of the aglucones is catalyzed by 2 specific β-glucosidases, Zm-GLU1 and Zm-GLU2, which were found in the vacuole and the plastids.[7,18]

For benzoxazinone-containing species, it is essential to develop mechanisms against autotoxification caused by self-produced phytotoxins. Maize has developed 2 different detoxification pathways for BOA and MBOA, resulting in the detoxification products BOA-6-O-glucoside, methoxyglucoside carbamate and glucoside carbamate, the latter arise from rearrangements of the *N*-glucosylated benzoxazolinones BOA and MBOA (Fig. 1). Since peroxidases are known to catalyze

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demethoxylation reactions which release methanol and the demethylated compound,[19] the intermediate BOA-6-OH may be a product of MBOA demethylation. BOA can get hydroxylated by enzymatic and non-enzymatic reactions. For instance, BOA-6-OH occurs during Fenton reactions with BOA.[14] Demethoxylation leads to BOA.

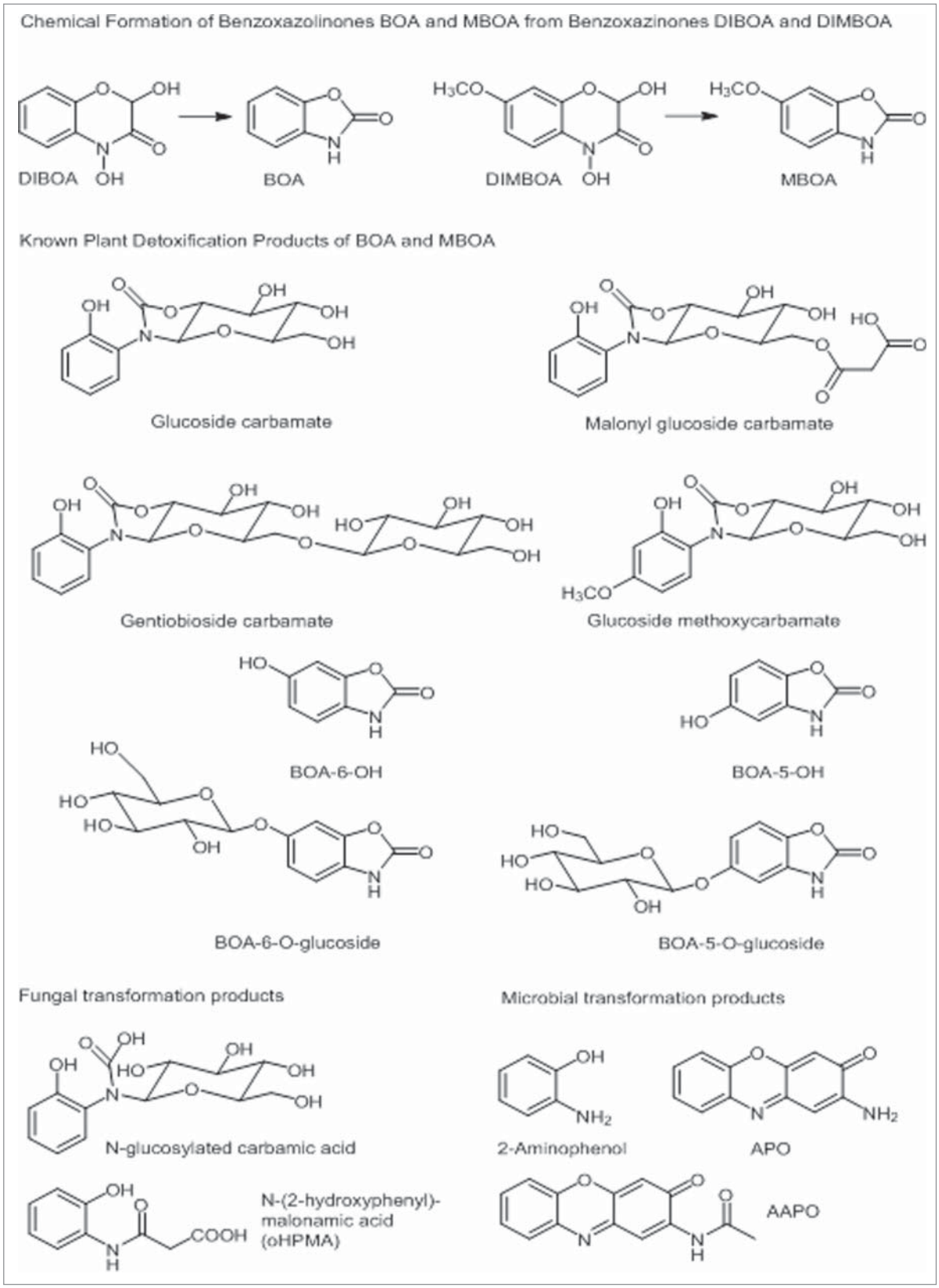

Figure 1. Structures of compounds mentioned in the text. Known BOA / MBOA plant detoxification products and the fungal degradation product of glucoside carbamate, *N*-glucosylated carbamic acid. N-(2-hydroxyphenyl)malonamic acid (oHPMA) is a microbial degradation product of BOA, which requires BOA heterocycle ring opening as first step of the reaction sequence. N-(3-oxo-3*H*-phenoxazin-2-yl)-acetamide (AAPO) is a fungal product derived from BOA degradation via 2-aminophenol and N-(3-oxo-3*H*-) phenoxazin-2-one.

Glucoside carbamate as a major detoxification product after BOA exposure is not only found in maize but in many other grasses, including those which do not contain benzoxazinoids. BOA-5-*O*-glucoside and in particular the isomer BOA-6-*O*-glucoside are more widespread in dicots, which often lack glucoside carbamate almost completely.[3,13,20-23] In contrast to easily hydrolysable BOA-6/5-*O*-glucoside, glucoside carbamate is not toxic up to concentrations of 1 mM. In maize, glucoside carbamate is subsequently modified by malonylation.[23] In plants, glucoside methoxycarbamate was presently only found in maize.

The BOA-detoxification process in BOA exposed maize roots starts with the production of BOA-6-*O*-glucoside. Depending on the cultivar and environmental conditions, glucoside carbamate can be detected in methanolic extracts as soon as 6–8 h after start of exposure. After 12–24h, this compound becomes the major extractable detoxification product, whereas the BOA-6-*O*-glucoside level stays constant or tends to decrease. Finally, malonyl-glucoside carbamate accumulates after 18–20 h as a late event in the detoxification process. The malonylated product becomes the major product after 48h. Another minor product is gentiobioside carbamate which is not always present. A portion of the detoxification products was found to be exuded during the next days after termination of the incubation.

Among the enzymes thought to be responsible for the different detoxification steps are oxi-reductases, glucosyltransferase (s) and malonyltransferase(s). However, the reaction sequence of the glucoside carbamate detoxification pathway is unknown, as are the sites where these steps can be linked. In addition, it is still unclear whether enzymes necessary for the biosynthesis, are recruited from other metabolic pathways or are specific for the carbamate detoxification pathway. In this work we focused on the elucidation of the highly complex pathway leading in maize to glucoside carbamate, the putative compartmentation of the synthesis and on a role of the endophyte *Fusarium verticillioides*. Results presented will help to understand how plants can withstand the inhibitory effects of benzoxazolinone. Such knowledge is important, when benzoxazinone derived allelochemicals will be utilized for weed control.

Results

Glucoside carbamate accumulation in transgenic arabidopsis lines

To get hints, whether the glucosyltransferases BX9, BX8 (maize) or Co-BX8 (*Consolida orientalis*) are not only the glucosylating enzymes in BX biosynthesis but catalyze also the glucosylation steps in the glucoside carbamate detoxification pathway, the transgenic *Arabidopsis* lines 35S::Bx8, 35S::Bx9 and 35S::Co-Bx8 were studied. *Arabidopsis* belongs to the dicots with a weaker developed ability to detoxify BOA. The major detoxification happened by BOA hydroxylation and subsequent glucosylation. Often, toxic BOA-6-OH accumulated as well, whereas glucoside carbamate was only marginally produced. Analyses of the methanolic extracts prepared from transgenic *Arabidopsis* plants revealed a significantly higher (2–3 fold) glucoside carbamate accumulation in the BX9–line compared to the wild type or

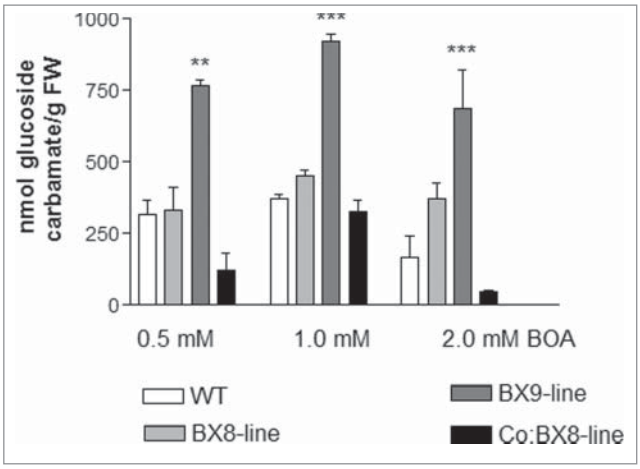

Figure 2. Accumulation of glucoside carbamate in the *Arabidopsis* lines 35S::Bx8, 35S::Bx9 and 35S::Co-Bx8 and the Wild Type after 24 h incubation with 0.5, 1.0, and 2.0 mM (250 ml/12 plants). Means ± SD are shown, asterisks indicate significant differences compared with the wild type Col-0 (*t*-test, ** $p \leq 0.001$; *** $p \leq 0.0001$).

BX8/Co-BX8 plants, but levels found in maize roots were never reached (Fig. 2; compare data later in the text). In the Co-BX8 line only a low amount of glucoside carbamate after incubation with 0.5 and 2.0 mM BOA was found, whereas the amount accumulating with 1.0 mM was similar to the wild type and the BX8 line. Thus, the benzoxazinoid-specific glucosyltransferase of *Consolida orientalis* and the maize UGT BX8 are less suitable for *N*-glucosylation.[7]

The experiments with the transgenic lines indicated that BX9 may account for the glucosylation step leading to BOA-*N*-glucoside/ glucoside carbamate, but the biosynthetic mechanism remained unclear. In recent studies, the *N*-glucosylating activity was found to be very low in the cytosolic protein fractions from maize. However, cytosolic BX9 catalyzes BOA-6-OH glucosylation (data not shown). With cytosolic protein fractions prepared from 35S::BX9 *Arabidopsis* roots or recombinant BX9, BOA-*N*-glucosylation was not possible. These findings led to several questions: 1. is it necessary to activate BOA molecules prior to glucosylation, 2. which cellular compartment is the site of carbamate production and 3. are there shifts of enzymes from the cytosol to the compartment where BOA-*N*-glucosylation takes place and are there alterations in enzymes abundances? It is also unclear whether BOA detoxification via glucoside carbamate is coupled with benzoxazinone synthesis.

Analyses of cell wall fractions and incubation media

A series of experiments was started to elucidate whether the extraplastic space could be the site of glucoside carbamate synthesis. Cell wall fractions from maize root tips were prepared after 6 and 24 h BOA incubation and after incubations without BOA. The 6 h BOA fraction and all control fractions contained only MBOA, the 24 h BOA fraction, however, glucoside carbamate, DIMBOA-glucoside, DIMBOA and about 100% more MBOA (Fig. 3). Assays of the salt extracted, rigorously washed and resuspended cell wall fraction with UDPglucose and BOA did not yield soluble glucoside carbamate, indicating the

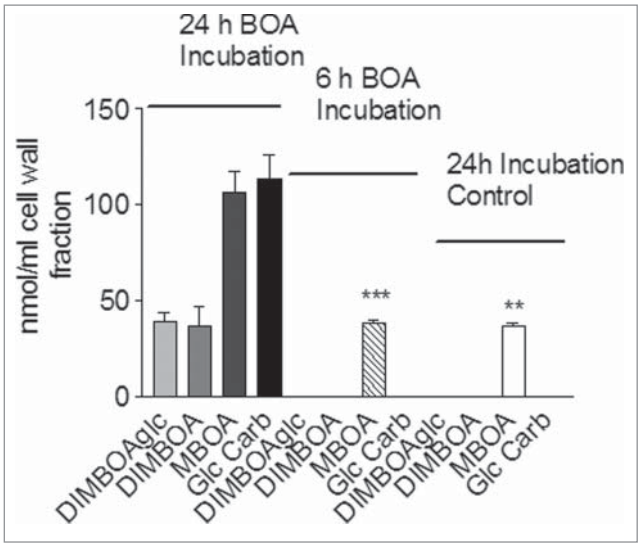

Figure 3. Benzoxoxazinone / benzoxazolinone contents of the cell wall fraction after 6 and 24 h incubation with BOA in comparison to the control. Glc Carb = glucoside carbamate. MBOA content after 24 h incubation with BOA is significantly different from the contents when the incubation was started and after 6 h (means ±SD, *t*-test $^{**}p \leq 0.001$; $^{***}p \leq 0.0001$).

participation of additional factors and that BOA might not be the direct substrate for glucoside carbamate synthesis.

BOA presents a compound which is chemically rather inert, due to the very low reactivity of the heterocyclic nitrogen neighbored to the C=O group. Heterocyclic ring opening, which results in the instable intermediate carbamic acid, is necessary for further transformations. Heterocycle ring cleavage was hypothesized for the synthesis of all known benzoxazinone and benzoxazolinone derived detoxification products.[24,25] For BOA, a hydrolysis of the lactone bond is assumed, resulting in carbamic acid. Consequently, N-glucosylation of BOA or MBOA should be not possible with the closed heterocycle or the molecule must be otherwise activated, for instance by radical formation.

Plant incubation media were analyzed to obtain hints whether microorganisms may produce a potential BOA-*N*-glucoside precursor. Analyses after 24 h of incubation revealed large amounts of the fungal BOA detoxification product oHPMA (*N*-(2-hydroxyphenyl)malonamic acid) and *N*-(3-oxo-3*H*-phenoxazin-2-yl)-acetamide (AAPO). The presence of the latter compound indicated oxidative processes in the rhizosphere and of microbial activities necessary for BOA heterocycle cleavage. To test an involvement of responsive heme containing proteins and lactonohydrolases, the effector 2-bromo-4′-nitroacetophenone, BNAP was supplemented to the medium.[26,27] BNAP is known to inhibit particularly certain heme containing enzymes such as monooxygenases but also some lactonohydrolases whereas others are activated. HPLC analyses of the media revealed a complete different pattern of compounds when the incubation was carried out in presence of 500 μM BNAP (Fig. 4).

No oHPMA or 2-aminophenoxazinones, but instead several benzoxazinoides, mainly DIMBOA, HMBOA (2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one) as well as the benzoxazolinones were present after 17 h of incubation. The content of BOA in the incubation medium stayed high, indicating that transformation reactions were inhibited. The presence of HMBOA (t_R = 5.09 min; $[M+H]^+$ = 196 Da, $[M+Na]^+$ = 218 Da) in incubation media supplemented with BNAP was ascertained by HPLC-PDA-MS. Aside from MBOA (t_R = 5.30 min; $[M+H]^+$=166 Da, $[M+Na]^+$=188 Da), DIMBOA (t_R = 2.35 min; $[M+H]^+$ = 212 Da, $[M+Na]^+$ = 234 Da; $[M+K]^+$ = 250 Da) and BOA (t_R = 4.85 min; $[M+H]^+$ = 136 Da) were identified. HMBOA was distinguished from its constitutional isomer M_2BOA (same molecular mass and sum formula) by its DAD spectrum (λ_{max} M_2BOA 282; HMBOA 268, sh292) and was further supported by the MS/MS spectrum (suppl. Table 1). BNAP did not lead to bromination of any compound.

Glucoside carbamate was detectable in the medium with a lower BNAP concentration (30 μM), (Fig. 5). The result indicates that defined concentrations of BNAP led to an inhibition of enzymes necessary for BOA heterocycle cleavage, yielding 2-aminophenol. Therefore, the syntheses of oHPMA and phenoxazinones are affected. The experiment indicates that HMBOA is, like DIBOA, a precursor for amino-3*H*-phenoxazin-3-one, as proposed by Zikmundová et al.[25] Fungal lactone cleavage of the glucoside carbamate molecule is apparently also inhibited.

2-Bromo-4′-nitroacetophenone affects the glucoside carbamate accumulation

Glucoside carbamate accumulation in the root was influenced by low BNAP concentration, whereas 100μM in the medium was a crucial concentration in combination with the time of exposure. Lower concentrations and shorter incubation times yielded a more than 2 fold increase of glucoside carbamate in the roots. On the other hand, higher BNAP concentrations also block the glucoside carbamate synthesis totally (Fig. 6). These findings are in line with the analyses of the incubation media. Although we could not identify a precursor molecule for glucoside carbamate synthesis, the study revealed the involvement of BNAP sensitive heme group containing redox enzymes. Incubations with MBOA resulted in high amounts of MBOA-*N*-glucoside and glucoside methoxycarbamate, which was completely inhibited in presence of BNAP (data not shown). Thus, both benzoxazolinones should be converted into *N*-glucosylated detoxification products by the same pathway reactions.

Fusarium verticillioides may produce an instable BOA-N-glucoside precursor

Unstable carbamic acid from BOA heterocycle cleavage may be produced by enzymatic activities of the fungal endophyte *Fusarium verticillioides*. A stable precursor molecule for glucoside carbamate synthesis could not be isolated from the culture medium. Only *N*-(3-oxo-3*H*-phenoxazin-2-yl)-acetamide (AAPO) was identified in the fungal culture media supplemented with BOA (compare MS and NMR analyses, Material and Methods), indicating that BOA heterocycle cleavage and subsequent decarboxylation of carbamic acid to 2-aminophenol took place. If carbamic acid is a precursor of BOA-*N*-glucoside,

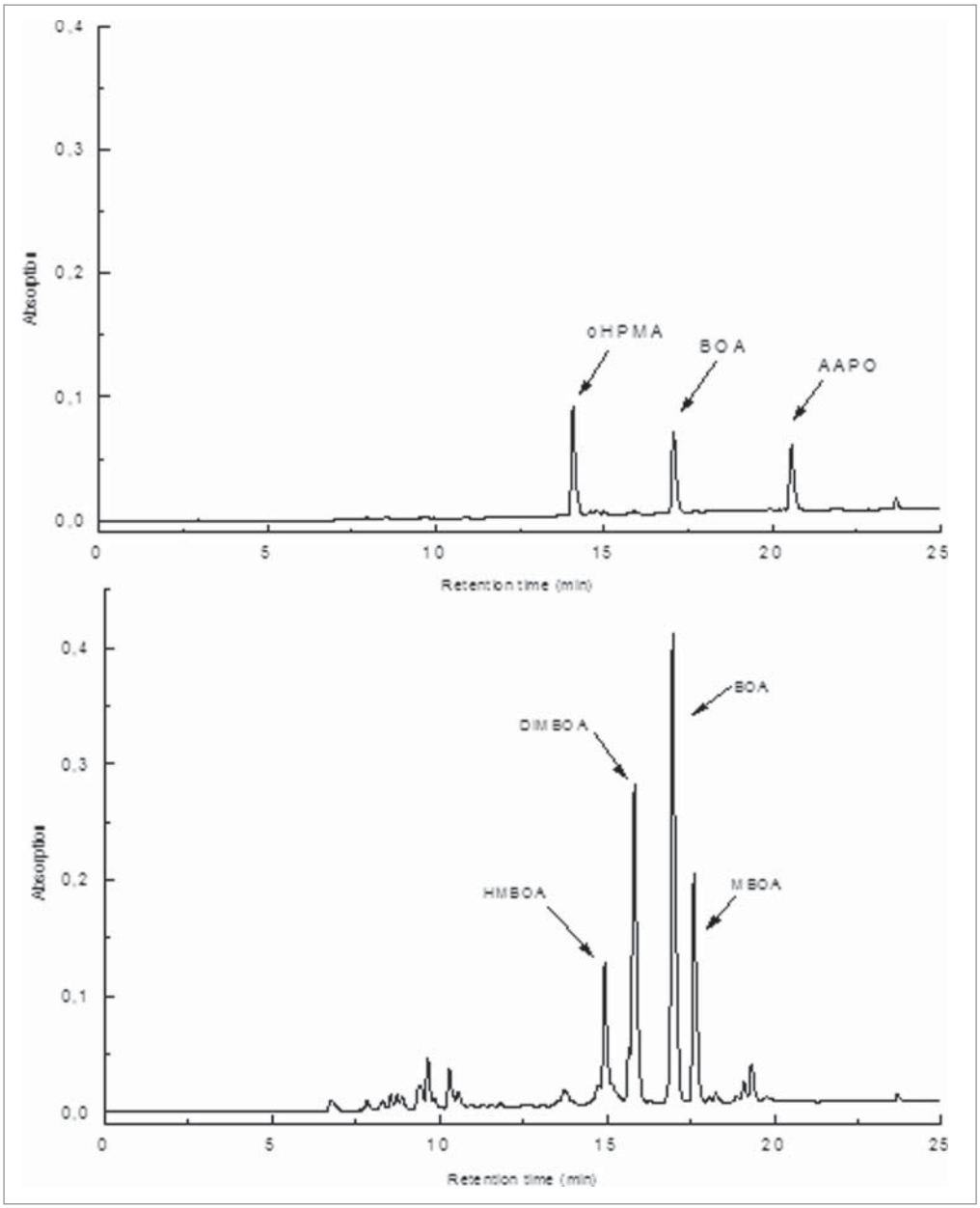

Figure 4. HPLC chromatograms of BOA media after 24 h incubation of maize seedlings. Above: Medium without BNAP (control), below medium with 500 μM BNAP. The inhibitor prevented the degradation/detoxification of benzoxazinones (HMBOA, DIMBOA) and benzoxazolinones (BOA, MBOA). The fungal BOA degradation product oHPMA (N-(2-hydroxylphenyl)malonamic acid) and the phenoxazinone, found in the control medium are not produced in presence of the inhibitor.

it has to be glucosylated in a fast channeling process to avoid decarboxylation and by masking reactive hydroxyl groups.

Effects of peroxidase inhibitor 2,3-butanedione

When the seedlings were incubated with 1 mM of the known peroxidase inhibitor 2,3-butanedione and BOA, the extractable amount of glucoside carbamate is significantly reduced (Fig. 7).[28] With regard to BOA-*N*-glucosylation, it is likely that peroxidases fulfill a function in glucoside carbamate production. These enzymes could be responsible for the generation of BOA radicals. The strong response to the inhibitor led again to the assumption that peroxidase generated BOA radicals are the major substrates for BOA-*N*-glucosylation. The versatile functions of peroxidases have been, for instance, investigated by Chen and Schopfer. [29]

Assays with highly concentrated whole cell protein extracts

Since all experiments indicate that the detoxification is a multicompartment process, we tested whether glucoside carbamate is produced when highly concentrated, whole cell protein extracts are used for assays. Reactions were run at pH 6 and in presence of BOA, UPDG, Fe^{2+} and H_2O_2. Assays with such protein extracts from roots after BOA incubation revealed considerable amounts of glucoside carbamate after 60 min incubation time, whereas in overnight assays, glycoside carbamate

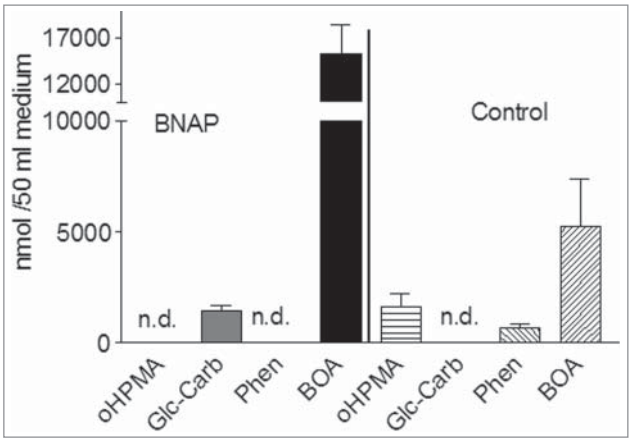

Figure 5. Compounds detected in incubation media supplemented with 30 μM BNAP and in control media. BNAP led to an accumulation of glucoside carbamate and a reduced degradation rate of BOA. In the controls, the fungal detoxification product oHPMA, AAPO (Phen) and only 50% of the applied BOA were found.

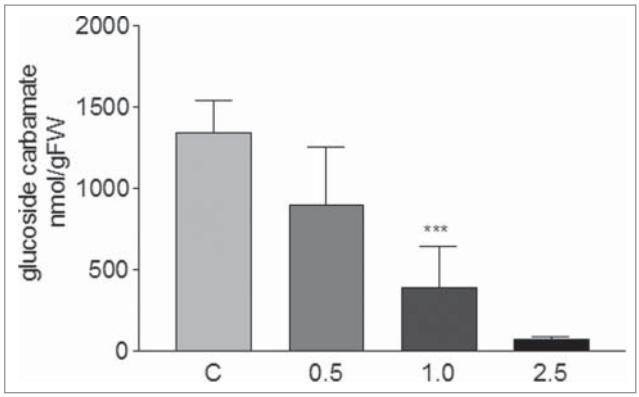

Figure 7. Reduced glucoside carbamate contents in roots which were incubated with 0.5, 1.0 and 2.5 mM 2,3-butanedione added to the BOA medium. Means ± SD are shown, asterisks indicate significant differences compared with the control (*t*-test, ** $p \leq 0.001$; ***$p \leq 0.0001$). 2.5 mM 2,3-butanedione led to almost no glucoside carbamate, but since 2.5 mM started to affect benzoxazinone biosynthesis, results obtained with 2.5 mM were not further considered. Means ± SD are shown, asterisks indicate significant differences compared with the control (*t*-test, ***$p \leq 0.0001$).

increased drastically (Fig. 8). The presence of peroxidase inhibitor 2,3 butandione in the assay mixtures completely prevented the generation of soluble glucoside carbamate. The presence of laminarin decreased the amount of glucoside carbamate. The disaccharide cellobiose had no effect (not shown). Laminarine either interfere with the pathway, for instance by quenching H_2O_2, or serve for fixation of BOA radicals. Without the addition of Fe^{2+}, the amount of glucoside carbamate is noticeably reduced and the absence of H_2O_2 prevented synthesis. These studies show again that peroxidases and also Fe ions have an essential role in glucoside carbamate production and that detoxification via glucoside carbamate production is a multicompartment process.

Presence of BX9 in the extraplastic space

As afore mentioned, cytosolic glucosyltransferase BX9 could be a candidate for catalyzing the *N*-glucose linkage. However, a contribution of this enzyme requires an altered compartmentation. Therefore, maize cell wall preparations were checked for the presence of BX9. Western blots with polyclonal BX9 antibodies indicated the presence of BX9 in the cell wall fraction and the apoplastic fluid prepared from BOA incubated maize roots (Fig. 9). BX9 was also found in the controls. In contrast to the cytosolic extract, the cell wall and the apoplastic fluid fractions contained, if at all, only low amounts of 2 stable BX9 degradation fragments which occur in high abundance in the cytosolic protein fraction after BOA incubations (Fig. 9). Once secreted, it is also possible that BX9 is tightly attached mainly to cell wall polysaccharides.

Analyses of apoplast fluid and cell wall proteins related to the glucoside carbamate pathway

The involvement of peroxidases in glucoside carbamate production was supported by analyses of apoplast fluid and cell wall proteins prepared from BOA incubated roots and controls, respectively. Most of the proteins identified hereafter have been described to be cell wall associated.[30] The proteomic analyses showed that many enzymes undergo shifting in response to

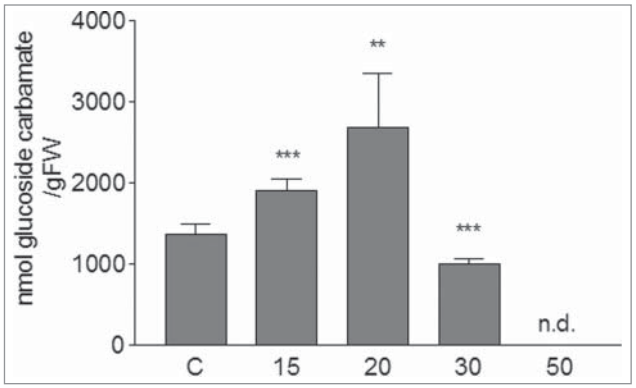

Figure 6. Influence of BNAP concentrations on glucoside carbamate accumulation in the root. An increase was detected with 15 and 20 μmol BNAP. 30 μmol of the effector reduced the amount of glucoside carbamate, 50 μmol led to a complete inhibition. Means ± SD are shown, asterisks indicate significant differences compared with the control (*t*-test, ** $p \leq 0.001$; ***$p \leq 0.0001$).

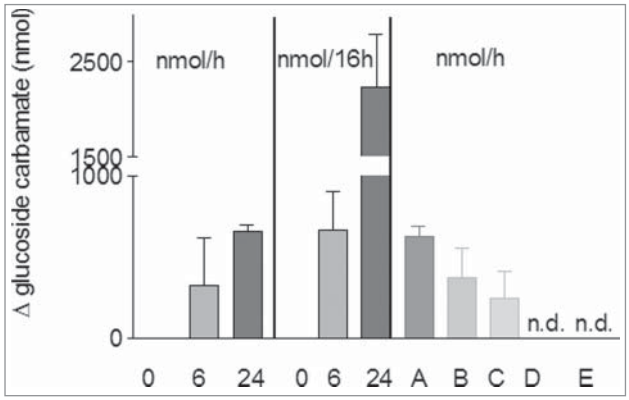

Figure 8. Glucoside carbamate synthesis within 60 min and in overnight-assays with highly concentrated total cell protein extracts. A: whole protein fraction from roots of 24 h BOA incubated seedlings, H_2O_2, Fe^{2+}. B: whole protein fraction + apoplast fraction + cellobiose, laminarin and cell wall material, H_2O_2, Fe^{2+}, C: as A, but without Fe^{2+}. D: as A but with 1 mM 2,3-butanedione; E: as A but without H_2O_2. A-E: assays were incubated for 1 h at 30°C.

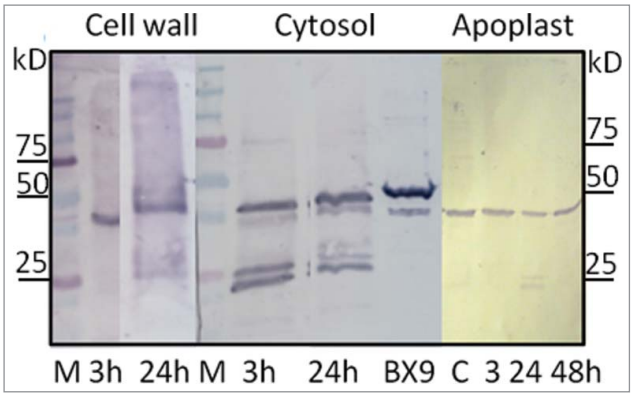

Figure 9. Western blots of cell wall, cytosolic and apoplast associated BX9. Fragments of BX9 are mainly located in the cytosol. Lane BX9: control, recombinant protein; M: marker proteins, protein mass in k_D. Samples were prepared after 3 and 24 h of BOA incubation, and an additional apoplastic sample after 48 h. The recombinant BX9 protein shows a major band at 42 kD and a weak degradation product at 40 kD. These bands are seen in the cytosol samples together with 2 other degradation products at 23 and 21 k_D. In the apoplast samples only the 40 k_D band is present, which also the major immune stained band in the wall samples.

BOA exposure. There are indications of an altered distribution of the proteins, the occurrence of new enzymes and the elimination of others, compared to the control.

1. Enzymes involved in carbohydrate-related reactions - In the apoplastic fluid of the controls, endo-1,3;1,4-β-D-glucanase GRMZM2G076348_P01 and exo-β-glucanase GRMZM 2G120962 were present, but the abundance was higher after BOA incubation. Glucan-endo-1,3-β-D- glucosidase GRMZ M2G111143 and exohydrolase II GRMZM2G017186 could be only detected in BOA samples (Table 1; suppl Table 2, Fig. 10). In turn, a protein belonging to the glycosylhydrolase family, GRMZM2G115065 was only found in the control. GDIMBOA glucosidase Zm GLU1 (GRMZM2G016890) was strongly present in the control but less in the BOA sample. Glucosyltransferase BX9 was only identified with low abundance in the apoplastic fluid of control plants but not in any other sample. Since this result is not in agreement with the immunological findings, the enzyme might be strongly attached to cell wall carbohydrates and amounts obtained during sample preparations were too low to allow proper identification by proteomic studies. In the cell wall preparations, β-glucosidase GRMZM2G118003 and endo1,3;1,4-β-D-glucanase GRMZM 2G076348_P01 was higher in the control. Thus, GRMZM 2G076348_P01 seems to be shifted into the apoplastic fluid. Glycosyl hydrolase GRMZM2G115065 and UDP-glucose-6-dehydrogenase GRMZM2G409642 were only detected in the control. Xyloglucan-endo-transglucosylase GRMZM 2G004699 was unchanged. BX9 was neither found in the cell wall samples of the controls nor in BOA samples. A possible explanation could be again a tight binding of the enzyme to cell wall carbohydrates, which may reduce the amounts drastically when protein samples were prepared for proteome analysis. Such limitations in cell wall protein analyses are known.[31] In the BOA cell wall samples, carbohydrate related enzymes are either unchanged or reduced.

3. Peroxidases - In all samples, peroxidases showed the highest diversity with a strong response to BOA treatment. BOA samples completely lacked several putative class III secretory peroxidases, which were highly abundant in the control. Three peroxidases were identified solely in the BOA samples. Cell walls, which were rigorously washed before salt extraction, showed as well a different abundance/distribution of peroxidases (Table 1, suppl. Table 2, Fig. 11). Three class III peroxidases (GRMZM2G044049, GRMZM2G048474 and GRMZM2G168073) and peroxidases GRMZM2G108219 and 410175 were only found in the apoplastic fluid of the control samples. The class III peroxidases have a function in lignification and stress reactions.[32] Peroxidases GRMZM2G108153 and GRMZM2G108207 were strongly reduced. In contrast, the peroxidases AC197758.3_FGP004, GRMZM2G070603 and GRMZM2G405459 were only detected in the apoplastic fluid of BOA samples, GRMZM2G070603 was found also in BOA cell wall samples but not in the control. Most of the peroxidases present in BOA samples showed a higher abundance than found in the controls. These enzymes have functions in redox processes and oxidative stress responses. GRMZM2G405459, a class III peroxidase with a role in phenoxyl radical formation (CornCyc MaizeCyc), was found in the apoplastic fluid only after BOA incubation. GRMZM2G405459 was also present in all cell wall samples. In the BOA cell wall fraction the abundance of several peroxidases was reduced or they disappeared

Table 1. BOA related occurrence of peroxidases and GSTs of the apoplastic fluid and the cell wall, with a possible function in the glucoside carbamate pathway.

Plant Proteins Peroxidases Apoplastic fluid		
AC197758.3_FG004	class III Peroxidase* (new)	phenoxyl radical production
GRMZM2G070603	Peroxidase* (new)	phenoxyl radical production
GRMZM2G405459	class III Peroxidase* (new)	phenoxyl radical production
Salt extractable Cell Wall Proteins		
GRMZM2G070603	Peroxidase*	phenoxyl radical production
GRMZM2G405459	class III Peroxidase* (increased)	phenoxyl radical production
GRMZM2G023840	peroxidase* (strongly increased)	phenoxyl radical production
GRMZM2G410175	peroxidase* (strongly increased)	phenoxyl radical production
Glutathione transferases		
Apoplastic Fluid		
GRMZM2G116273	Glutathione transferase *(increased)	GSH-toxin conjugation
GRMZM2G132093	Glutathione transferase * (increased)	GSH-toxin conjugation
Salt extractable Cell Wall Proteins		
GRMZM2G119499	putative Glutathione transferase* (new)	GSH-toxin conjugation

* involvement of enzyme class ascertained by inhibitor studies.

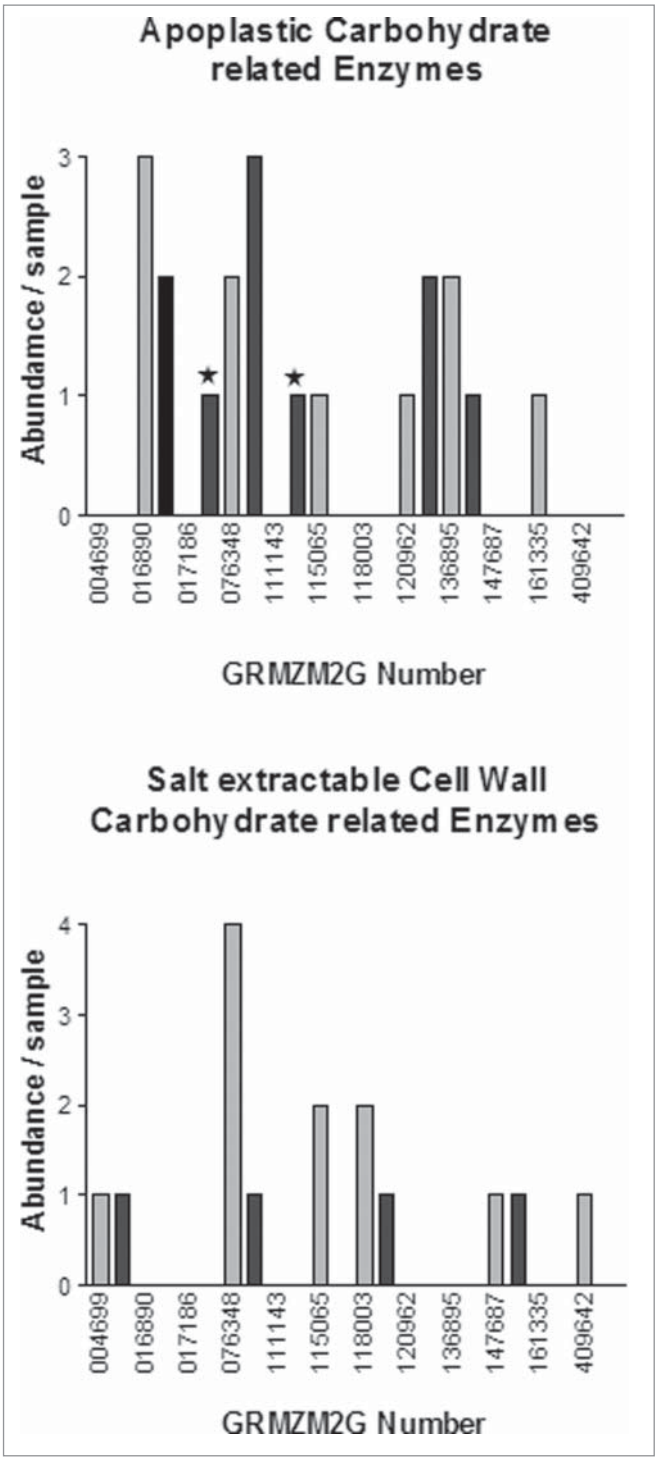

Figure 10. Carbohydrate related enzymes in the apoplastic fluid and cell wall. Gray bars: control, black bars: BOA. Genome Sequence IDs are below the bars. **Figure 10**: Carbohydrate related enzymes in the apoplastic fluid and cell wall. Gray bars: control, black bars: BOA; Genome Sequence IDs are below the bars (MaiZeGBD maize genetics and genomics data base).

(GRMZM2G055487, GRMZM2G089982, GRMZM2G044049, GRMZM2G108207, GRMZM2G135108), whereas peroxidases GRMZM2G023840, GRMZM2G085967, GRMZM2G070603, GRMZM2G405459 and GRMZM2G410175 were increased. Generally, apoplast fluid and cell wall fractions showed noticeable differences in their peroxidase patterns. The peroxidases AC197758.3_FGP004, GRMZM2G070603, GRMZM2G405459 and GRMZM2G023840 could have a special function in the BOA response. Almost all peroxidases are described as heme binding (pathway.gramene.org/gramene/Maizecyc.shtml). Therefore, we assume that BNAP is not only an effector of monooxygenases and certain lactonohydrolases but also of peroxidases. The alteration in the peroxidase patterns may be indicative for a changed cell wall polymer metabolism in BOA exposed roots.

4. Glutathione transferases - The patterns of glutathione transferases show a lower diversity and were less affected than the carbohydrate related proteins or particularly, the peroxidases. Nevertheless, specific differences were found. Glutathione transferases GRMZM2G116273 and GRMZM2G132093 were more abundant in the apoplastic fluid after BOA incubation. GST GRMZM2G126781 was only in the cell wall preparations of the control, whereas putative GST GRMZM2G119499 was new in the cell wall after BOA exposure (Fig. 12). Glutathione transferases are known to have functions in detoxification processes.[33] These enzymes may serve as a transporter system during the import of BOA-*N*-glucoside / glucoside carbamate into the protoplast. The GSTs involved in the export could be located in the cytoplasm, which was not directed in the proteome analyses. Incubation of maize seedlings in presence of the GST inhibitor ethacrynic acid and the multi-drug resistance transporter inhibitor nifedipine,[34,35] but not the transporter inhibitor verapamil, resulted in a 3 to 5 fold increase of glucoside carbamate in methanolic extracts from the root tips (Fig. 13). It is therefore likely that the exudation of the demalonylated compound depends on glutathione transferases and nifedipine sensitive transporters, although respective conjugates were not yet identified.

The proteomic analyses strongly support the previous results and underline the involvement of peroxidases and glutathione transferases in the maize root responses to BOA. Several of them may have overlapping activities, which could optimize the management of the detoxification process. A function of BX9 is concluded from the studies with the BX9 transgenic *Arabidopsis* line and from immunological experiments verifying the presence of this enzyme in the extraplastic space. Glucanases and hydrolases could indirectly contribute to the detoxification by releasing glucose or cell wall bound BOA-*N*-glucoside. Enzymes thought to be involved in the glucoside carbamate production are listed in Table 1.

Compositional analysis of the cell wall

BOA treatment is known to enhance cell wall rigidity.[36] The above described alterations in carbohydrate-related enzymes support the assumption that BOA interferes in the cell wall glucan metabolism and favors lignifications. An intensified glucose release should be correlated to a change in the overall composition of the root cell wall polymers. The root sections from BOA-treated and control material showed different contents for the acid insoluble lignin (Klason lignin) and for the polysaccharides (Fig. 14). By tendency, Klason lignin content of BOA-pretreated roots is higher in the youngest root sections. Although only significant for root tip material, polysaccharides are affected by the BOA-treatment and the detectable amount of released sugars from cellulose and hemicelluloses is higher

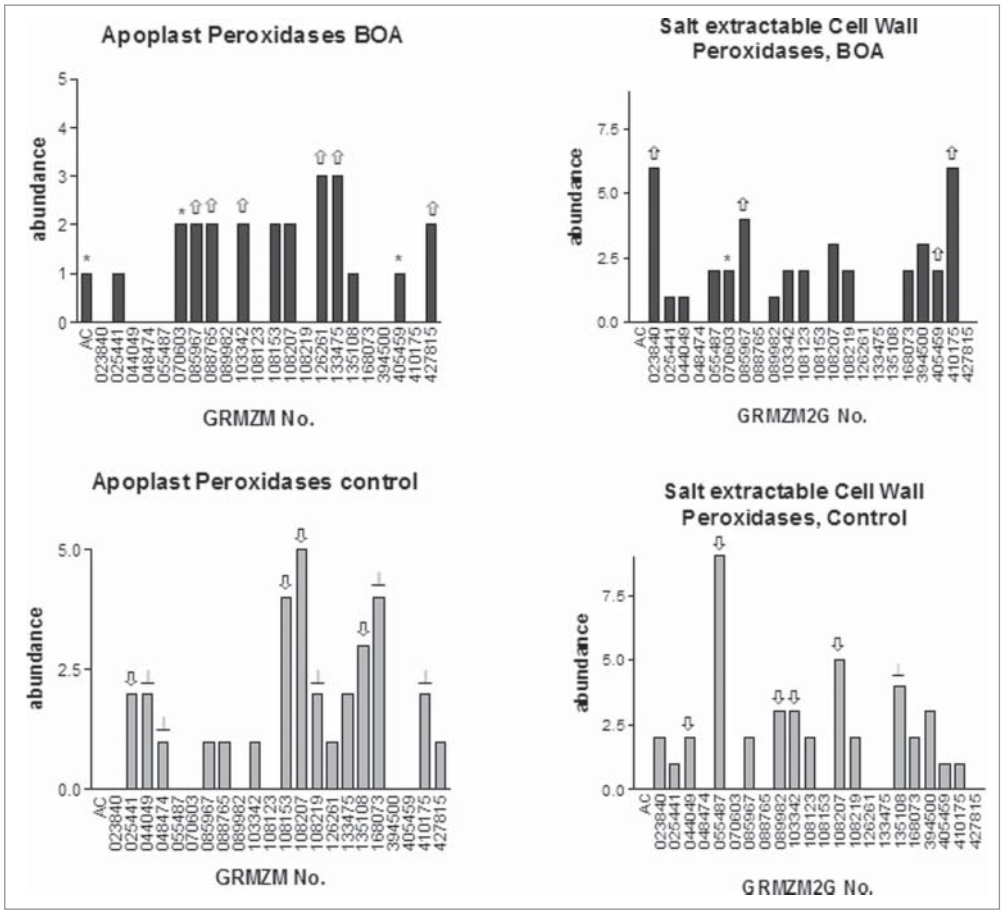

Figure 11. Peroxidases in the apoplastic fluid (A,B) and the salt extracted cell wall fraction (C,D). Gray bars: control, black bars: BOA. Genome Sequence IDs are below the bars. *: new, ⊥: not present in BOA samples. Arrows upwards: increased abundance in BOA samples; arrows downwards: decreased abundance in BOA samples.

in the controls. The results obtained for the root tips are in agreement with the data of the proteomic analyses exhibiting alterations in defined carbohydrate related enzymes. The ratio of cellulose/hemicelluloses was markedly decreased in BOA samples (1.6/1.3), and the ratio of Klason lignin to celluloses was higher in the older BOA sections (0.28/0.09). The changed proportions could contribute to the described higher rigidity of BOA treated roots.[37] Presently it is not known whether only the outer layers of root tissue or the entire sections are affected by BOA. The content of soluble lignin was not clearly affected by the BOA treatment and additional studies are necessary. Under the acidic conditions used for the compositional analysis,[38-40] sugar degradation can occur with 5-hydroxymethylfurfural (5-HMF) as one of several sugar degradation products. Those degradation products may explain the high standard deviations of the measurements.

BOA detoxification via glucoside carbamate synthesis is uncoupled from BX biosynthesis

Incubation media of maize mutant BX-less contained the fungal detoxification product oHPMA, but no 2-aminophenoxazinone(s). Addition of 50 μmol BNAP led to a complete absence of oHPMA. The medium contained numerous phenolic compounds, which were not further investigated in this study as they were not directly related to detoxification (Fig. 15). In methanolic extracts of the BOA incubated BX-less mutant, the known detoxification products gentiobioside carbamate, glucoside carbamate, BOA-*N*-glucoside and malonyl-glucoside carbamate were found but no BOA-6-*O*-glucoside. The presence of BOA-*N*-glucoside in similar amounts than glucoside carbamate seems to be a feature of the mutant, since in BX containing maize cultivar "Cassila" BOA-*N*-glucoside does not accumulate, due to a rapid rearrangement to glucoside carbmate. Malonyl-glucoside carbamate was the major detoxification product in the control and BNAP samples. It accumulated earlier than found with BX containing maize. In the controls without BNAP, all detoxification products were present in much higher concentrations than in maize cultivar "Cassila." Addition of BNAP reduces the amounts of the detoxification products drastically, BOA was only found in BNAP samples in similar amounts as glucoside carbamate and BOA-*N*-glucoside. The results indicate that the glucoside carbamate pathway is not obligatorily linked to the benzoxazinone synthesis. However, the course of the process seems to be differently organized in BX containing maize than in the BX less mutant, since malonyl glucoside accumulation is started simultaneously with glucoside carbamate and BOA-*N*-glucoside accumulation. An enzyme responsible for the rearrangement cannot be excluded.

Discussion

Studies with 3-week-old transgenic BX9 and BX8 *Arabidopsis thaliana* plants indicated that maize glucosyltransferase BX9

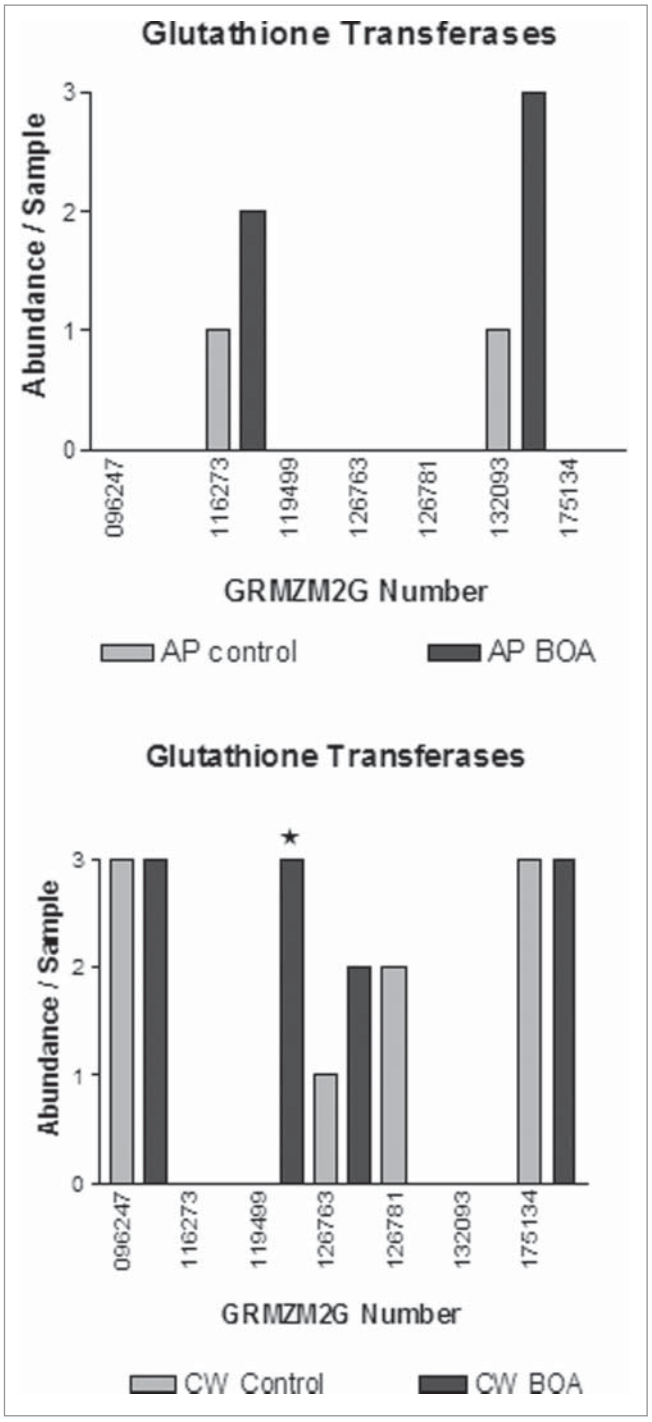

Figure 12. Glutathione transferases in the apoplastic fluid, AP (A) and the salt extracted cell wall fraction, CW (B). Gray bars: control, black bars: BOA. Genome Sequence IDs are below the bars. *: new in the fraction.

may play a role in the BOA detoxification via glucoside carbamate. Only the BX9 transgenic plants accumulate glucoside carbamate in significantly higher amounts, compared to the wild type or the BX8 transgenic *Arabidopsis* line. The Co:BX8 line with the UGT from the dicot *Consolida orientalis* and maize UGT BX8 cannot catalyze BOA-*N*-glucosylation sufficiently for a successful detoxification via glucoside carbamate synthesis. Although *C. orientalis* contains benzoxazinones, the plant detoxifies BOA mainly by BOA-6-O-glucosylation, as

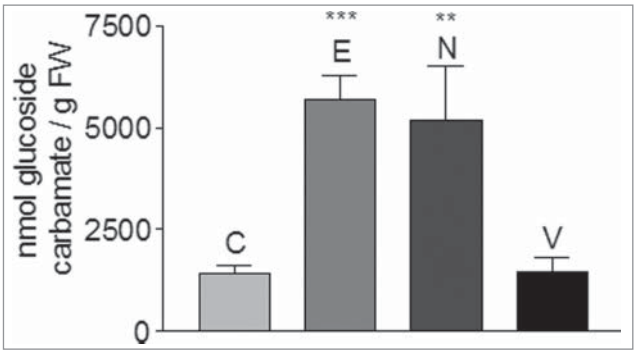

Figure 13. Influence of ethacrynic acid (E, GST inhibitor) and of the transporter inhibitors Nifedipine (N), Verapamil (V) on glucoside carbamate accumulation. Ethacrynic acid and nifedipine increase the accumulation significantly compared to the control (means ±SD; E: $p < 0.0001$; N: $p < 0.001$). C: control.

most of the dicots studied. The low accumulation of glucoside carbamate in the Co:BX8 line is therefore in accordance with the detoxification behavior of *C. orientalis*.[21]

As shown, activities or biochemical characteristics of single enzymes catalyzing defined reactions steps of the BOA-*N*-glucoside/glucoside carbamate detoxification pathway could not be determined. A direct precursor of BOA-*N*-glucoside was not identifiable since mainly unstable BOA radicals seem to be necessary for *N*-glucosylation. Even dilution of the highly concentrated protein extracts or desalting led to a loss of glucoside carbamate synthesis. BOA radicals should be preferentially produced by peroxidases. The Fenton reaction, occurring naturally in the cell wall, could present another source for BOA radicals.[41] BOA derived, unstable carbamic acid can be considered as a third possibility to serve as reactive precursor molecule. An immediate fixation of reactive BOA species directly to cell wall glucans during the early detoxification and later release as

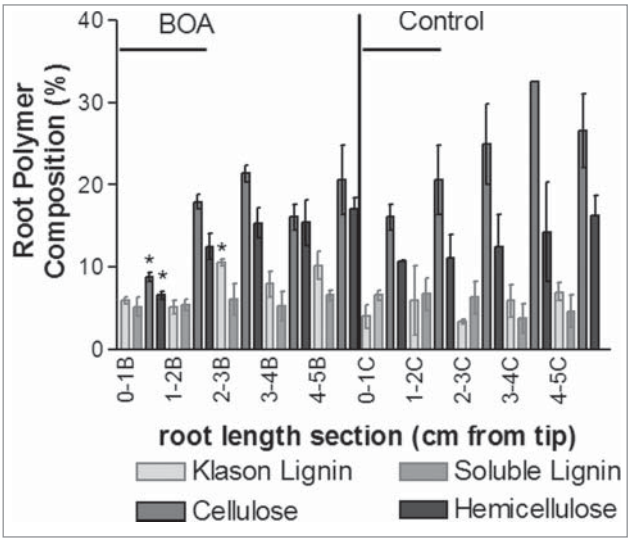

Figure 14. Cell wall components in different zones of roots of BOA treated and untreated plants. The amount of Klason lignin (light gray) is increased in the younger root parts of BOA treated plants (left hand side). Soluble lignin (gray), cellulose (dark gray) and hemicellulose (black) do not show significant differences in the older root sections but within the first 1 cm from the root tip. The horizontal bars indicate the root sections which were used for the proteome analyses. Shown are means ±SD; *$p < 0.005$.

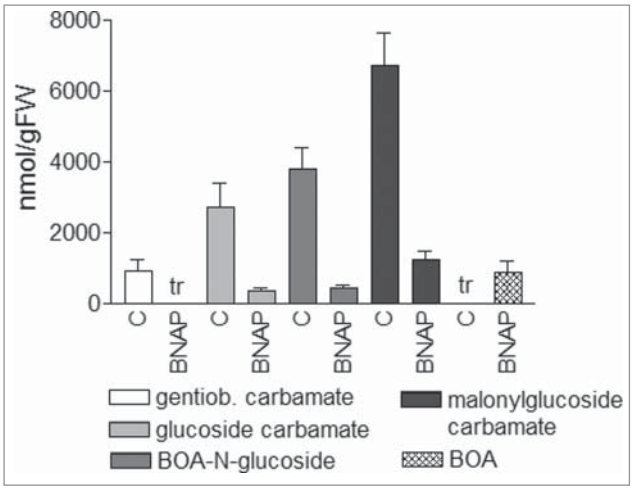

Figure 15. BOA and BOA detoxification products in the roots of the maize mutant BX-less without and in presence of 50 μM BNAP. gentiob. carbamate = gentiobioside carbamate; tr = traces. C: controls; BNAP: incubations with BNAP. Glucoside carbamate is slightly more hydrophilic (retention time 12.2 min) than BOA-*N*-glucoside (retention time 15.25 min). Shown are means ± SD of 3 biological repetitions.

BOA-*N*-glucoside, catalyzed by glucanases/glucosidases is thought to present the major source of *N*-glucosylated BOA. Thus, a large portion of the detoxification via BOA-*N*-glucosylation is possible without catalysis of specific UGTs.

The role of UGT BX9 stays unclear. A modified, oxidized form of the enzyme may catalyze the glucosylation of unstable carbamic acid in a channeled reaction sequence without release of the unstable molecule. This possibility is regarded as an alternative but ancillary mechanism. UDPG, the donor substrate for UGT catalyzed glucosylations, can be synthesized by plasma membrane or cell wall bound UGPase using glucose from hydrolyzed glucans.[42,43]

The pathway seems to be regulated to a large extent on the protein level with extensive protein shifting between compartments and takes place in defined sections of the root, mainly the elongation and maturation zones. Protein shifting into the extraplastic space requires their excretion. A leaderless, unconventional protein secretion is increasingly recognized in plants.[44,45] The authors assume an involvement of plant leaderless secretory proteins (LSPs) in stress responses, including different roles and functions of the same protein depending on its localization, either intercellular or in the extraplastic space. Glycosylation is an important post translational modification for trafficking of secretory proteins, but also post-secretional processing is discussed.[46,47] Zhu et al.[30] detected that 33% of their cell wall fraction I proteins belonged to the nonclassical secretory proteins. BX9 might belong to the LSPs with different functions in the cytosol and in the extraplastic space. GDIM-BOA glucosidase has been regarded as another LSP protein which can be secreted into the extraplastic space, most likely for the fast release of bioactive DIMBOA. Ahmad et al.[48]

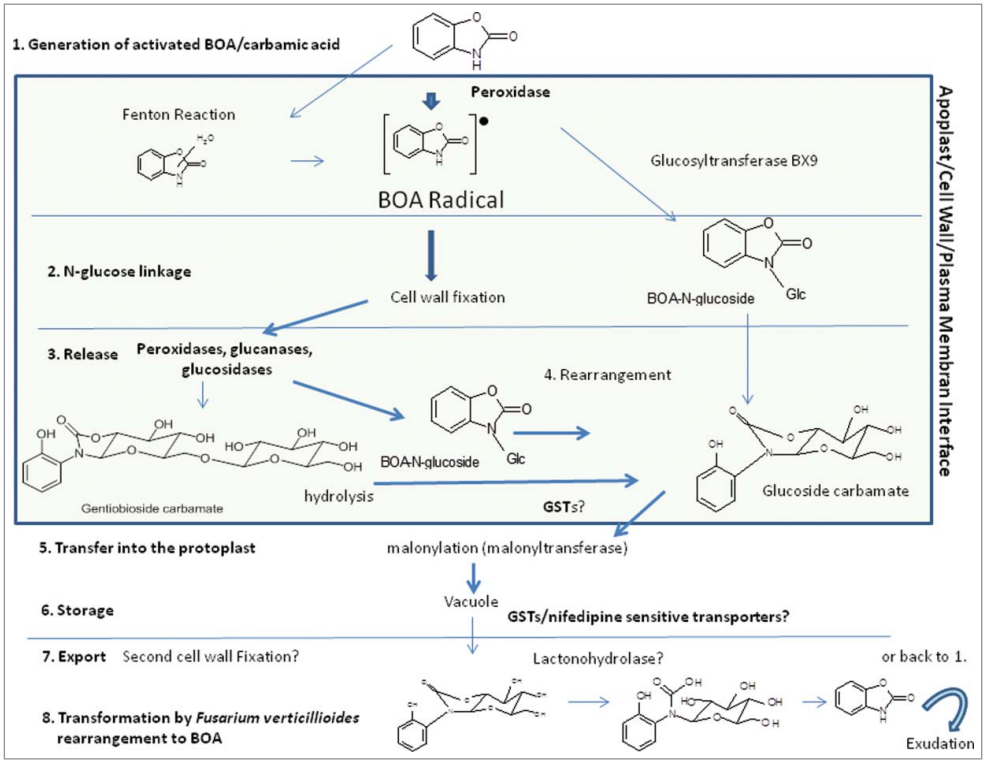

Figure 16. Partly hypothetical pathway of glucoside carbamate synthesis in the extraplastic space, malonylation and import into the vacuole for temporary storage, export and transformation/reconstitution to BOA by the endophyte *Fusarium verticillioides*. Peroxidase generated BOA radicals and subsequent fixation of the radicals at carbohydrate cell wall components are thought to present the major start reactions for later N-glucosylated BOA release by hydrolytic enzymes. A rearrangement of the N-glucosylated molecule results in glucoside carbamate, which can be malonylated and stored within the vacuole. This pathway prevents not only entering of toxic benzoxazolinone into the cytosol, but is competitive to phenoxazinone synthesis. An auxiliary pathway may start after generation of instable carbamic acid and *N*-glucosylation by maize glucosyltransferase BX9. *Fusarium verticillioides* can open the lactone ring of glucoside carbamate, resulting in glucosylated carbamic acid. This relatively stable compound was recently identified as a fungal degradation product of glucoside carbamate. It can be further degraded resulting finally in phenoxazinones or is recycled to BOA.

reported the glucose hydrolysis of BX glucosides in the apoplast and capture of the aglyca in a callose matrix, but the identity of the hydrolyzing enzyme was not determined. Also Frébortova et al.[49] assumed a hydrolysis of DIMBOA-glucoside by a cell wall located glucosidase. GDIMBOA glucosidase, however, is not involved in the glucoside carbamate pathway and its abundance is reduced upon BOA exposure.

Fig. 16 depicts all aspects of the partly hypothetical pathway of glucoside carbamate synthesis. The entire network is apparently redox-regulated. The cell wall might be regarded as an allelochemical depot, when a second fixation of detoxification products takes place. The endophyte *Fusarium verticillioides* could mobilize the allelochemical via glucosylated carbamic acid.[50] The fungus is able to hydrolyse glucose from this compound and can rearrange carbamic acid to BOA, if no decarboxylation to 2-aminophenol occurs. The depot also remained in dead root material, but can be mobilized by fungi. In natural habitats the mobilization may improve the survival of the plants'offspring by suppressing other species that try to colonize the same place but are not adapted to the compounds.

Exposure to BOA is known to enhance cell wall rigidity. In soybean, BOA increases the production of lignin monomers and the degree of polymerization, leading to higher lignin contents.[36,37] In maize root tips, the enhanced rigidity seems to be more the result of a changed ratio of cell wall carbohydrates to lignin. Deposition of BOA and a second fixation of BOA-*N*-glucosylated detoxification products within the cell wall of older root parts may contribute to the enhanced rigidity.

BOA-*N*-glucosylation is much more abundant in monocots than in dicots. It is assumed that most dicots do not have comparable efficient strategies to detoxify BOA within the cell wall or are not provided with secretory, cooperating glucosyltransferases able to perform the *N*-glycosylation. Moreover, the unique cell wall composition of Poaceae may favor BOA-*N*-glucoside detoxification. Mixed-linkage (1/3), (1/4)-β-D-glucan, the substrates for 1,3;1,4 β-glucanase, are only found in the cell walls of grasses, with few exceptions.[51-53] These β-glucans accumulate during cell enlargement in all growing tissues and are hydrolyzed for cell wall expansion by endo- and exo-glucanases. This process may be enhanced by BOA activated endo1,3;1,4-β-D-glucanase (GRMZM2G076348), glucan-endo-1,3-β-glucosidase (GRMZM 2G111143), exohydrolase II (GRMZM2G017186) and exo-β –glucanase (GRMZM2G120962). High amounts of glucose are released, also caused by radical attacks.[54] Another advantage of Poaceae could be the ability to start an efficient over flow rescue with a temporary vacuolar deposition of malonylated glucoside carbamate(s), when the cell wall is oversaturated. The lack of 1,3;1,4 β-glucan and perhaps of suitable secretory glucosyltransferases would explain why most of the dicots, including WT *Arabidopsis thaliana* and *Consolida regalis*, do not accumulate extractable glucoside carbamate in similar amounts as maize, oat, or other grasses, although other β-glucans present in Type I cell walls of the dicots, might be compensatory to some amount. This interpretation is supported by the fact, that BOA detoxification is uncoupled from BX biosynthesis in maize. Interestingly, most of the dicot weeds have been found to be more sensitive to benzoxazolinones (and other allelochemicals) than grasses. They seem to be highly dependent on suitable glucosyltransferases. If these are the general reasons why grasses exhibit a less pronounced sensitivity toward many allelochemicals has still to be proved.

Conclusions

This study revealed a high complexity and dynamic of the redox dependent BOA detoxification by *N*-glucosylation. Glucoside carbamate synthesis bases mainly on peroxidase catalyzed generation of BOA radicals as precursor molecules for attachment to cell wall material and subsequent release by hydrolases. An alternative mechanism may present BX9-catalyzed glucosylation of carbamic acid, the later produced by the endophyte *Fusarium verticillioides*. A trapping of carbamic acid by glucosylation is competitive to the production of phenoxazinone, a phytotoxic compound. It is the question whether mainly Poaceae displace relevant first steps of detoxification pathways into the extraplastic space, which is not completely in line with the common concept of the 3 phased course of detoxification events with cytosolic phase II glucosylations of modified toxic molecules.[55]

Material and methods

Plant material, incubation conditions and analyses of extracts

Seven-day-old, hydroponically grown maize seedlings (cultivar *Zea mays* L. "Cassila" a gift from KWS SAAT SE (Kleinwanzlebener Saatzucht GmbH, Einbeck Germany) were incubated for 24 hours in tap water containing 0.5 mM BOA. Other incubation times were 3, 6, 24 and 48h. Controls were incubated without BOA. In several incubations, the following effectors were added to benzoxazolinone containing mediums: 2-bromo-4′-nitroacetophenone (effector of mono-oxygenases and other cytochrome P450 iron-containing hemoproteins), peroxidase inhibitor 2,3-butanedione, ethacrynic acid, a common glutathione transferase inhibitor, and the transporter inhibitors nifedipine and verapamil. Effectors were purchased from Sigma. Controls were without effectors. The applied concentrations are given in the further text.

Arabidopsis thaliana Col-0 and the transgenic lines 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 4 weeks in pots with a Vermiculite/soil mixture (1:3).[7,17] Plants were harvested and soil particles were removed from the root system by repeated water baths. Twelve plants were incubated with 250 ml incubation medium containing 0, 0.5, 1.0 and 2.0 mM BOA for 24h. Plants were washed, extracted with 50% methanol (w/v:1/6) and analyzed by HPLC as described in Schulz et al.[56]

Caryopses of the maize mutant BX-less were imbibed overnight, hydroponically grown for 7 days, then the seedlings were incubated with 0.5 mM BOA, extracted and extracts analyzed as afore mentioned for maize cultivar Cassila. 50 μmol 2-bromo-4′-nitroacetophenone (BNAP) was used for inhibition studies.

Analyses of incubation media

BNAP supplemented BOA incubation mediums from maize were further analyzed by Ultra Performance Liquid Chromatography (UPLC)-electrospray(ESI)-mass spectrometry (MS) using an ACQUITY UPLC system including a PDA detector (190–330 nm) coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Eschborn, Germany). UPLC analyses were run at 40°C using a reversed-phase PFP column (100 × 2.1 mm, 2.5 μm core-shell Kinetex, Phenomenex, Aschaffenburg, Germany). Solvent A was water, solvent B methanol (Biosolve, Valkenswaard, The Netherlands). The elution was performed at a flow rate of 0.2 ml/min using a linear gradient from 5 %B to 100%B in 12 min. The injection volume was 10 μl for each sample. The mass spectra were recorded in positive mode. Full scan MS were recorded in the mass range 50–800 Da, MS/MS spectra in the mass range 50–350 Da with argon as collision gas.

Cultivation of the maize endophyte Fusarium verticillioides

F. verticillioides was isolated and cultured as described in Knop et al. [57] Plugs of the mycelium were cultured in liquid Czapek medium, 100 μmol BOA was added when the oxygen concentration was below 0.4 mg/ml. After 4 days the fungus was removed and the culture liquid was concentrated under vacuum. The concentrate was immediately transferred into Falcon tubes, sealed and stored at −20°C until used for MS and NMR analyses of BOA derived compounds.

MS and NMR analyses of compounds in F. verticillioides culture medium

The concentrate obtained from the BOA culture medium of *Fusarium verticillioides* was evaporated to dryness in vacuo to yield 10 mg of a reddish solid. Analytical TLC on TLC sheets ALUGRAM Xtra SIL G/UV254 (n-hexane:ethyl acetate 2:1 v/v) proved this solid to consist of at least 4 constituents: compound 1, colorless spot with quenching of fluorescence, Rf = 0.16; compound 2, orange spot, Rf = 0.23; compound 3, yellow spot, Rf = 0.30; compound 4, colorless spot, quenching of fluorescence, Rf = 0.36. The mixture was subjected to preparative TLC on MERCK glass TLC plates Silica gel 60F254 20 × 20 cm × 0.25 mm with concentrating zone, using the same eluent. The four zones described above were collected and the compounds contained therein extracted with ethyl acetate and isolated from the solutions. The yields obtained were: compound 1: 0.9 mg, compound 2: 0.6 mg, compound 3: 3.8 mg, compound 4: 3.8 mg. Overview ^{1}H-NMR spectra were measured and it was decided to elucidate the structures of compounds 3 and 4, resp. Compound 4 was found to be BOA by identity of Rf value, mass spectrum, ^{1}H and ^{13}C NMR spectra with those of an authentic reference of BOA. Compound 3 was most likely assigned to be N-(3-oxo-3H-phenoxazin-2-yl)-acetamide (CAS 1916-55-8, acronym in this field = AAPO) by means of NMR spectra and MS in this manner.

The mass spectrum of 3 gives the correct molecular ion (m/z = 254) for $C_{14}H_{10}N_2O_3$ in accordance to literature.[58] The base peak m/z = 212 corresponds to a fragment caused by loss of ketene CH_2=C=O (M^+- 42) which is typical for elimination from N-acetyl derivatives. I.a., the ^{1}H NMR spectrum in $CDCl_3$ shows a singlet for the protons of the N-acetyl group at 2.30 ppm and 4 signals in 1:1:1:1 ratio at 7.93, 7.63, 7.57, and 7.49 ppm, respectively, with the characteristic coupling pattern of a 1,2-disubstituted aromatic ring. Because of the small quantity of substance the carbon absorptions were extracted from 2 D correlations in the HMBC spectrum. The values are in coincidence with literature results to the greatest extent. [59,60]

Concentration of apoplastic proteins and salt extractable cell wall proteins

The apoplastic fluid was prepared as described by Lohaus et al. [61] and Witzel et al.[62] Root tips (3 g) were cut and placed in plastic syringes containing 10 ml extraction buffer with the Sigma protease inhibitor cocktail (5 μl / ml). The cell wall fraction of ionically bound proteins (further on denoted as cell wall fraction) was prepared according to Sukalovic et al.,[63] including 100 mM NaCl and protease inhibitors (5 μl/ml). Proteins of the apoplastic fluid and of the cell wall fraction were concentrated with StrataClean^{TM} resin (Agilent), (1 ml / 10 μl resin), according to the manufacturer's instruction. The cell wall fraction was first diluted with extraction buffer to reduce the NaCl concentration. BX9 was immunologically identified in apoplastic fluid and cell wall protein fractions by SDS-PAGE and Western blot with polyclonal BX9 antibodies from rabbit.[17] Protein concentrations were determined with the Bradford method.

Assays with whole cell protein concentrates

Seven day-old maize (Cassila) seedlings were incubated with tap water (control) or with 0.5 mM BOA for 0, 6 and 24 h, respectively. 3 g root tips (1–1.5 cm) were harvested and homogenized with extraction buffer (1ml/g FW) including 10μl protease inhibitor cocktail per ml buffer. The homogenate was centrifuged for 20 min at 4°C and 5000 *g*. The supernatant was adjusted to 1 mg protein/ml. The assay mixture contained 150 μl of the highly concentrated whole-cell-protein extract, 500 μmol $FeSO_4$, 200 μmol H_2O_2, 10 mM UDPG and 100 μmol BOA. In other series of assays, either $FeSO_4$ or H_2O_2 or both were omitted. Additional assays were run in presence of 2 mg cellobiose and 5 mg laminarine, a (1→3)-β-D-glucan. Another set of assays contained 1 mM 2,3-butanedione. Assays were incubated for 1 h or overnight at 37°C. The reaction was stopped by 5 min boiling. Assays were analyzed by HPLC.

SDS-PAGE, in-gel digests and protein identification by LC-MS/MS

The concentrated target proteins were separated by PAGE according to Laemmli on an Ettan DaltSix electrophoresis system (GE Healthcare) using 12% Tris-Glycine SDS gels. After visualization of proteins with Coomassie the gel tracks were completely sliced into single bands, excised from the gel and subjected to tryptic in-gel digestion according to Shevchenko et al.[64] The extracted peptides were purified and concentrated

using the StrataClean™ resin and subsequently analyzed by LC-MS/MS on a Bruker HCTultra ion trap mass spectrometer coupled to a Proxeon Easy nano liquid chromatograph. The resulting spectra were processed using the Compass 1.3 software package and peak data were imported for protein identification into the ProteinScape database system version 3.0 (Bruker), which initiated Mascot version 2.3 (Matrix Science) searches against the NCBI nonredundant database (http://www.ncbi.nlm.nih.gov) and UniProt knowledgebase (http://www.uniprot.org/).

Cell wall analyses

For cell wall component analyses, 150 7-day-old maize seedlings were incubated with 500 ml 0.5 mM BOA or with tap water (control) for 24h. After the incubation, the roots were rinsed with tap water, dabbed with paper and cut in 1 cm pieces from the tip to 5 cm length. The pieces were weighed and collected in a tube, then dried at 114°C for 48 h and the dry weights determined. The procedure was repeated until 1 g dry matter/section was obtained. For the samplings, about 1000–1200 roots/ treatment were prepared.

The cell wall components cellulose, hemicellulose and lignin of *Zea mays* L. were determined using a 2-step sulfuric acid hydrolysis protocol described by Sluiter et al.[39] The non-hydrolysable component lignin is determined after washing gravimetrically. The hydrolysable components cellulose and hemicellulose were determined as monosaccharides using high performance anion exchange chromatography coupled to pulsed amperometric detection (ICS-5000+, ThermoScientific, USA). The alkaline eluent composition and the gradient for monosaccharide separation were applied according to Anders et al. [40]

Statistical Data Analysis – If not otherwise mentioned, each data point is based on at least 3 biological replicates from 3 independent experiments. Error bars are based on SD. For statistical analysis of all data, the *t*-test was used.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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