Running head: Role of Benzoxazinoids in Maize Innate Immunity

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Benzoxazinoid Metabolites Regulate Innate Immunity against Aphids and Fungi in Maize.

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Abstract (250 words)

Benzoxazinoids (BXs), such as 2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one (DIMBOA), are secondary metabolites in grasses. The first step in BX biosynthesis converts indole-3-glycerol phosphate into indole. In maize, this reaction is catalysed by either BENZOXAZINELESS 1 (BX1), or INDOLE GLYCEROL PHOSPHATE LYASE (IGL). The Bx1 gene is under developmental control and is mainly responsible for BX production, whereas the Igl gene is inducible by stress signals, such as wounding, herbivory, or jasmonates. To determine the role of BXs in defence against aphids and fungi, we compared basal resistance between Bx1 wild-type and bx1 mutant lines in the igl mutant background, thereby preventing BX production from IGL. Compared to Bx1 wild-type plants, BX-deficient bx1 mutant plants allowed better development of the cereal aphid Rhopalosiphum padi, and were affected in penetration resistance against the fungus Setosphaeria turcica. At stages preceding major tissue disruption, R. padi and S. turcica elicited increased accumulation of DIMBOA-glucoside, DIMBOA and HDMBOA-glucoside, which was most pronounced in apoplastic leaf extracts. Treatment with the defence elicitor chitosan similarly enhanced apoplastic accumulation of DIMBOA and HDMBOA-glucoside, but repressed transcription of genes controlling BX biosynthesis downstream of BX1. This repression was also obtained after treatment with the BX precursor indole and DIMBOA, but not with HDMBOA-glucoside. Furthermore, BX-deficient bx1 mutant lines deposited less chitosan-induced callose than Bx1 wild-type lines, whereas apoplast infiltration with DIMBOA, but not HDMBOA-glucoside, mimicked chitosan-induced callose. Hence, DIMBOA functions as a defence regulatory signal in maize innate immunity, which acts in addition to its well-characterised activity as a biocidal defence metabolite.
Introduction

Induced plant defence against pests and diseases encompasses a wide variety of mechanisms, ranging from deposition of callose-rich papillae at the cell wall to accumulation of biocidal defence metabolites. Defensive metabolites can be synthesised *de novo* in response to microbe or insect attack, such as phytoalexins, but can also be produced constitutively and stored as an inactive form in the plant cell. These so-called phytoanticipins can be activated by β-glucosidase activity during herbivory, which allows for a very rapid release of biocidal aglycone metabolites (VanEtten et al., 1994; Morant et al., 2008). Well-characterised examples of phytoanticipins are glucosinolates, (GSs), which are hydrolysed by endogenous β-thioglucoside glucohydrolases, called myrosinases. Although GSs have traditionally been associated with defence against herbivores, recent insights have revealed that they can also play an important role in resistance against microbes. For instance, Betnarek et al. (2009) demonstrated in Arabidopsis that early-acting penetration resistance against powdery mildew requires intact biosynthesis of the indolic GS 4-methoxyindol-3-ylmethylglucosinolate (4MI3G) and subsequent hydrolysis by the atypical myrosinase PEN2. Furthermore, penetration resistance of Arabidopsis against *Phytophthora brassicae* depends on the sequential action of the same class of indolic GSs and the indolic phytoalexin camalexin (Schlaeppi et al., 2010; Schlaeppi and Mauch, 2010). Interestingly, Clay et al. (2009) reported that callose deposition after treatment with the flagellin epitope flg22 requires intact biosynthesis and breakdown of 4MI3G. This finding uncovered a novel signalling role by indolic GSs in Arabidopsis innate immunity, but also raises the question of how callose deposition is regulated in non-*Brassicaceous* plants, which do not produce glucosinolates.

Benzoxazinoids (BXs) are widely distributed phytoanticipins amongst *Poaceae*. It is commonly assumed that BX-glucosides are hydrolysed by plastid-targetted β-glucosidases upon tissue disruption, which results in the release of biocidal aglycone BXs (Morant et al., 2008). Since their discovery as plant secondary metabolites, many investigations have focussed on their role in plant defence against herbivorous insects and pathogens (Niemeyer, 1988; Niemeyer, 2009). Most of these studies revealed positive correlations between resistance and BX levels in cereal varieties or inbred populations, or biocidal activity when added to artificial growth medium (for review see: Niemeyer, 2009). In maize, defence elicitation by pathogenic fungi or treatment with the defence regulatory hormone jasmonic acid (JA) influences BX metabolism by promoting the conversion of DIMBOA-glucoside
(DIMBOA-glc) into N-O methylated HDMBOA-glucoside (Oikawa et al., 2002; Oikawa et al., 2004). When supplied to artificial growth medium, this di-methylated BX compound is more effective than DIMBOA-glc in reducing survival rates of the aphid *Metopolophium dirhodum* (Cambier et al., 2001).

The biosynthesis of BXs is mostly under developmental control and leads to accumulation of inactive BX-glucosides that are stored in the vacuole (Frey et al., 2009). In rye and wild barley, 2,4-dihydroxy-2H-1,4-benzoazin-3(4H)-one (DIBOA) is the dominant BX, whereas the methoxy derivative 2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one (DIMBOA) is more prevalent in maize and wheat (Niemeyer, 2009). The *BENZOXAZINELESS1 (Bxl)* gene mediates the first dedicated step in the BX pathway and encodes a close homolog of the tryptophan synthetase α-subunit (TSA), which catalyses the formation of indole from indole-3-glycerole phosphate (Frey et al., 1997; Melanson et al., 1997). This compound is oxidised by four cytochrome P450 monooxygenases, BX2 – BX5, into DIBOA (Frey et al., 1997), which can subsequently be glucosidated by the glucosyltransferases BX8 and BX9 (von Rad et al., 2001). Elucidation of the final reactions towards DIMBOA-glc revealed that the cytosolic di-oxygenase BX6 and methyltransferase BX7 mediate conversion of DIBOA-glc via TRIBOA-glc into DIMBOA-glc (Jonczyk et al., 2008). Upon tissue disruption, DIBOA-glc and DIMBOA-glc can be hydrolysed by two plastid-targetted β-glucosidases, ZmGLU1 and ZmGLU2 (Cicek and Esen, 1999; Czjzek et al., 2001), which causes the release of biocidal DIBOA and DIMBOA aglycones. This mode of action is consistent with a role for BXs in resistance against chewing herbivores that cause major tissue damage. However, BXs have also been implicated in defence against aphids and pathogenic fungi that cause relatively little tissue damage (Niemeyer, 2009), which suggests an alternative mechanism of BX-dependent resistance.

Apart from BX1, the BX1 homologue *INDOLE-3-GLYCEROL PHOSPHATE LYASE (IGL)* can also convert indole-3-glycerole phosphate into free indole (Frey et al., 2000). The enzymatic properties of IGL are similar to BX1, but the transcriptional regulation of their corresponding genes is profoundly different. Like other *Bx* genes, *Bxl* is constitutively expressed during the early developmental stages of the plant, which correlates with endogenous benzoxazinoid levels. Plants carrying the mutant alleles of the *Bxl* gene produce only a fraction of the BXs that are found in *Bxl* wild-type plants (approximately 1.5%; Figures S1 and S2). Hence, the BX1 enzyme is accountable for the bulk of BX biosynthesis, whereas the functionally equivalent IGL enzyme appears to have a minor contribution in unstressed maize seedlings. Indeed, the *Igl* gene is expressed at much lower levels than *Bxl*.
during seedling development (Frey et al., 2000), explaining why it largely fails to complement BX production in Bx1 seedlings. Unlike Bx1, the expression of Igl correlates tightly with the emission of volatile indole: defence-eliciting stimuli, such as herbivore feeding, wounding, the insect elicitor volicitin and JA, all stimulate Igl expression and indole emission (Frey et al., 2000; Frey et al., 2004), suggesting transcriptional regulation by the JA pathway. It has been proposed that herbivore-induced indole emission contributes to attraction of natural enemies, such as parasitoid wasps. Nevertheless, using pharmacological treatments to inhibit indole production, D’Alessandro et al. (2006) reported no or even a repellent effect by indole on parasitoid behaviour. Interestingly, the bx1 single mutant can accumulate up to up to 20% of wild-type BX levels after caterpillar infestation (Nathalie Veyrat, personal communication). Furthermore, treatment of the bx1 single mutant with indole can rescue DIMBOA production (Frey et al., 1997; Melanson et al., 1997). Hence, IGL has the potential to complement the bx1 mutation and contribute to in planta BX biosynthesis during expression of JA-dependent plant defence.

In this study, we have investigated the role of BXs in maize defence against plant attackers that do not cause major tissue disruption. We compared basal resistance against the bird cherry oat aphid, Rhopalosiphum padi, and the pathogenic fungus Setosphaeria turcica between wild-type and bx1 mutant lines in the igl mutant background, thereby blocking BX production from stress-induced IGL. We demonstrate that Bx1-dependent BXs play a critical role in basal resistance against aphids and fungi, which manifests itself as increased deposition of BXs in the apoplast. Moreover, we provide evidence that extracellular DIMBOA regulates pathogen-associated molecular pattern (PAMP)-induced callose and Bx gene expression, thereby uncovering a novel regulatory function of this compound in cereal innate immunity against pests and diseases.
Results

Selection of single and double mutants in Igl and Bx1.

The Igl gene is inducible by herbivory, tissue wounding, volicitin and JA (Frey et al., 2000; Frey et al., 2004; Ton et al., 2007; Erb et al., 2009), suggesting transcriptional regulation by the JA response pathway. It is, therefore, plausible that plants with a dysfunctional Bx1 gene can accumulate BX levels from the stress-inducible IGL enzyme. Consequently, the bx1 single mutant is unsuitable to assess the contribution of BXs to resistance against JA-eliciting attackers, such as R. padi and S. turtica (Delp et al., 2009; Erb et al., 2009). We, therefore, created Bx1 wild-type and bx1 mutant plants in the genetic background of a dysfunctional Igl gene. To this end, a Mutator (Mu)-induced mutant in the Igl gene was crossed with the original bx1bx1 mutant (Hamilton, 1964). Homozygous mutants in progenies from 2 independent crosses were selected and confirmed by high performance liquid chromatography (HPLC) analysis of BX leaf content, and gas chromatography (GC) analysis of wound-inducible indole emission (Figures S1 and S2). This selection resulted in three confirmed genotypes from each cross: the indole-producing bx1 single mutant (bx1 Igl), the indole-deficient igl single mutant (Bx1 igl), and the BX- and indole-deficient double mutant (bx1 igl).

Bx1 is required for basal resistance against aphids.

To establish the role of BXs in basal resistance against aphids, we compared growth and survival rates of R. padi between Bx1 wild-type lines (Bx1 igl) and bx1 mutant lines (bx1 igl and bx1 Igl) from both crosses. After 7 days of feeding from the first leaf, aphids gained significantly less weight when placed on BX-producing Bx1 igl lines compared to BX-deficient bx1 igl and bx1 Igl lines (Figure 1A). In addition, the percentage of aphid survival was less than 10% after feeding from the Bx1 wild-type lines, whereas over 70% remained alive after feeding from the bx1 mutant lines (Figure 1B). These differences were similar in progenies from both crosses and indicate a major contribution from the Bx1 gene to basal resistance against R. padi. Interestingly, aphids reared on the bx1 single mutant from the second cross showed marginally lower levels of survival than the corresponding bx1 igl double mutant (Student’s t-test; P = 0.048), suggesting a relatively small contribution from the Igl gene.
Aphid infestation stimulates apoplastic BX accumulation.

To examine which BX compounds contribute to BxI-dependent resistance against R. padi, we quantified BX profiles after 2 d of feeding from BX-producing wild-type plants. Analysis of whole-leaf extracts by HPLC-diode array detection (HPLC-DAD) identified three major BX compounds: DIMBOA-glc, DIMBOA and HDMBOA-glc (Figure 2A), which were confirmed by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS; Glauser et al., 2011) and nuclear magnetic resonance analysis (NMR; Figure S3). Surprisingly, analysis of these compounds in whole-tissue extracts did not reveal major differences between mock- and aphid-infested plants (Figure 2).

However, defence-related proteins and metabolites often accumulate in the apoplast of stressed tissues, and the aphid stylet must pass through the spaces between the epidermal and mesophyll cells in order to reach the phloem and establish a parasitic interaction. We, therefore, considered the possibility that BxI-dependent resistance against aphids depends on extracellular accumulation of active BX compounds. Indeed, targeted HPLC analysis of apoplastic leaf extracts revealed a statistically significant increase of DIMBOA-glc and DIMBOA at 48 h of feeding (Figure 2B). HDMBOA-glc levels were also increased in apoplastic leaf extracts from R. padi-infested plants, although this effect was more variable and borderline statistically significant (Student’s t-test; P = 0.051; Figure 2B). Together, these results indicate that infestation by R. padi boosts BX accumulation in the apoplast.

BxI contributes to penetration resistance against the necrotrophic fungus S. turcica.

To study the role of BXs in resistance against pathogens, we quantified levels of colonisation by the fungus S. turcica in leaves of BxI wild-type lines (BxI igl) and bxl mutant lines (bxl igl and bxl Igl). This hemi-necrotrophic fungus penetrates the leaf tissue directly and colonises the leaf apoplast before inducing necrosis (Agrios, 1997; Chung et al., 2010). S. turcica-inoculated leaves were collected at 3 days post inoculation (dpi), stained with lactophenol trypan-blue, and examined for hyphal growth by light microscopy. In the progenies from both crosses, BX-deficient lines carrying the bxl mutant alleles allowed significantly more hyphal growth than the BX-producing lines carrying the BxI wild-type alleles (Figure 3A). This difference in resistance was also reflected by the fraction of arrested spores in the epidermal cell layer, which ranged between 14% - 23% in bxl mutant lines, to 47% - 49% in BxI wild-type lines (Figure 3B). Although the indole-producing bxI lines from both crosses expressed marginally higher levels of resistance than the indole-deficient double mutant lines, this difference was not statistically significant (0.3 < P < 0.05). Hence, BxI
contributes to slowing down and/or arresting *S. turcica* colonisation during the relatively early stages of infection, whereas the defensive contribution of *Igl* appears marginal at this stage.

*S. turcica* elicits apoplastic BX accumulation during early stages of infection. Based on our finding that *Bx1*-expressing plants express enhanced penetration resistance against *S. turcica*, we profiled levels of DIMBOA-glc, DIMBOA, and HDMBOA-glc from mock and *S. turcica*-inoculated wild-type plants. At the relatively early stage of 2 dpi, whole-tissue extracts from *S. turcica*-infected plants showed statistically increased levels of DIMBOA in comparison to mock-inoculated plants, whereas DIMBOA-glc and HDMBOA-glc remained similar between both treatments (Figure 4A). As observed during the interaction with *R. padi* (Figure 2A), apoplastic extracts from *S. turcica*-infected leaves showed a more dramatic response and contained significantly enhanced amounts of DIMBOA-glc, DIMBOA and HDMBAO-glc compared to mock-inoculated leaves (Figure 4A). By 5 dpi, BX profiles from *S. turcica*-infected leaves had changed, which coincided with the occurrence of disease symptoms. At this stage, *S. turcica*-inoculated leaves still produced more HDMBOA-glc than mock-inoculated leaves, but there was no longer a difference in DIMBOA-glc (Figures 4A-B). DIMBOA showed an even more dramatic increase than HDMBOA-glc, but was also more variable and not statistically significant in comparison to mock-treated leaves (Figures 4A-B). Hence, maize deposits increased levels of DIMBOA-glc, DIMBOA and HDMBOA-glc in the apoplast during the relatively early stages of *S. turcica* infection, and continues to accumulate HDMBOA-glc and DIMBOA during later symptomatic stages of the interaction.

DIMBOA regulates transcriptional feedback inhibition of BX biosynthesis. To elucidate further the involvement of the BX pathway in maize innate immunity, we profiled BX production and *Bx* gene expression after leaf infiltration with chitosan, a PAMP that is common in fungal cell walls and insect shells (Iritri and Faoro, 2009). At 24 h after infiltration with chitosan, whole-tissue extracts showed increased levels of HDMBOA-glc in comparison to mock-treated leaves (Figure 5A). Apoplastic extracts from chitosan-treated leaf segments displayed a more pronounced increase of both DIMBOA and HDMBOA-glc (Figure 5B), thereby resembling the BX response to *R. padi* or *S. turcica*. Surprisingly, however, reverse-transcriptase quantitative PCR (RT-qPCR) analysis of *Bx* gene expression revealed reduced expression of *Bx4*, *Bx5*, *Bx6*, *Bx7*, *Bx8* and *Bx9* after treatment with chitosan (Figure 6A). To investigate whether this transcriptional repression is due to negative
feed-back inhibition from BX compounds, we profiled Bx gene expression after exposure to
the volatile BX precursor indole. As is shown in Figure 6B, indole triggered similar patterns
of Bx gene repression as chitosan. Furthermore, comparison of Bx gene transcription between
BX-producing igt single mutant lines and BX-deficient bxl igt double mutant lines revealed
enhanced Bx expression in BX-deficient plants (Figures 6C-D), providing further genetic
evidence for the transcriptional feedback inhibition. Since chitosan boosts accumulation of
both DIMBOA and HDMBOA-glc in the apoplast (Figure 5B), we purified DIMBOA and
HDMBOA-glc by preparative HPLC and examined which of both compounds is responsible
for feed-back response. At 24 h after infiltration of the purified compounds in the leaf
apoplast, DIMBOA repressed Bx gene expression in a dose-dependent manner (Figures 6E-
F), whereas HDMBOA-glc had no statistically significant effect (Figure 6G). We, therefore,
conclude that DIMBOA acts as an extracellular signal for transcriptional feedback of BX
biosynthesis.

**DIMBOA regulates callose deposition**

PAMP-induced callose deposition is commonly used as a marker for plant innate immunity
(Luna et al., 2011). Chitosan elicits callose deposition in a wide variety of plants (Iritri and
Faoro, 2009). To optimise this PAMP response in maize, we quantified callose after leaf
infiltration with increasing concentrations of chitosan. At 24 h after infiltration, maize leaves
deposited callose in a dose-dependent manner (Figure S4), confirming that chitosan-induced
defence of maize is marked by callose deposition. In Arabidopsis, PAMP-induced callose
requires endogenous production of indolic GSs (Clay et al., 2009), suggesting a signalling
function by these secondary metabolites. Since monocots do not produce indolic GSs, we
tested whether BXs fulfil a similar role in maize innate immunity. To this end, we treated
leaves with non-saturating concentrations of chitosan (0.05%) and assayed callose deposition
in leaves of BX-producing bxl igt lines and BX-deficient bxl igt lines, which revealed that
the BX-deficient genotypes are severely affected in chitosan-induced callose (Figure 7).
Hence, PAMP-induced callose requires regulation by one or more bxl-dependent
metabolites. Since chitosan increases DIMBOA and HDMBOA-glc in the apoplast (Figure
5), we examined which of these two compounds are responsible for bxl-dependent callose.
To this end, we quantified callose intensities at 24 h after apoplast infiltration with either of
both compounds. As observed for Bx gene repression (Figure 6), DIMBOA was active and
triggered callose deposition in a dose-dependent manner, whereas HDMBOA-glc was
inactive and failed to boost callose deposition (Figure 8). Hence, DIMBOA functions as an extracellular signal for PAMP-induced callose.

**Discussion**

The role of BXs in plant defence against pests and diseases has been studied for decades. Most of these studies are based on either *in vitro* evidence, where BX compounds had been supplemented to artificial growth medium, or on correlative evidence between resistance and BX levels among cereal varieties and/or inbred lines (reviewed by Niemeyer, 1988; Niemeyer, 2009). For instance, *in vitro* supplied DIMBOA has been demonstrated to affect a broad spectrum of herbivorous insects and microbes, including aphids (Cambier et al., 2001) and *S. turcica* (Rostás, 2007). Other studies revealed negative correlations between endogenous BX concentrations and aphid performance in populations of cereal varieties (Argandoña et al., 1980; Leszczynski and Dixon, 1990; Niemeyer and Perez, 1994). In the present study, we have investigated the defence function of BXs further, by comparing resistance phenotypes of wild-type and *bx1* maize lines in the background of the *igl* mutant. Since IGL controls herbivore-induced indole production, the *igl* mutation prevents production of IGL-dependent BXs and, therefore, allows for a more accurate assessment of the defence contribution by BXs against IGL-inducing pests and diseases. Comparison between BX-producing and BX-deficient progenies from 2 independent crosses between the *igl* mutant and the *bx1* mutant confirmed a major role for BXs in resistance against the cereal aphid *R. padi* (Figure 1). Using these lines, we also discovered a significant contribution of BXs to early-acting post-invasive penetration resistance against the necrotrophic fungus *S. turcica* (Figure 3). Hence, BXs play an important role in basal resistance against aphids and fungi. Interestingly, the expression of this BX-dependent defence occurred independently from large-scale tissue disruption.

The involvement of BXs in penetration resistance against *S. turcica* (Figure 3B) suggests an alternative mode of action that contradicts the classical notion that BX defence requires tissue damage to hydrolyse vacuole-localised BX-glc compounds by plasmid-localised β-glucosidases (Morant et al., 2008). Chromatographic profiling of BX compounds revealed increased accumulation of apoplastic DIMBOA-glc, DIMBOA and HDMBOA-glc during the relatively early stages of infestation by either *R. padi*, or *S. turcica* (Figures 2 and
4). Notably, during both interactions, these effects preceded major tissue disruption or symptom development. Since aphid stylets and fungal hyphae must colonise the host apoplast before the host-parasite interaction can be established, enhanced deposition of biocidal BXs in this compartment is consistent with a role in penetration resistance. Based on our findings, we propose an alternative mode of BX-dependent defence, which depends on the accumulation of DIMBOA-glc and HDMBOA-glc into the apoplast, where they contribute to penetration resistance upon subsequent activation by plant- or attacker-derived β-glucosidases (Figure 9). In support of this, we observed increased accumulation of DIMBOA aglycone in the apoplast of challenged leaves (Figures 2 and 4). The lack of HDMBOA aglycone in this fraction can be explained by the highly unstable nature of this metabolite (Maresh et al., 2006). Although we cannot exclude that apoplastic BX accumulation during the early stages of aphid or fungal infestation occurs entirely without tissue damage, our experiments with chitosan demonstrate that this response can occur independently of tissue damage in maize. The mock treatments of these experiments involved a similar leaf infiltration as the chitosan treatments, but failed to increase apoplastic BX content (Figure 5). Hence, the difference in apoplastic BX accumulation between both treatments is unrelated to possible tissue damage due to leaf infiltration. Future research will be necessary to identify the molecular transportation mechanisms underpinning this PAMP-induced accumulation of BXs in the apoplast.

In addition to the defensive contribution from Bxl, we were also able to determine the role of Igl by comparing resistance levels between indole-producing bxl single mutants and indole-deficient bxl igl double mutants. Although aphid performance and fungal colonization were consistently lower in Igl wild-type lines compared to igl mutant lines, this difference was only statistically significant for aphid survival in the progeny from one cross, and was not proportional to the relatively major contribution from the Bxl allele (Figure 1B). Nonetheless, these relatively weak effects by Igl suggest a minor contribution from IGL-derived BXs. It is possible that IGL has a more prominent contribution during the later stages of the interaction, when more IGL-derived indole is channelled into the BX pathway and replenishes the rapidly declining pool of DIMBOA-glc. The extent of this contribution requires further investigation and can be addressed by comparing BX profiles between indole-producing Igl lines and indole-deficient igl lines at different stages of the plant-parasite interaction. These lines will also prove useful to assess the role of IGL-dependent indole emission in tritrophic interactions and indirect defence against herbivores.
Early-acting post-invasive plant defence is marked by a rapid accumulation of reactive oxygen species followed by deposition of callose-rich papillae (Ton et al., 2009; Luna et al., 2011). Our finding that bx1 mutant lines are affected in PAMP-induced callose suggests a regulatory function of BXs in this defence response (Figure 7). Recently, it was reported that DIMBOA acts as an electron acceptor for apoplastic cytokinin dehydrogenase, thereby contributing to the degradation of cytokinins (Frebortova et al., 2010). Since cytokinins can antagonise abscisic acid (ABA)-regulated plant processes (Shkolnik-Inbar and Bar-Zvi, 2010; Subbiah and Reddy, 2010; Vysotskaya et al., 2010), it is tempting to speculate that DIMBOA-catalysed cytokinin degradation contributes to ABA-dependent priming of callose deposition (Ton et al., 2009). In support of this, we found that apoplast infiltration with DIMBOA boosts callose deposition (Figure 8). Interestingly, Frebortova et al. (2010) proposed that DIMBOA-dependent degradation of cytokinins depends on the –N-OH group of DIMBOA at the indolic ring, which is absent in HDMBOA after O-methylation. Indeed, HDMBOA-glc failed to elicit callose deposition in our experiments (Figure 8). Future research will be necessary to decipher the interplay between extracellular DIMBOA, cytokinins and ABA in the regulation of post-invasive cereal defence against pests and diseases.

PAMP-induced callose in Arabidopsis requires intact biosynthesis of the 4-methylated indolic GS 4MI3G and subsequent hydrolysis by the atypical myrosinase PEN2 (Clay et al., 2009). This discovery revealed an important regulatory function of breakdown products of indolic glucosinolates in Arabidopsis innate immunity, but at the same time raised the question how non-Brassicaceous plants regulate callose deposition. In this study, we have examined whether BXs fulfil a similar regulatory function in maize, and found that BX-deficient bx1 lines are indeed dramatically reduced in their capacity to deposit PAMP-induced callose compared to BX-producing Bx1 lines (Figure 7). Moreover, of the two chitosan-inducible BXs, only DIMBOA elicited callose deposition upon infiltration into the apoplast, whereas infiltration with similar amounts of HDMBOA-glc failed to trigger callose depositions (Figure 8). A similar compound-specificity was found for the transcriptional feed-back regulation of the BX pathway (Figure 6). Since DIMBOA and HDMBOA-glc are both degraded into MBOA and BOA (Maresh et al., 2006; Macias et al., 2007), it can be concluded that extracellular DIMBOA, rather than its successive break-down products, is responsible for the regulation of callose deposition and Bx gene expression (Figure 9). We, therefore, hypothesize that this signalling function originates from a conserved detoxification response, which prevents auto-toxic build-up of BXs by translocation to the apoplast. Once
deposited into the apoplast, BX-glucosides become hydrolysed and captured in a matrix of callose to provide targeted chemical defence against invading parasites. The striking analogy with IG metabolites in Arabidopsis points to a conserved regulatory function of indole-derived secondary metabolites in innate immunity across the plant kingdom.

Conclusions

BX-deficient maize is more susceptible to aphids and is affected in penetration resistance against fungal pathogens. The difference in resistance between BX-deficient and BX-producing maize lines occurs before the onset of major tissue damage and manifests itself as increased accumulation of BX compounds in the apoplast. In addition to its contribution as a biocidal defence metabolite, extracellular DIMBOA regulates Bx gene expression and PAMP-induced callose, which reveals a novel regulatory function of BX metabolites in cereal innate immunity against pests and diseases.
Materials & Methods

Plant material and cultivation.

The Pioneer Hi-Bred collection of 42,300 F1 maize plants, mutagenized by means of Robertson’s Mutator element, was screened for Mu-containing alleles of Igl by a reverse genetics approach (Bensen et al., 1995). PCR amplification was performed as described previously (Mena et al., 1996). Mu-Integration in the non-translated 5’-leader region of IGL was identified and Mendelian segregation was confirmed within the progeny. Heterozygous progeny was selfed from which one homozygous mutant was identified (cv. B73). This plant was used as female (cross 307) and male (cross 308), respectively, in crosses with the homozygous bx1 reference allele mutant (cv. GeHu Yellow Dent; Hamilton, 1964). Individual heterozygous progeny (plants 308-1, 308-5 and 307-1) of the two reciprocal crosses were used to generate segregating progeny for the wild-type and mutant alleles of BX1 and IGL. Homozygous lines “22” (bx1 igl), “7” (bx1 Igl), and “25.13” (Bx1 igl) were selected from a cross between 308-1 x 307-1 (cross A), whereas lines “32R” (bx1 igl), “16R” (bx1 Igl) and “24R” (Bx1 igl) were selected from a cross between 308-5 x 307-1 (cross B). Genotypes from these crosses were initially identified by phenotyping (bx1bx1 mutants; FeCl3 root staining; Bailey and Larson, 1991), or PCR genotyping (igl mutants), and were propagated by selfing. Resulting F3 and F4 lines were further selected and confirmed by HPLC-DAD analysis of BX leaf content and GC-MS analysis of indole emission from Spodoptora littoralis-infested or wounded plants as described previously (Ton et al., 2007; Figure S1). Plant responses to R. padi, S. turcica, chitosan, indole, DIMBOA, or HDMBOA-glc were performed with the BX- and indole-producing wild-type cultivar Delprim (Delley Semences, Switzerland). Seeds were germinated at 22°C in petri-dishes in the dark. After 2 – 3 d, germinated seedlings of similar size were transplanted to pots containing compost soil and cultivated under controlled conditions (16:8 h L:D, 22°C).

Aphid and fungus bioassays.

Rhopalosiphum padi were obtained from a single field-collected apterous virginopara, reared under controlled conditions (16:8 h L:D, 22°C) on maize (cv. Delprim) for at least 10 generations. No-choice development assays were performed by placing 15 replicated groups of six adult apterae in clip cages, attached to the first leaf of 8 d old plants and left overnight to larviposit. The adult apterae were then removed and neonate nymphs were counted. A
maximum of 10 nymphs per clip cage were retained. For each assay, seedlings were maintained in a controlled climate chamber for 6 d (20° +/- 2°C, 16:8 h L:D, 40% RH) after which surviving nymphs were counted and then weighed in their batches in a 0.2 ml microfuge tube on a microbalance (Cahn C33, Scientific and Medical Products Ltd, Manchester, UK). Data were expressed as average weight per aphid or as percentage of aphid survival, and subjected to ANOVA. To determine aphid-induced BX production, at least 10 replicated batches of 25 late instar nymphs were enclosed in clip cages on 8 d old plants and left to feed for 48 h. Aphids were then removed and the leaf tissue extracted as described below. Mock-treated plants had clip cages only. For HPLC analysis of BX content, leaf material was collected from 4 to 6 infested leaves from different plants. *Setosphaeria turcica* cultivation, spore collection and inoculation of 8-day-old seedlings (5 x 10^4 spores.ml^-1) were performed as described by (Rostas et al., 2006). Sixteen randomly collected segments (2 - 3 cm) from inoculated leaves of 4 different plants per line were collected at indicated time-points and divided for HPLC analysis and microscopic analysis following trypan-blue lactophenol-blue staining (Koch and Slusarenko, 1990). Colonisation by *S. turcica* was examined microscopically. Penetration resistance was expressed as the fraction of arrested spores, or the average hyphal length emerging from germinating spores, which was determined from digital photographs, using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

**BX extraction, quantification and verification.**

BX extraction and analysis by high performance liquid chromatography coupled to diode array detection (HPLC-DAD) were adapted from Baumeler et al. (2000). Briefly, weighed plant material was frozen in liquid nitrogen and pulverised by vortexing in microfuge tubes containing 4 ball bearings (3 mm Ø). After addition of 1 mL extraction buffer (EB; methanol/HAc; 49/1; v/v), samples were sonicated (10 min) and centrifuged (12.600 g, 10 min). Supernatants were collected for analysis by a Shimadzu prominence HPLC system (Shimadzu Corporation, Kyoto, Japan) with BetaSil C18 column (250mm X 4.6 mm; 5 µ particle size; Thermo Scientific, USA) and diode array detector set at 254 nm. The mobile phase consisted of a mixture of pure water (solution A) and Methanol/Isopropanol/HAc (3800/200/1; v/v; solution B). The flow rate was maintained at 1 mL.min^-1, starting with isocratic conditions at 10% B for 2 min, linear gradient to 50% B from 2 - 27 min, isocratic conditions at 50% B from 27 - 29 min, linear reverse gradient to 10% B from 29 - 31 min, and isocratic conditions at 10% B from 31 - 35 min. Retention times of the different BXs
were established from synthetic standards (kindly provided by Prof. Dieter Sicker, University of Leipzig). BX tissue content (μg.g⁻¹ FW) was estimated from standard curves, which showed linear relationships between peak area and concentration. Mass identities of DIMBOA-glc, DIMBOA and HDMBOA-glc were confirmed by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS; Glauser et al., 2011). Since DIMBOA and HDMBOA-glc elute closely together using the above HPLC separation protocol, we performed additional verification of both compounds by nuclear magnetic resonance (NMR) analysis after preparative HPLC purification. ¹H, GCOSY and GHSQC spectra were recorded with a Varian VNMRS 500 MHz spectrometer; the chemical shifts were reported in ppm from tetramethylsilane with the residual solvent resonance taken as the internal standard. For DIMBOA, ¹H NMR spectrum revealed five resonances (Figure S3A), which were readily assigned to the OCH₃, aliphatic CH and three aromatic CH groups. For HDMBOA-glc, six proton resonances from the aglycone framework (Figure S3B) were detected whereas proton resonances from the glycoside moiety were observed as broad singlets or multiplets with chemical shifts comparable to closely related glycosides (Rashid et al., 1996). ¹H-¹H gCOSY and single bond ¹H-¹³C gHSQC experiments were used to further confirm the identity of the HDMBOA-glc (data not shown). Although BX compounds can be unstable during extraction procedures, our extraction method yielded recovery rates of >98% when purified BX compounds were added to plant tissues before grinding.

**Extraction of apoplastic fluids.**

The method was adapted from Yu et al (1999) and Boudart et al. (2005). Briefly, collected leaf tissues were weighted and submerged into 14 μg.ml⁻¹ proteinase K solution (Sigma) under a glass stopper in Greiner tubes. Vacuum infiltration was performed using a desiccator at -60 kPa for 5 min. After infiltration, leaf tissues were blotted dry, carefully rolled up, and placed in a 12-mL tubes, containing 20 ball bearings (3 mm Ø) and 0.5 mL EB supplemented with 14 μg.ml⁻¹ proteinase K. After centrifugation for 5 min at 2,300 g (4°C), tissues were removed and the collected liquid comprising EB and apoplastic fluid was collected from beneath the ball bearings with a pipette and subjected to HPLC analysis. Leaf segments infiltrated with chitosan solution were incubated for 24 h in sealed petri-dishes before centrifugation.

**Chemical treatments and callose quantification.**
Indole exposure was performed with 10-d old plants of similar size. Plants (n=9) per treatment were placed in air-tight glass chambers and exposed to indole that had been dissolved in dichloromethane, or dichloromethane only (mock), applied on filter paper discs. After 24 h of exposure, leaf segments (2 - 3 cm) from the second leaf were collected and divided for analysis of gene expression and BX content. Analyses of gene expression, BX content and callose deposition after chemical leaf infiltration were based on 9 - 18 randomly collected leaf segments (2 - 3 cm; second leaf) from at least 3 different 10-d old plants per treatment. Chitosan (Sigma) was dissolved to 1% (m/v) in 1% HAc initially, and diluted to 0.2% Chitosan (0.2% HAc) with water. Subsequent dilutions to 0.1 or 0.05% chitosan were performed with 0.2 % HAc, and adjusted to pH = 5.5 - 5.7. Chitosan solutions were infiltrated in leaf segments (2-3 cm) as described above, and left for 24 h under standard growth conditions before further analysis. Mock treatments were performed similarly with 0.2 % HAc (pH = 5.5 -5.7). DIMBOA and HDMBOA-glc were purified using a semi-preparative BetaSil C18 column (250mm X 10 mm; 5 µ particle size; Thermo Scientific, USA) at a flow rate of 4 mL.min⁻¹. Collected elutes were lyophilised and re-suspended in EB. After verification by HPLC-DAD and UHPLC-QTOFMS, compounds were diluted to indicated concentrations (1.96% methanol; 0.04% HAc; pH – 5.5 – 5.7) and infiltrated into leaf segments, as described above. Mock treatments were performed similarly using 1.96% methanol 0.04% HAc (pH = 5.5 – 5.7). Aniline-blue staining and quantification of callose by epi-fluorescence microscopy were performed, as described by Luna et al. (2011).

**RNA isolation, cDNA preparation and RT-qPCR analysis.**

Gene expression analyses were based on three biologically replicated samples from the 2nd leaf of 10-d old plants. Total RNA was extracted as described previously (Matthes et al. 2010). Genomic DNA was digested according to manufacturer’s guidelines (RQ1 RNase-Free DNase; Promega, UK). Synthesis of cDNA was performed as described by Ton et al. (2007). Two technical replicates of each cDNA sample were subjected to RT-qPCR analysis, as described previously (Ahmad et al., 2011). Primer sequences are listed in Table S1. PCR efficiencies (E) of primer pairs were estimated from multiple amplification plots using the equation (1+E) = 10^{slope} (Ramakers et al., 2003), and were confirmed to provide (1+E) values close to 2. Transcript levels were calculated relative to the reference genes GAPC or Actin-1 (Erb et al., 2009), using the 2^{ΔΔCt} method, as described (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), or the 2^{ΔCt} method, where ΔCt = Ct (reference gene) - Ct (gene of interest).
Acknowledgements

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Figure Legends

Figure 1: Contribution of Bx1 and Igl to basal resistance against the cereal aphid Rhopalosiphum padi. Batches of neonate nymphs in clip cages were allowed to feed for 7 d from the first leaf of igl mutant lines, bx1 mutant lines, and bx1 igl double mutant lines, which had been selected from two independent crosses between the bx1 mutant and igl mutant of maize. A, Average weights (± SEM; n = 15) of neonate nymphs after 7 d. B, Average percentages of batch survival (± SEM) after 7 d. Different letters indicate statistically significant differences (ANOVA, followed by Fisher’s LSD test; α = 0.05). Wild-type alleles are indicated in black and mutant alleles in grey. The comparison between igl and bx1 igl mutant lines was repeated in two additional experiments with similar results.

Figure 2: HPLC-DAD quantification of DIMBOA-glc, DIMBOA and HDMBOA-glc in whole-tissue extracts (A) and apoplastic extracts (B) from mock- and R. padi-infested maize leaves. Material was collected at 48 h after aphid feeding in clip cages. Mock treatments consisted of clip cages without aphids. Data represent mean values in μg.g⁻¹ F.W. (± SEM) from 4 biologically replicated leaf samples. Asterisks indicate statistically significant differences compared to mock-treated leaf samples (Student’s t-test; α = 0.05). The experiment was repeated with similar results.

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Figure 5: HPLC-DAD quantification of DIMBOA-glc, DIMBOA and HDMBOA-glc in whole-tissue extracts (A) and apoplastic extracts (B) at 24 h after infiltration of leaf segments with 0.2% chitosan or mock buffer. Data represent means in μg.g⁻¹ F.W. (± SEM) from three biologically replicated samples. Asterisks indicate statistically significant differences compared to mock-treated leaves (Student’s t-test; α = 0.05). The experiment was repeated twice with similar results.

Figure 6: Transcriptional feed-back regulation of the benzoxazinoid pathway by Bx1-dependent DIMBOA. Apoplastic infiltration with benzoxazinoid-inducing concentrations of chitosan (0.2 %) (A) and exposure to the volatile benzoxazinoid precursor indole (B) repressed *Bx* gene expression. benzoxazinoid-deficient *bx igl* double mutant lines displayed enhanced *Bx* gene transcription compared to benzoxazinoid-producing *igl* single mutant lines (C, D). Apoplastic leaf infiltration with DIMBOA (E, F) repressed *Bx* gene expression, whereas HDMBOA-glc had no effect (G), suggesting transcriptional feed-back regulation by apoplastic DIMBOA. Shown are average fold-change values (± SEM) of genes with a statistically significant level of induction (red) or repression (green) compared to mock treatments (A, B, E – G) or BX-producing *igl* single mutant lines (C, D). Differences in expression between three biologically replicated samples from independent experiments were tested for statistical significance, using Student’s t-tests or a non-parametric Wilcoxon Mann–Whitney test when values did not follow normal distributions (α = 0.05). n.d.: not determined.

Figure 7: Bxl regulates chitosan-induced callose deposition. Leaf segments from *igl* single mutant lines and *bxl igl* double mutant lines were infiltrated with chitosan (0.05%) or mock solution. At 24 h after infiltration, leaf segments were collected for aniline-blue staining, UV-epifluorescence microscopy, and digital quantification of callose intensity. Shown are fold-
induction values of callose (± SEM; n = 15), relative to the average callose intensity in mock-treated Bxl/igl lines from each cross. Photographs show representative differences in fluorescent callose signals under UV-epifluorescence microscopy.

**Figure 8:** DIMBOA-induced callose deposition. Infiltration with 20 μg.mL⁻¹ DIMBOA elicits similar levels of callose deposition as infiltration with chitosan (0.1%), whereas infiltration with 20 μg.mL⁻¹ HDMBOA-glc had no effect in comparison to the corresponding mock treatment. Shown are fold-induction values of callose deposition (± SEM; n = 15), relative to average callose intensities in mock treatments at 24 h after infiltration treatment. Different letters indicate statistically significant differences (ANOVA, followed by Fisher’s LSD test; α = 0.05). Photographs show representative differences in fluorescent callose signals by UV-epifluorescence microscopy.

**Figure 9:** Model of BX-dependent innate immunity against aphids and fungi. Activation of maize innate immunity leads to apoplastic deposition of HDMBOA-glc and DIMBOA-glc. Subsequent hydrolysis into biocidal aglycones can provide chemical defence against pests and diseases (Cambier et al., 2001; Rostás, 2007) Both HDMBOA and DIMBOA are degraded into MBOA and BOA (Maresh et al., 2006), indicating that DIMBOA (red), and not HDMBOA, has an additional function in the regulation of Bx gene expression and callose deposition. I: intracellular space; A: apoplast.
Supplemental files

**Figure S1:** Confirmation of mutant phenotypes of *bx1* and *igl* carrying maize lines. Shown are typical HPLC-DAD chromatograms from un-stressed leaves of 7-d old seedlings (in pink), and GC-FID chromatograms of head-space collections from wounded leaves of 10-d old seedlings (in blue). Arrows in HPLC chromatograms indicate peaks corresponding to DIMBOA-glc or HDMBOA-glc; arrows in GC chromatograms indicate peaks corresponding to indole.

**Figure S2:** HPLC-DAD quantification of basal and chitosan-induced DIMBOA-glc, DIMBOA and HDMBOA-glc in whole-leaf extracts from the *igl* single mutant, the *bx1* single mutant, and the *bx1 igl* double mutant (cross B). Leaf samples were collected at 24 h after infiltration of leaf segments with mock buffer or 0.2% chitosan. Data represent means in μg.g⁻¹ F.W. (± SEM) from three biologically replicated samples. Asterisks indicate statistically significant differences compared to mock-treated leaves (Student’s t-test; α = 0.05). nd: not detected.

**Figure S3:** ¹H nuclear magnetic resonance (NMR) spectrogram of DIMBOA (A) and HDMBOA-glc (B). A, DIMBOA: ¹H NMR (500 MHz, CD₃OD): δ 3.76 (s, 3H), 5.67 (s, 1H), 6.61 (s, 1H), 6.67 (d, 8.5 Hz, 1H), 7.28 (d, 8.5 Hz, 1H). B, HDMBOA-glu: ¹H NMR (500 MHz, CDCl₃): δ 3.34 (broad singlet), 3.37 (broad singlet), 3.43 (broad singlet), 3.52 (broad singlet), 3.72 (3.72 s, 3H), 3.78 (broad multiplet, 2H), 3.90 (s, 3H), 4.72 (broad singlet, 1H), 5.84 (s, 1H), 6.59 (d, 8 Hz, 1H), 6.66 (s, 1H), 7.05 (d, 8Hz, 1H).

**Figure S4:** Dose-dependent callose deposition at 24 h after pressure infiltration with different concentrations of chitosan. Shown are fold-induction values of callose deposition (± SEM; n = 15), relative to mock treatments at 24h after infiltration treatment.
**Table S1:** Primers used for RT-qPCR analysis of gene expression

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<td>5′-AAAAACATGGGACCTCGTG-3′</td>
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**Figure 7:** *Bx1* regulates chitosan-induced callose deposition. Leaf segments from *igl* single mutant lines and *bx1 igl* double mutant lines were infiltrated with chitosan (0.05%) or mock solution. At 24 h after infiltration, leaf segments were collected for aniline-blue staining, UV-epifluorescence microscopy, and digital quantification of callose intensity. Shown are fold-induction values of callose (± SEM; n = 15), relative to the average callose intensity in mock-treated *Bx1 igl* lines from each cross. Photographs show representative differences in fluorescent callose signals under UV-epifluorescence microscopy.
Figure 8: DIMBOA-induced callose deposition. Infiltration with 20 μg.mL⁻¹ DIMBOA elicits similar levels of callose deposition as infiltration with chitosan (0.1%), whereas infiltration with 20 μg.mL⁻¹ HDMBOA-glc had no effect in comparison to the corresponding mock treatment. Shown are fold-induction values of callose deposition (± SEM; n = 15), relative to average callose intensities in mock treatments at 24 h after infiltration treatment. Different letters indicate statistically significant differences (ANOVA, followed by Fisher’s LSD test; α = 0.05). Photographs show representative differences in fluorescent callose signals by UV-epifluorescence microscopy.
Figure 9: Model of BX-dependent innate immunity against aphids and fungi. Activation of maize innate immunity leads to apoplastic deposition of HDMBOA-gluc and DIMBOA-gluc. Subsequent hydrolysis into biocidal aglycones can provide chemical defence against pests and diseases (Cambier et al., 2001; Rostás, 2007). Both HDMBOA and DIMBOA are degraded into MBOA and BOA (Maresh et al., 2006), indicating that DIMBOA (red), and not HDMBOA, has an additional function in the regulation of Bx gene expression and callose deposition. I: intracellular space; A: apoplast.