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Highly localised and persistent induction of *Bx1*-dependent herbivore resistance factors in maize

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Summary

The induced production of secondary metabolites in herbivore-attacked plants varies in space and time. However, the consequences of these spatiotemporal patterns for herbivore performance are not well understood. This is particularly true for 1,4-benzoxazin-3-ones (BXs), the major induced defensive metabolites of maize. Here we report on the spatiotemporal dynamics of BX induction and its consequences for the leaf feeder

Spodoptera littoralis. Defence-related phytohormones and transcript levels of BX biosynthetic genes were upregulated locally at the wound site within 12 hours of herbivory. Within another 12 hours, the insecticidal BX HDMBOA-Glc started to accumulate in a highly localised manner at the feeding site. Changes in BX metabolism away from the feeding site within the same leaf were much weaker and were undetected in systemic leaves. Following the removal of the caterpillars, local HDMBOA-Glc levels remained elevated for seven days. Caterpillars that were forced to feed directly on locally induced leaf parts, but not on adjacent leaf parts, suffered from reduced growth. This effect was abolished in the BX deficient *bx1* mutant. We did not find any evidence that BXs regulate defensive phytohormones or their own accumulation. In summary, this study shows that induced herbivore resistance in maize is highly localised and dependent on BXs.

Introduction

Plants respond to herbivore attack by mobilising specific chemical defences (Karban and Baldwin, 1997). These defences are orchestrated by an array of signalling molecules like salicylic acid (SA) or jasmonic acid (JA) and its derivatives (Howe and Jander, 2008; Bonaventure, 2014). Jasmonates in particular regulate a broad set of plant defences against herbivores, including toxic secondary metabolites (Kessler et al., 2004; Bodenhausen and Reymond, 2007; Schmelz et al., 2011).

As defence induction patterns may influence the outcome of the interaction between plants and attacking herbivores, the spatiotemporal dynamics of secondary metabolite accumulation have gained significant attention over the last years (Karban, 2011). Locally restricted induction of defence responses for instance may promote herbivores to move away from an induced site, thereby increasing associated foraging costs (Edwards and Wratten, 1983;

Paschold et al., 2007; Karban, 2011; Perkins et al., 2013). To date, several studies have explored the spatial and/or temporal dynamics of defence induction in different plant species including systemic responses (Pearce and Ryan, 2003; Glauser et al., 2008; Glauser et al., 2009; Mathur et al., 2011; Hettenhausen et al., 2014; McCormick et al., 2014; Tzin et al., 2015b). Feeding by *Pieris rapae* caterpillars on radish leaves, for instance, leads to an increased variability of glucosinolate distribution in subsequently developing leaves (Shelton, 2005). Moreover, locally defined treatment of *Nicotiana attenuata* leaves with caterpillar oral secretions (OS) elicits a specific spatial pattern of defence-related kinase activity and jasmonic acid accumulation presumably leading to a non-uniform defence induction within treated leaves (Wu et al., 2007). Similarly, within individual maize leaves the jasmonate burst was found to be most pronounced at the site of induction. However, JA levels were also elevated towards the leaf tip (Engelberth et al., 2007; Engelberth et al., 2012). The same pattern of unidirectional defence induction was observed for sesquiterpene emission from caterpillar-infested maize foliage (Köllner et al., 2013).

The predominant class of jasmonate-regulated, herbivory-induced secondary defence metabolites in maize are indole-derived 1,4-benzoxazin-3-ones (BXs), which are stored as inactive glucosides in the vacuole (Macias et al., 2009; Niemeyer, 2009). Upon tissue disruption, e.g. caused by leaf-chewing insects, the glucosides come into contact with β-glucosidases that cleave off the sugar moiety and release the active aglucone (Czjzek et al., 2000; Morant et al., 2008). The most abundant BX metabolites in undamaged maize leaves are 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc) and 2-β-D-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA-Glc). Caterpillar feeding induces enzymatic transformation of DIMBOA-Glc to HDMBOA-Glc (Glauser et al., 2011) by several *O*-methyltransferases (Meihls et al., 2013; Handrick et al.,

2016). Agluconic HDMBOA seems to be more toxic than DIMBOA, which can be detoxified by several insect species (Rostás, 2007; Sasai et al., 2009; Glauser et al., 2011; Wouters et al., 2014).

The first committed step in the BX biosynthetic pathway is the enzymatic conversion of indole-3-glycerol phosphate (IGP) to indole by BX1 (Frey et al., 2009) (Figure S1). The synthesis of DIMBOA-Glc from indole then requires the activity of eight additional enzymes (BX2-BX9). Unexpectedly, homozygous *bx1* mutant lines are not completely devoid of BXs (Köhler et al., 2015). This has been ascribed to the activity of indole-3-glycerol phosphate lyase (IGL). IGL catalyses the conversion of IGP to free indole that is being released from infested plants as part of the herbivore-induced plant volatile (HIPV) bouquet and serves as a priming signal (Frey et al., 2000; Erb et al., 2015). Thus, BX levels may also slightly increase in infested *bx1* mutants due to channelling of IGL-derived indole into the BX pathway. Nevertheless, *bx1* mutant lines were found to be more susceptible to the aphid *Rhopalosiphum padi* and leaf blight *Setosphaeria turcica* (Ahmad et al., 2011). Interestingly, DIMBOA also serves as an elicitor of callose deposition and was found to mediate callose-dependent aphid resistance suggesting that certain BX metabolites may exert additional signalling functions *in planta* (Ahmad et al., 2011; Meihls et al., 2013; Maag et al., 2015).

In contrast to the emission of terpenoid volatiles from herbivore-attacked maize leaves little is known about the spatiotemporal dynamics of non-volatile defence induction in infested maize leaves and their consequences for herbivore performance. Here we report on the within-leaf distribution of defence-related metabolites and gene transcripts in maize following varying periods of spatially constrained feeding by larvae of the moth *Spodoptera littoralis*. As the inducibility of BXs represents an important aspect of maize defence

responses (Glauser et al., 2011) we were particularly interested in their spatiotemporal induction and relaxation dynamics. Including BX-deficient *bx1* mutants allowed us to estimate the contribution of BXs to the observed localised resistance phenotype. In addition, we assessed the putative effects of *Bx1*-dependent metabolites on herbivory-triggered phytohormone accumulation during the interaction between maize leaves and a chewing herbivore as an important phenomenon that may contribute to highly localised, persistent induction patterns.

Results

Early induction of phytohormones and 1,4-benzoxazin-3-ones is highly localised

Within-leaf gradients of defence-related metabolites and gene transcripts were measured following different periods of herbivore feeding. Clip cages were used to restrict caterpillar feeding to the leaf centre (corresponding to leaf segment C), allowing us to estimate the spatial extent of metabolite induction (Figure 1). Jasmonic acid and its isoleucine conjugate JA-Ile were strongly increased by herbivory, with sharp concentration increases in the leaf centre (Figure 1a,b). Although both hormones tended to increase further over the course of the infestation, no statistically significant differences were detected between 12, 24 and 24+24 hours post-infestation (hpi). Likewise, removal of the caterpillars did not result in significant changes of JA and JA-Ile at the damage site (segment C, 24+24 hpi). Abscisic acid (ABA) showed an induction pattern similar to those of JA and JA-Ile with a pronounced local increase upon herbivore feeding and declining levels once herbivory had ceased (Figure 1c). SA levels were not affected by herbivore treatment (Figure 1d).

All of the measured gene transcripts were upregulated after herbivore infestation and most of them exhibited maximum induction levels at the feeding site (Figure 2). Transcripts of *Bx1*, whose expression product catalyses the first committed step of BX biosynthesis, reached their maximum accumulation already at 12 hpi (segment C, 45.0 ± 2.0-fold; mean ± SE) and dropped slightly thereafter (Figure 2a). During the entire course of the experiment the feeding site was significantly enriched for *Bx1* transcripts compared to the four adjacent leaf segments. Upregulation of *Bx1* also occurred towards the leaf base, albeit to a much lower extent (e.g. segment A at 12 hpi, 4.1 ± 0.8-fold). In contrast to *Bx1*, *Igl* was transiently upregulated throughout the whole leaf at 24 hpi with the upper leaf part (corresponding to segments D and E) showing significantly higher transcript levels at the respective time point compared to the remaining three segments (Figure 2b). Within 24 hours after removal of the caterpillars *Igl* transcript levels returned to control levels, except at the feeding site where they decreased even further (segment C, 0.4 ± 0.1-fold). In addition, we characterised the expression profiles of two genes coding for the DIMBOA-Glc-*O*-methyltransferases BX10 (GRMZM2G311036) and BX11 (GRMZM2G336824) and the DIM2BOA-Glc-*O*-methyltransferase BX14 (GRMZM2G127418). Due to the high sequence similarity between *Bx10* and *Bx11*, they were co-amplified by the same primer pair (Meihls et al., 2013). *O*-methyltransferase expression was highly responsive to herbivore feeding. Transcripts of *Bx10,11* started to significantly accumulate at the feeding site within the first 12 hours of herbivore feeding (Figure 2c). During the following 12 hours of infestation, transcript levels continued to increase, with levels being highest in the leaf centre and the adjacent segment towards the leaf tip (333.9 ± 58.8-fold and 278.3 ± 75.7-fold in segments C and D, respectively). Significantly upregulated transcript levels were also detected at the leaf tip itself after 24 hours of caterpillar feeding (segment E, 34.2 ± 15.3-fold). Following caterpillar removal, transcript levels dropped off, but were still significantly enhanced around the

feeding site (10.3 ± 1.5-fold and 5.7 ± 2.7-fold in segments C and D, respectively). The expression profile of *Bx14* was highly similar to that of *Bx10,11* (Figure 2d). Accordingly, *Bx14* showed the strongest response to herbivory after 24 hours of feeding and was still significantly upregulated at the feeding site at 24+24 hpi (270.6 ± 53.2-fold).

Among the six BX glucosides that were detected in the plant samples, HDMBOA-Glc and 2-*β*-D-glucopyranosyloxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one (HDM2BOA-Glc) displayed the strongest concentration increases following caterpillar infestation (Figure 3a,b). Similar to jasmonate levels, the concentrations of these two BXs showed a strong spatially restricted peak around the feeding site. Inducible BXs did not start to accumulate before 24 hours of caterpillar feeding. Both HDMBOA-Glc and HDM2BOA-Glc concentrations tended to increase further following the removal of the insects. In contrast, DIBOA-Glc was induced only slightly, but across the entire leaf. DIMBOA-Glc and DIM2BOA-Glc were slightly suppressed by herbivory across the different leaf segments (Figure 3c,e,f). In addition, the two BX aglucones HMBOA and DIMBOA were found to accumulate exclusively at the feeding site (Figure S2). Interestingly, no significant increase of BX aglucones was detected before 24 hours of caterpillar feeding. Due to its high reactivity it was impossible to reliably quantify HDMBOA in the leaf samples. Relative water contents did not differ between control and herbivore attacked leaf segments (Figure S3).

Induced 1,4-benzoxazin-3-one levels are maintained in absence of herbivory

The observation that HDMBOA-Glc and HDM2BOA-Glc concentrations tended to increase further during the 24 hours after caterpillar removal prompted us to investigate how induced levels of these two metabolites evolve in absence of herbivory over a longer time period. As before, HDMBOA-Glc and HDM2BOA-Glc were strongly upregulated around the feeding site (corresponding to leaf segment C in the previous experiment) in response to *S. littoralis*

attack (Figure 4a,b). Following caterpillar removal, HDMBOA-Glc concentrations did not significantly increase further but remained constant for 72 hours and started to decline slightly afterwards. At all time points over the 7-day period, HDMBOA-Glc levels were significantly higher than in control plants. As observed before, HDM2BOA-Glc was only detectable in induced plant tissues (Figure 4b) and its levels remained constantly high for five days after herbivore removal before starting to decline. Besides the BX glucosides, we also detected BX breakdown products in this experiment. Levels of the active DIMBOA aglucone and its breakdown product MBOA were consistently higher in damaged leaf tissue than in intact control plants at all time points (Figure 4c,d).

Herbivory does not affect systemic 1,4-benzoxazin-3-one accumulation

Next, we investigated whether local herbivory affects BX levels in non-attacked leaves. Based on the current literature on systemic induced resistance in maize, we selected the next youngest leaf as systemic leaf (Turlings and Tumlinson, 1992; Balmer et al., 2013; Erb et al., 2015). Infestation of a local leaf by eight *S. littoralis* caterpillars for either 24 or 48 hours did not have any effect on BX levels in the next younger leaf (Figure 5a). To determine whether local herbivory primes the next younger leaf for HDMBOA-Glc induction, a defence response was elicited in the systemic leaf by mechanical damage and application of caterpillar OS 24 hours after infestation of the local leaf. Treatment with OS caused a strong increase of HDMBOA-Glc in the systemic tissue. However, this increase was independent of preceding herbivore feeding on the local leaf (Figure 5b).

Induced resistance is highly localised and partially dependent on *Bx1*

Based on the observed within-leaf distribution of inducible responses following feeding by *S. littoralis*, we hypothesised that the performance of subsequently feeding caterpillars should be suppressed locally by induced BXs. To test this hypothesis, we designed a no-choice feeding assay during which caterpillars were forced to feed either directly on a previously caterpillar-induced leaf segment or right next to it, i.e. between leaf segments C and D (Figure 6). After 24 hours of feeding, caterpillar weight gain was compared to that of caterpillars that were attached to the centre of a non-induced leaf. To estimate the contribution of inducible BXs to the resistance phenotype we included a *bx1* mutant line that is impaired in the first committed step in the BX biosynthetic pathway. This near-isogenic mutant line was obtained by introgression of a non-functional *bx1* allele into B73 maize. Overall, *S. littoralis* caterpillars gained approximately 30% more weight on the *bx1* mutant line than on wild-type B73 plants. When feeding locally on previously induced B73 leaf material, *S. littoralis* gained approximately 22% less weight compared to caterpillars feeding on intact leaves or on a distal segment of the induced leaf. By contrast, larval weight was reduced by only 12% when feeding at the induction site of the *bx1* mutant, and caterpillar growth did not differ significantly between feeding sites.

1,4-benzoxazin-3-one induction is dependent on *Bx1*

In parallel to the caterpillar performance assays, we also analysed the respective within-leaf gradients of BX metabolites in the *bx1* mutant and the corresponding wild-type B73 plants following herbivore infestation in the leaf centre. Leaf segments were collected at 24+24 hpi, which was the time point that the focal caterpillars of the bioassay were transferred onto the leaves. As expected, total BX levels in the mutant line were 95% lower than in B73 (Table S1, Figure 7a,b). In contrast to B73, total BX levels in the *bx1* mutant were highest at the leaf

base and decreased to nearly below detectable levels towards the tip. Induction of HDMBOA-Glc followed a similar pattern as observed before with a highly localised concentration increase upon feeding damage in the leaf centre (Figure 7c,d). This was the case for both the wild-type and the *bx1* mutant line, although in the latter induced levels were about four times lower than in B73. HDM2BOA-Glc was not detected in the *bx1* mutant (Figure 7e,f). Compared to the first experiment, accumulation of HDMBOA-Glc was slightly lower and appeared more strongly restricted to the leaf centre. As maize plants for the two experiments were grown in a greenhouse at different time points these differences may be the result of slightly differing environmental conditions.

Herbivory-induced phytohormone accumulation is not influenced by BXs

Within-leaf gradients of JA, JA-Ile, ABA and indole-3-acetic acid (IAA) were measured around the feeding site immediately after feeding by two *S. littoralis* caterpillars in the leaf centre for 24 hours (Figure 8). This experiment was carried out to determine whether the products of *Bx1* have any feedback effects on hormone induction. In contrast to previous experiments, leaves were not elongated enough to provide enough plant material for analysis of the most distal segments. Data are therefore only available for the three central segments. All four hormones showed highly localised induction patterns following herbivory and their concentration patterns were nearly identical for the *bx1* mutant and B73 (Figure 8). Accordingly, plant genotype did not have a significant effect on hormone levels (Table S2).

As plants were induced by caterpillar feeding, contrasting, genotype-dependent damage patterns could not be excluded. An additional experiment was therefore conducted where plants were induced in a standardised manner by wounding and application of BX-free caterpillar OS. Only leaf tissue directly from the wounding site was analysed, and time post-elicitation was introduced as an additional factor. All of the measured hormones responded to

the induction treatment in a time-dependent manner (Table S3). Levels of JA, JA-Ile, and ABA were induced most strongly one hour after OS application and decreased thereafter (Figure 9a-f). OS-specific IAA induction was somewhat slower and reached its maximum at 6 hours post-elicitation treatment (Figure 9g,h). No genotype-dependent effects were detected except for IAA, for which slightly higher concentrations were measured in the *bx1* mutant. In an additional experiment, we tested whether the BXs in the OS have any influence on induced BX responses. Treatment with *S. littoralis* OS led to a significant induction of HDMBOA-Glc and HDM2BOA-Glc, and this induction was independent from the presence or absence of BX-derived metabolites in the OS (Figure S4).

Discussion

The results presented in this study allow for three major conclusions regarding herbivory-induced defence responses in maize. Firstly, HDMBOA-Glc induction follows a temporal sequence during which an initial jasmonate burst is followed by the transcription of genes encoding for DIMBOA-Glc-*O*-methyltransferases and subsequent metabolite accumulation. Secondly, the induced BX responses and the *Bx1*-dependent induced resistance are highly localised. And thirdly, phytohormone signalling is not affected by the presence of a non-functional *Bx1* allele.

The importance of the jasmonate burst for the reconfiguration of both the plant transcriptome and metabolome in response to herbivory is well established (Howe and Jander, 2008; Wu and Baldwin, 2010). In our experiments, we observed a significant increase of JA and its isoleucine conjugates within 12 hours of caterpillar feeding that was accompanied by enhanced transcript levels of *Bx10,11* and *Bx14*. Induced accumulation of HDMBOA-Glc in response to fungal elicitors, external JA or herbivore infestation has been reported before

(Oikawa et al., 2002; Oikawa et al., 2004; Glauser et al., 2011; Huffaker et al., 2011). In addition, we also observed a significant upregulation of ABA following caterpillar feeding. Interestingly, ABA previously has also been associated with priming of the BX pathway (Erb et al., 2009b; Erb et al., 2009a).

The temporal dynamics of HDMBOA-Glc induction during herbivory, however, as well as the expression of associated biosynthetic genes had not been studied yet. While transcript levels of the underlying biosynthetic genes were already upregulated after 12 hours of caterpillar feeding, HDMBOA-Glc only started to accumulate during the next 12 hours of infestation. Interestingly, JA and JA-Ile as well as the *Bx10,11* and *Bx14* transcripts were still upregulated 24 hours after removal of the caterpillars. This translated into a continued accumulation of HDMBOA-Glc and HDM2BOA-Glc at the induction site. Besides DIMBOA-Glc, BX14 also accepts DIM2BOA-Glc as a substrate leading to the formation of HDM2BOA-Glc, thus likely contributing to HDM2BOA-Glc induction (Handrick et al., 2016). However, whether this on-going induction in absence of herbivory has an adaptive function or simply represents physiological constraints on the cessation of the underlying enzymatic activity remains to be determined. Similar findings were reported recently by Tzin et al. (2015a), who investigated the intraspecific variation of caterpillar-induced aphid resistance in maize and observed elevated *Bx10* and *Bx11* transcript levels almost three days after removal of the caterpillars from the plants. Interestingly, the within-leaf gradients of HDMBOA-Glc and HDM2BOA-Glc did only partially correlate with the distribution of *Bx10,11* and *Bx14* transcripts in non-infested leaves (Figure S5), and HDMBOA-Glc and HDM2BOA-Glc accumulation in the leaf tip was much lower than one would expect based on the distribution patterns of *Bx10,11* and *Bx14* transcripts in caterpillar infested plants. This divergence could be the result of either posttranscriptional regulation of BX biosynthesis or

the transport of BXs to the wound site. Many secondary metabolites can be transported across plants (van Dam et al., 1995; Chen et al., 2001; Andersen et al., 2013), and BXs are well known to be exuded from maize roots and have been reported to accumulate in the waxy layers of maize whorls (Hedin et al., 1993) and the apoplast of aphid infested plants (Ahmad et al., 2011). Understanding if and how BXs are transported to the wound site of attacked leaves is an exciting prospect of this work.

Recently, Karban (2011) identified a gap of knowledge regarding the relaxation of induced defence responses. Although in our study we did not follow induced BX levels until complete relaxation, it became apparent that relaxation takes place on a much longer time scale than induction. While induction of HDMBOA-Glc and HDM2BOA-Glc occurred within 24 hours, their levels remained almost constantly high for seven days after herbivory had ceased. Surprisingly, levels of free DIMBOA also remained higher in damaged leaves during the seven days following caterpillar removal. This was contrary to our expectations as the reported half-life for DIMBOA at physiological pH is below 24 hours (Woodward et al., 1978), and we expected DIMBOA to be produced exclusively by wounding, which together should have resulted in a rapid reduction in DIMBOA concentrations in the absence of herbivory. The observed elevated levels of DIMBOA coincided with the accumulation of its breakdown product MBOA over time. Together these observations suggest continuous induced β-glucosidase activity and a concomitant release of DIMBOA for at least one week after herbivore feeding. Besides their toxic effects on insects, DIMBOA and MBOA also possess antifungal properties (Oikawa et al., 2004; Rostás, 2007; Niemeyer, 2009). Thus, the continuous release of BX aglucones may serve as a countermeasure to prevent pathogen infection at the feeding site.

The induction of most defence-related genes and metabolites was restricted to the damage site. Defence-related gene expression, although mostly localised, could be divided into three groups depending on the respective expression patterns: (I) transcript upregulation exclusively at the feeding site (*Bx1*); (II) localised upregulation at the feeding site with transient induction towards the leaf tip (*Bx10,11* and *Bx14*); and (III) equally enhanced transcript levels throughout the whole leaf (*Igl*). Interestingly, *Bx1* and *Igl* showed very distinct expression profiles. Like BX1, IGL catalyses the formation of indole from indole-3-glycerol phosphate, however, indole derived from IGL activity is only partly available for BX biosynthesis and mainly supplies the pool of free, volatile indole (Frey et al., 2000; Köhler et al., 2015). Recently, it was shown that indole emission from infested plants primes neighbouring plants for enhanced phytohormone induction and HIPV release upon herbivore attack (Erb et al., 2015). Furthermore, it serves as a within-plant signal priming systemic leaves of infested plants for increased mono- and homoterpene emission. Thus, leaf-wide upregulation of *Igl* transcripts following spatially confined herbivory may result in an amplified signal by facilitating indole emission from the entire leaf blade.

Three other studies have investigated the spatial dynamics of defence induction in maize, but with a focus on differing defensive traits. Application of caterpillar OS or volicitin, an insect-derived defence elicitor, in the leaf centre resulted in a strong local increase of JA (Engelberth et al., 2007). However, JA levels were also enhanced towards the leaf tip. Interestingly, mechanical wounding alone caused a less pronounced jasmonate burst that was limited to the induction site. This unidirectional propagation of the JA signal was reflected at the transcriptomic level, with increased transcript levels of defence-related genes towards the leaf tip, but to a much lesser extent at the leaf base (Engelberth et al., 2012). In another study, caterpillars were allowed to feed on varying positions of maize leaves, revealing that the

unidirectional induction pattern is also true for sesquiterpene production (Köllner et al., 2013). In contrast to these observations, we only detected a slight increase of HDMBOA-Glc levels towards the leaf tip following *S. littoralis* feeding.

Despite the rather localised induction pattern, we hypothesised that the onset of the defence response may not be limited to the infested leaf as systemic effects of herbivory have been reported for several plant species including maize (Turlings and Tumlinson, 1992; Pearce and Ryan, 2003; Engelberth et al., 2012; Hettenhausen et al., 2014). However, in a series of additional experiments we did not observe any changes in systemic BX levels nor did we observe any priming for enhanced HDMBOA-Glc production following a second induction treatment on systemic tissue within the 48 hours following the first feeding bout. During the course of our experiments all plants were kept openly and in close proximity to each other. Thus, airflow between plants did occur and may have resulted in interplant communication via HIPVs thereby possibly confounding our measurements for a systemic effect (Ton et al., 2007; Erb et al., 2015).

Following the characterisation of the spatiotemporal dynamics of BX induction we investigated their possible effects on foraging herbivores. The foraging behaviour of herbivorous arthropods is affected by the constitutive distribution of defence-related metabolites, but also their inducibility (Edwards and Wratten, 1983; Paschold et al., 2007; Shroff et al., 2008; Perkins et al., 2013; Köhler et al., 2015). In our performance assays, *S. littoralis* larvae that were forced to feed on induced leaf segments gained significantly less weight compared to caterpillars that fed on non-induced leaves. Furthermore, caterpillars that were forced to feed right next to the damage site gained as much weight as those on non-induced leaves, which indicates that defence induction in maize is extremely localised. Our

experimental set-up did not allow for measuring the amount of consumed leaf material. Thus it is impossible to determine whether the contrasting growth rates on adjacent leaf segments were due to direct toxic effects or whether they resulted from differential food intake. Interestingly, the growth-impairing effect of preceding induction was attenuated on *bx1* mutant plants. Together with the fact that growth-impairing effects of various BX metabolites including HDMBOA have been demonstrated *in vitro* (Rostás, 2007; Sasai et al., 2009; Glauser et al., 2011; Maag et al., 2014), we conclude that the observed reduction in caterpillar growth was likely mediated by induced BXs like HDMBOA-Glc and HDM2BOA-Glc. Based on our observations we predict that caterpillars foraging on maize should move frequently to avoid intoxication due to local formation of the highly insecticidal HDMBOA-Glc (Glauser et al., 2011). Indeed, caterpillars of *S. littoralis* establish multiple feeding sites on maize leaves when they are allowed to forage freely (Köhler et al., 2015). In addition, caterpillar foraging patterns within individual maize seedlings are strongly influenced by BXs (Köhler et al., 2015), thus suggesting that within-leaf movement may also be affected by the distribution of these metabolites.

Several observations suggest that DIMBOA may act as a modulator of defence-related signalling events in addition to its direct defensive properties (Ahmad et al., 2011; Meihls et al., 2013; Maag et al., 2015). For instance, aphid-induced callose deposition in maize is mediated by apoplastic DIMBOA (Ahmad et al., 2011). Interestingly, in *A. thaliana,* callose accumulation also depends on the presence of certain indolic glucosinolates (Clay et al., 2009; Bednarek, 2012). To date, nothing is known about the underlying mechanisms. Therefore, we hypothesised that DIMBOA may interact with herbivory-triggered phytohormone accumulation during infestation by *S. littoralis* caterpillars. However, we did not find any consistent differences in induced levels of JA, JA-Ile, ABA and IAA between

wild-type maize plants and *bx1* mutant lines following herbivore feeding or mimicked herbivory. The transient increases of JA, JA-Ile and ABA that were observed following application of *S. littoralis* OS resembled the temporal dynamics that were reported earlier (Erb et al., 2015). In addition, we also observed a significant increase of IAA concentrations irrespective of the induction treatment. Interestingly, IAA levels remained nearly constant during the first three hours after OS application and increased only afterwards when levels of the other three hormones had already started to decline. The role of IAA during plant-herbivore interactions only started to become apparent in recent years (Erb et al., 2012). In tobacco, for instance, IAA was found to accumulate within minutes following defence elicitation and this induced accumulation was required for the onset of specific jasmonate-dependent defence metabolites (Machado et al., 2016). In addition, IAA appears to be a major regulator of herbivore tolerance (Machado et al., 2013). Furthermore, 2,4-dichlorophenoxyacetic acid (2,4-D), a structural homolog of IAA, was found to act as a defence elicitor in rice, suggesting similar properties of IAA itself (Xin et al., 2012). However, infestation of maize plants with larvae of *Helicoverpa zea* has been shown to reduce IAA levels (Schmelz et al., 2003). Thus, the exact role of IAA may be highly species-specific and likely depends on the interaction partners. In summary, we did not find any evidence for a role of DIMBOA or other BX-related metabolites as modulators of caterpillar-induced phytohormone levels. Moreover, the induced accumulation of HDMBOA-Glc and HDM2BOA-Glc did not depend on the presence of BX metabolites in caterpillar OS following mimicked herbivory (Figure S4), suggesting that caterpillar-derived BX metabolites do not have any feedback effects on BX biosynthesis. Hence, the observed regulatory properties of DIMBOA may be limited to maize-aphid interactions or fungal infections as both interaction types are facilitated, at least partly, via the apoplastic space, in contrast to the non-specific tissue maceration inflicted by caterpillar feeding. Alternatively,

DIMBOA may play a role in downstream defence signalling rather than hormone biosynthesis.

Conclusions

This study demonstrates that BX and resistance induction in maize are highly localised. The creation of small induction halos around feeding sites has been suggested to provide sufficient protection against herbivory by leading to a dispersal of feeding damage (Edwards and Wratten, 1983). Our data supports this notion. By studying BX-impaired mutant plants we further demonstrate that the locally restricted resistance phenotype is largely mediated by inducible BXs.

Experimental Procedures

Plants and insects

Seeds of the maize (*Zea mays* L.) variety B73 were obtained from Delley semences et plantes SA (Switzerland). The original *bx1* mutant was backcrossed 5 times into B73 followed by selfing, resulting in a homozygous near isogenic *bx1* mutant line in a B73 background. The *bx1* NIL and B73 wild type plants were multiplied together in the field in summer 2013. The BX-deficient *bx1 igl* double mutant line 32R was originally derived from a cross between the two corresponding single mutant lines (Ahmad et al., 2011). Plants were grown under natural light conditions inside a greenhouse (25 ± 4 °C) using plastic pots (Ø 4 cm, 11 cm deep) and commercial potting soil (Aussaaterde, Ricoter Erdaufbereitung AG, Switzerland). After approximately 14 days, when the fourth leaf was growing out of the whorl, plants were transferred to a climate-controlled room (24 ± 2 °C, 40 ± 5% r.h.) and kept under light benches (16:8 h L:D, app. 150 µmol m^{-2} s^{-1}) for the duration of the experiments.

Eggs of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) were obtained from Syngenta (Stein, Switzerland) and larvae reared on artificial diet (Turlings et al., 2004).

Collection of BX-free caterpillar oral secretions

Benzoxazinone-free OS was collected from L4-L5 *S. littoralis* caterpillars that were allowed to forage on excised plants of the BX-deficient maize line 32R for at least 12 hours prior to OS collection. To provoke regurgitation caterpillars were gently squeezed using storkbill forceps. Regurgitate droplets were directly collected from the caterpillars using a micropipette, transferred to 1.5 ml tubes and stored at -20 °C until use. Prior to freezing the regurgitant was diluted with an equal volume of water (Milli-Q). Absence of BXs from the collected regurgitate was verified by UHPLC-QTOF-MS (see below).

Within-leaf gradients of defence-related metabolites in B73 and *bx1* mutant plants

To examine the spatio-temporal dynamics of defence-related metabolite induction in leaves of the maize line B73, two L2 *S. littoralis* larvae were placed inside a circular clip cage (Ø 1.5 cm) and attached to the adaxial side in the centre of the third leaf. Following different treatment periods the leaves were excised and cut into five segments, of which the three central ones were of 4 cm length, whereas the two outside segments consisted of the remaining tissue and thus were of varying length. Leaves were harvested after 12 or 24 hours of caterpillar feeding (12 and 24 hpi, respectively) or 24 hours after a 24-hour feeding period (24+24 hours). In addition, intact plants were sampled at the beginning of the experiment (0 hpi, n=5). Leaf samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until further use. The experiment was replicated twice. Plant samples from the first experiment were used for phytohormone measurements. Samples from the second experiment were used for gene expression analyses and BX quantitation.

Phytohormone levels and BX gradients in the *bx1* mutant were measured at 24 hpi (phytohormones, n=7-8) and 24+24 hpi (BXs, n=4-6), respectively. B73 was included as the corresponding wild type control. In both experiments plants that had received empty clip cages served as control groups. Leaf segments were collected as described above.

Temporal persistence of induced 1,4-benzoxazin-3-ones

Two L2 *S. littoralis* caterpillars were attached to the adaxial side in the centre of the third leaf of B73 plants and allowed to feed for 24 hours as described above. After 24 hours, caterpillars were carefully removed from all plants and the 4 cm leaf segment enclosing the feeding site was collected at different time points over a period of seven days. Control plants did not receive any treatment and were sampled at the same time intervals (n=5). Effects of the clip cages themselves on BX accumulation were determined in a separate experiment (Figure S6).

Effect of local feeding on systemic 1,4-benzoxazin-3-one accumulation

Two experiments were conducted to examine putative effects of local herbivory on BX levels in non-attacked leaves in the maize variety B73. A previous study demonstrated that mechanical wounding and treatment with caterpillar OS results in the systemic release of herbivore induced volatiles from non-infested maize leaves (Turlings and Tumlinson, 1992). Furthermore local infection by *Colletotrichum graminicola* was found to increase pathogen resistance in both younger and older leaves (Balmer et al., 2013). Based on these findings, we selected the next younger leaf to detect potential systemic responses. As in the experiments described above, the third leaf was subjected to herbivore feeding and the next younger, fourth leaf was used to measure systemic effects. In the first experiment, the local leaf received feeding damage from eight L2 *S. littoralis* caterpillars for 24 or 48 hours,

respectively. The caterpillars were confined to the third leaf using mesh sleeves that were attached to the leaf base using cotton pads and metal clips. Caterpillars were allowed to forage freely across the entire leaf. In both treatment groups, whole systemic leaves were harvested 48 hours after onset of herbivory, i.e. in the 24-hour feeding group there was a 24-hour delay between removal of the caterpillars and sampling of systemic leaf tissue. Control plants received empty mesh sleeves (n=4-5).

While the first experiment focused on constitutive BX accumulation in systemic tissue, the second experiment explored whether local herbivory primes systemic tissues for increased BX production upon a subsequent induction event. To do so, a factorial experimental design was established consisting of a local and a systemic treatment. For the local treatment, 2 L2 *S. littoralis* caterpillars were attached to the centre of the third leaf using clip cages as described above and allowed to feed for 24 hours when they were carefully removed. Twenty-four hours after removal of the caterpillars, defence responses in the systemic leaf were elicited by wounding the leaf blade on either side of the midvein using forceps, thereby creating a wound of approximately 1 cm^2 surface area. Ten µl of pure caterpillar OS were subsequently pipetted onto the wound. The elicitation treatment was repeated after 12 hours and another 12 hours later, the entire systemic leaf was harvested for BX analysis. Control plants did not receive any treatment (n=5). Caterpillar OS for this experiment was collected from B73-fed *S. littoralis* larvae as described above. The application of caterpillar OS was preferred over natural infestation in this experiment as it allows for a standardised induction treatment. Treatment with caterpillar OS resulted in a BX induction pattern that was comparable to the one elicited by natural infestation, albeit at a lower magnitude (Figure S7).

Early phytohormone signalling in the *bx1* mutant

To test whether Bx1-dependent metabolites affect early induction of any of the major stress-related phytohormones we devised a short-term time course experiment using mechanical wounding and application of BX-free caterpillar OS as a mimic for herbivory. The third leaf of *bx1* mutant plants or the corresponding wild-type, B73, was wounded as described above and 10 µl of 50% OS (diluted with water) were pipetted onto the wound. Control plants were either left intact or received 10 µl of water (Milli-Q) instead of OS. Leaf segments of 8 cm length enclosing the wounding site were excised after 1, 3 and 6 hours for phytohormone measurements and immediately snap-frozen in liquid nitrogen (n=5).

Phytohormone profiling

Jasmonic acid, JA-Ile, ABA, SA and IAA were quantitated by UHPLC-MS/MS according to Glauser et al. (2012) with slight modifications. In brief, 80 ± 2.5 mg of frozen plant powder was extracted twice with ice-cold ethyl acetate/formic acid (99.5:0.5, v/v). The two fractions were combined and subsequently evaporated to dryness using a centrifugal evaporator (CentriVap, Labconco, Kansas City, MO, USA) and the dry residue was suspended in 100 µl of aqueous methanol (methanol/H_2O, 70:30, v/v). Following centrifugation the supernatant was transferred to a conical glass insert placed inside an LC vial and stored at -80 °C until analysis. Isotopically labelled d_5-JA, $^{13}C_6$-JA-Ile, d_6-ABA, d_6-SA and d_5-IAA served as internal standards and were added prior to extraction. The extracts were analysed using a Dionex RSLC Ultimate 3000 UHPLC instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Waters Acquity BEH C18 column (2.1 x 50 mm, 1.7 µm particle size; Waters, Milford, MA, USA) and connected to a 4000 QTRAP mass spectrometer (AB Sciex, Framingham, MA, USA). All analytes were detected in negative ionisation mode except for IAA, which was detected in positive mode applying the following parent ion/product ion

transitions in multiple reaction monitoring (MRM) mode: 176/130 and 181/135 for IAA and d_5-IAA, respectively (Machado et al., 2013). Pure hormone mixtures containing isotopically labelled standards were injected at five concentrations to establish external calibration curves. All hormone peak areas were normalised to those of the corresponding labelled standards. JA-Ile was quantified using the sum of different isomers.

RNA extraction and gene expression analysis

RNA extractions and gene expression analyses were performed as described in Balmer et al. (2013). RNA was extracted from plant tissue using the RNeasy plant mini kit (Qiagen, Venlo, The Netherlands) and treated with DNase (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. An amount of 400 ng of total RNA was transcribed into cDNA using SuperScript III reverse transcriptase and oligo (dT_{20}) primers (Invitrogen, Carlsbad, CA, USA). All primers were obtained from Sigma-Aldrich (St. Louis, MO, USA) and are listed in Table S4. Quantitative real-time PCR (qRT-PCR) was performed on a Rotor Gene 6000 cycler (Qiagen, Venlo, The Netherlands) using the SensiMix SBYR kit (Bioline, London, UK). Reactions were set up with 0.3 µl forward and reverse primer (10 µM each), 5 µl SensiMix SYBR, 2 µl cDNA (corresponding to an equivalent of 22.6 ng total RNA) and amended to a final volume of 10 µl with water (Milli-Q). Amplification of cDNA was accomplished during 45 cycles with the following conditions: 95 °C for 15 s, 60 °C for 20 s and 72 °C for 15 s. A dissociation curve analysis was performed by ramping from 68 °C to 95 °C (1 °C increase every 5 s). A mix containing equal amounts of cDNA from every sample was created, diluted three times (10-fold each) and the resulting dilution series included in the analysis to determine the primer efficiencies. Threshold cycle (C_T) values were determined using the Rotor Gene 6000 software (Version 1.7) and normalised to C_T values of the two reference genes Zm-Actin and Zm-GAPc. Expression levels of target genes

in the respective leaf segments were calculated relative to those in the same segments of control plants (0 hpi) and expressed as $\log_2$ ratios.

Extraction and analysis of 1,4-benzoxazin-3-ones

For extraction of 1,4-benzoxazin-3-ones approximately 27.5 ± 2.5 mg of frozen plant powder was suspended in 1 ml of methanol/H_20 (50:50, v/v; 0.5% formic acid) and five to eight glass beads added. Following agitation in a bead mill at 30 Hz for 3 min, the samples were centrifuged at 20 800 *g* for 5 min and 500 µl of the supernatants recovered for analysis and transferred to glass vials. BXs were analysed using a Waters Acquity UPLC™ system equipped with an Acquity BEH C18 column (2.1 x 50 mm, 1.7 µm particle size) coupled to an eλ photodiode array (PDA) detector and a Waters Synapt G2 QTOF mass spectrometer (Waters, Milford, MA, USA) according to Meihls et al. (2013). Pure standards of DIMBOA-Glc, DIMBOA and HDMBOA-Glc were injected in at least four concentrations to obtain external calibration curves. When signal intensities exceeded the linear range of the MS detector, which was the case for highly concentrated plant extracts, quantitation was achieved using the PDA detector (λ = 264 ± 1.2 nm) of the UPLC™ system.

Caterpillar performance assay

Larval weight gain was measured on intact maize leaves (control), and on induced leaves, with larvae feeding either directly on the induction site or next to it. Two thirds of the plants were therefore induced for 24 hours by two second-instar *S. littoralis* feeding in a clip cage in the centre of the third leaf. The remaining third received empty clip cages as a control. Following the induction period, plants were left for another 24 hours before new second-instar larvae were weighed and placed individually in a clip cage in the centre of intact leaves, directly on the induction site or next to the induction site towards the leaf tip. Larvae

were re-weighed after 24 hours of feeding. The centre of intact leaves was selected as control treatment. During initial herbivore performance assays weight gain of *S. littoralis* caterpillars did not differ between the centre and the tip of intact maize leaves. Sample sizes were n = 22 per group for B73 and n = 25 per group for the *bx1* mutant.

Statistical analyses

Within-leaf gradients of phytohormones, gene expression profiles and BXs in B73 were analysed by two-way analyses of variance (ANOVAs) using treatment and leaf segment as factors. Data on the temporal persistence of induced BX metabolites were analysed by two-way ANOVAs using treatment and time as factors. Effects of local herbivory on systemic BX levels were analysed by two-way ANOVAs with the two factors treatment and treatment duration in case of the first experiment and local and systemic treatment for the second experiment, respectively. Caterpillar performance was analysed by two-way ANOVA using plant genotype and feeding site as factors. Three-way ANOVAs were used to examine putative effects of a non-functional *bx1* allele on phytohormone and BX concentrations with the factors being genotype, treatment and leaf segment or plant genotype, treatment and time, respectively. In all cases, data were analysed for normal distribution and homoscedasticity prior to ANOVA. Where necessary, ANOVAs were performed on transformed or ranked data. Holm-Sidak tests were used for *post-hoc* pairwise comparisons. Whenever metabolites could not be detected in a given plant sample, their concentration was set to the respective limit of detection. All statistical analyses were performed with SigmaPlot 12.5. (Systat Software Inc., San Jose, CA, USA).

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The authors declare no conflicts of interest.

Short Supporting information Legends

Figure S1. Schematic representation of the BX biosynthetic pathway

Figure S2. Within-leaf distribution of 1,4-benzoxazin-3-one aglucones after different periods of caterpillar feeding

Figure S3. Relative water content in local leaf during *S. littoralis* feeding

Figure S4. Induction of HDMBOA-Glc and HDM2BOA-Glc following application of BX-containing or BX-free caterpillar oral secretions

Figure S5. Within-leaf distribution of BX biosynthesis gene transcripts after different periods of caterpillar feeding normalised to segment C at 0 hpi.

Figure S6. Effect of empty clip cages on BX levels

Figure S7. Induction of HDMBOA-Glc and HDM2BOA-Glc following caterpillar feeding or application of caterpillar oral secretions

Table S1. ANOVA table for herbivory-induced 1,4-benzoxazin-3-one levels in B73 and *bx1* mutant

Table S2. ANOVA table for herbivory-induced phytohormones in B73 and *bx1* mutant

Table S3. ANOVA table for herbivory-induced phytohormones over time in B73 and *bx1* mutant

Table S4. Primers used for qRT-PCR in this study

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Figure legends

Figure 1. Within-leaf distribution of herbivory-induced phytohormones. Concentrations of JA (a), JA-Ile (b), ABA (c) and SA (d) in five different leaf segments before *S. littoralis* caterpillar infestation (0 hpi), after 12 hours (12 hpi) or 24 hours (24 hpi) and 24 hours after the end of a 24-hour feeding period (24+24 hpi) are shown (means ± SE, n=5). Caterpillar infestation was restricted to leaf segment C. Statistically significant effects of treatment and leaf segment on phytohormone levels were determined by two-way ANOVAs (*$P \leq 0.05$, ***$P \leq 0.001$, ns: not significant). Significant differences within leaf segments as detected by *post-hoc* tests are indicated by different letters ($P \leq 0.05$).

Figure 2. Spatiotemporal expression profiles of defence-related genes in herbivory-induced maize leaves. Relative expression levels of *Bx1* (a), *Igl* (b), *Bx10,11* (c) and *Bx14* (d) in five different leaf segments before *S. littoralis* caterpillar infestation (0 hpi), after 12 hours (12 hpi) or 24 hours (24 hpi) and 24 hours after the end of a 24-hour feeding period (24+24 hpi) are shown. Caterpillar infestation was restricted to leaf segment C. Transcript abundance is shown on a log2-scale relative to the abundance in the respective leaf segment at the beginning of the experiment (0 hpi) (means ± SE, n=3-5). Statistically significant effects of treatment and leaf segment on gene expression were determined by two-way ANOVAs (**$P \leq 0.01$, ***$P \leq 0.001$). Significant differences within leaf segments as detected by *post-hoc* tests are indicated by different letters ($P \leq 0.05$).

Figure 3. Within-leaf distribution of herbivory-induced 1,4-benzoxazin-3-ones. Concentrations of HDMBOA-Glc (a), HDM2BOA-Glc (b), DIBOA-Glc (c), HMBOA-Glc (d), DIMBOA-Glc (e) and DIM2BOA-Glc (f) in five different leaf segments before *S. littoralis* caterpillar infestation (0 hpi), after 12 hours (12 hpi) or 24 hours (24 hpi) and 24 hours after the end of a 24-hour feeding period (24+24 hpi) are shown (means ± SE, n=5). Caterpillar infestation was restricted to leaf segment C. Statistically significant effects of treatment and leaf segment on BX levels were determined by two-way ANOVAs (**$P \leq 0.01$, ***$P \leq 0.001$, ns: not significant). Significant differences within leaf segments as detected by *post-hoc* tests are indicated by different letters ($P \leq 0.05$). LOD: limit of detection.

Figure 4. Temporal persistence of induced 1,4-benzoxazin-3-one levels after herbivore removal. *S. littoralis* caterpillars were attached to the centre of the third leaf of the maize variety B73 and allowed to feed for 24 hours (arrows indicate caterpillar removal). Central

leaf segments were harvested at different time points and concentrations of HDMBOA-Glc (a), HDM2BOA-Glc (b), DIMBOA (c) and MBOA (d) were determined (closed circles). Identical leaf segments were collected from untreated plants as control (open circles). Concentrations are given in µg / g FW (means ± SE, n=5). Statistically significant effects of treatment and time period on metabolite concentrations were determined by two-way ANOVAs (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Student's *t* tests were used to detect significant differences within individual time points (**$P \leq 0.01$, ***$P \leq 0.001$). LOD: limit of detection.

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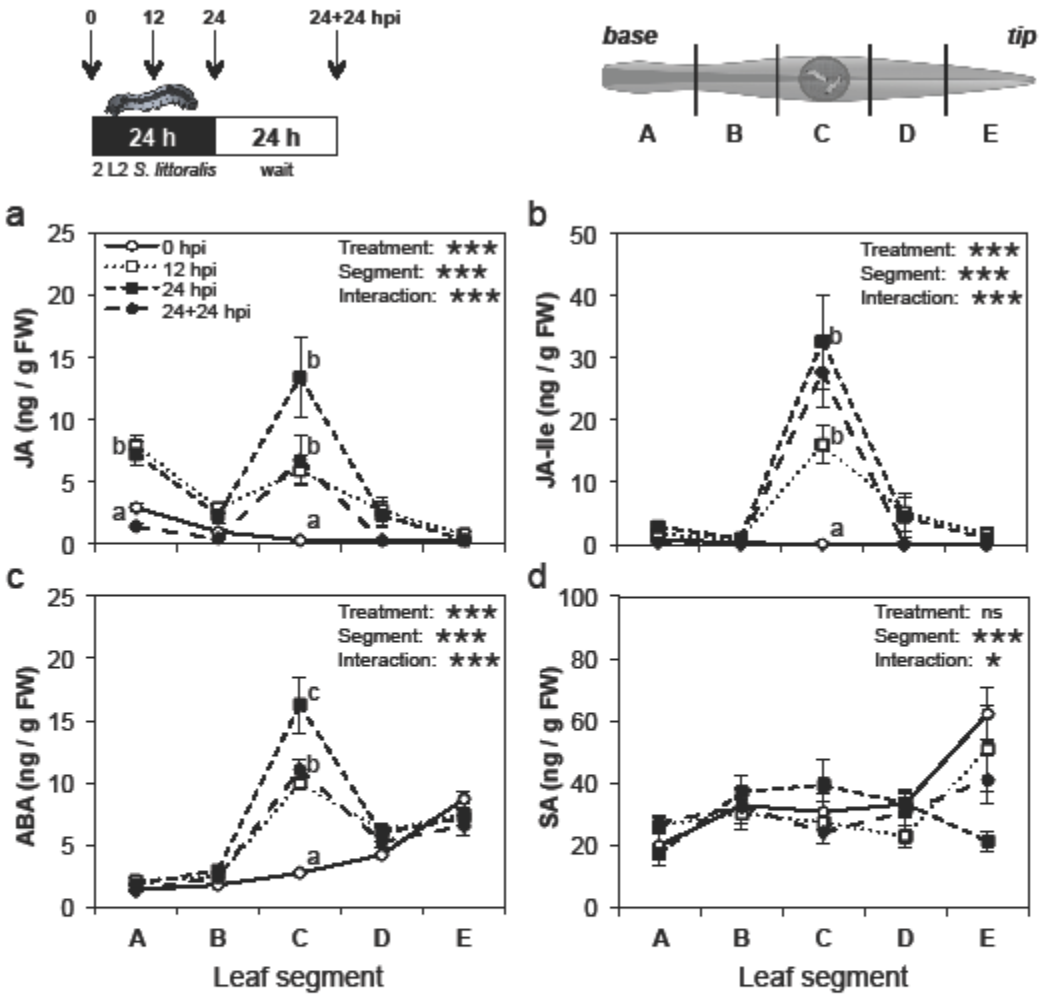
Figure 1



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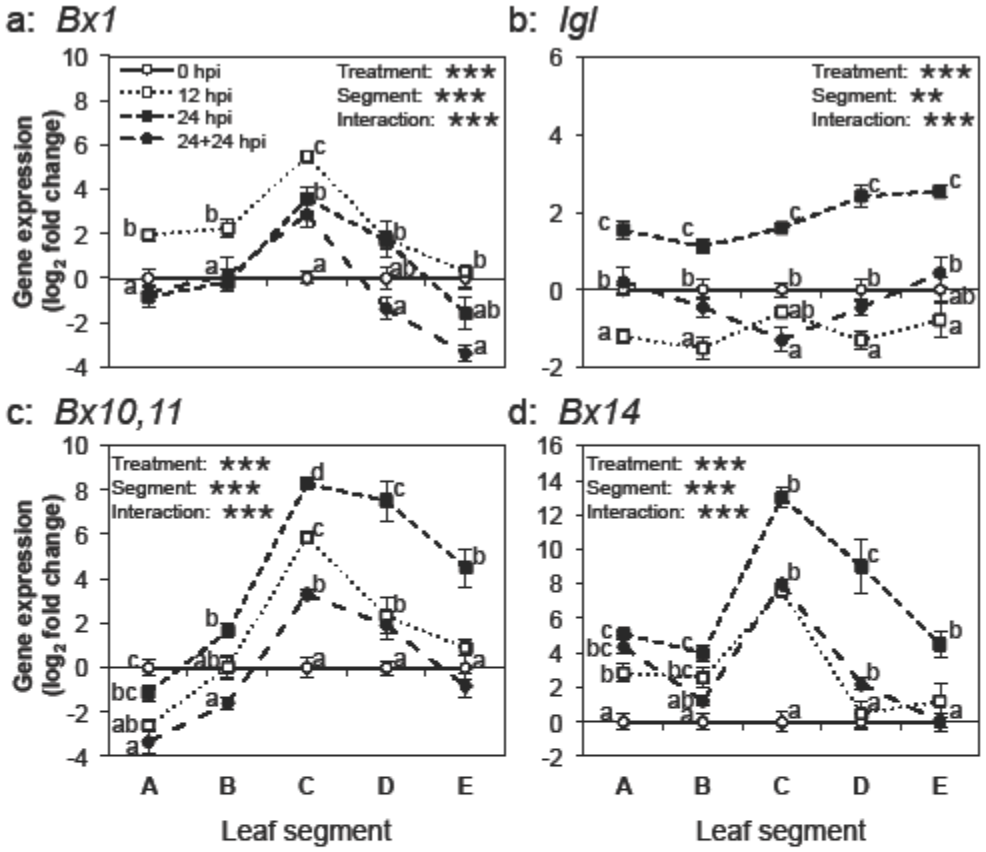

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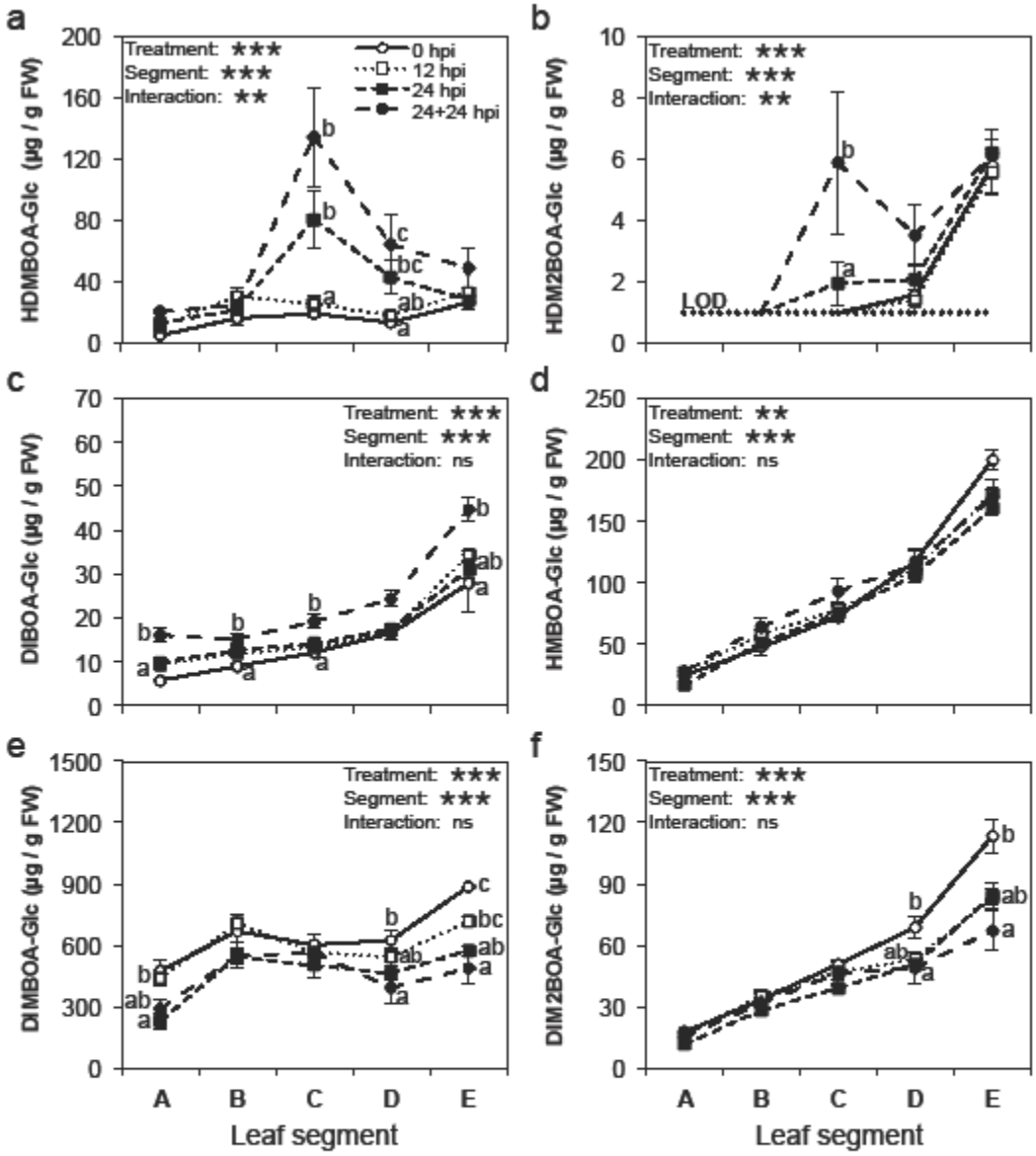

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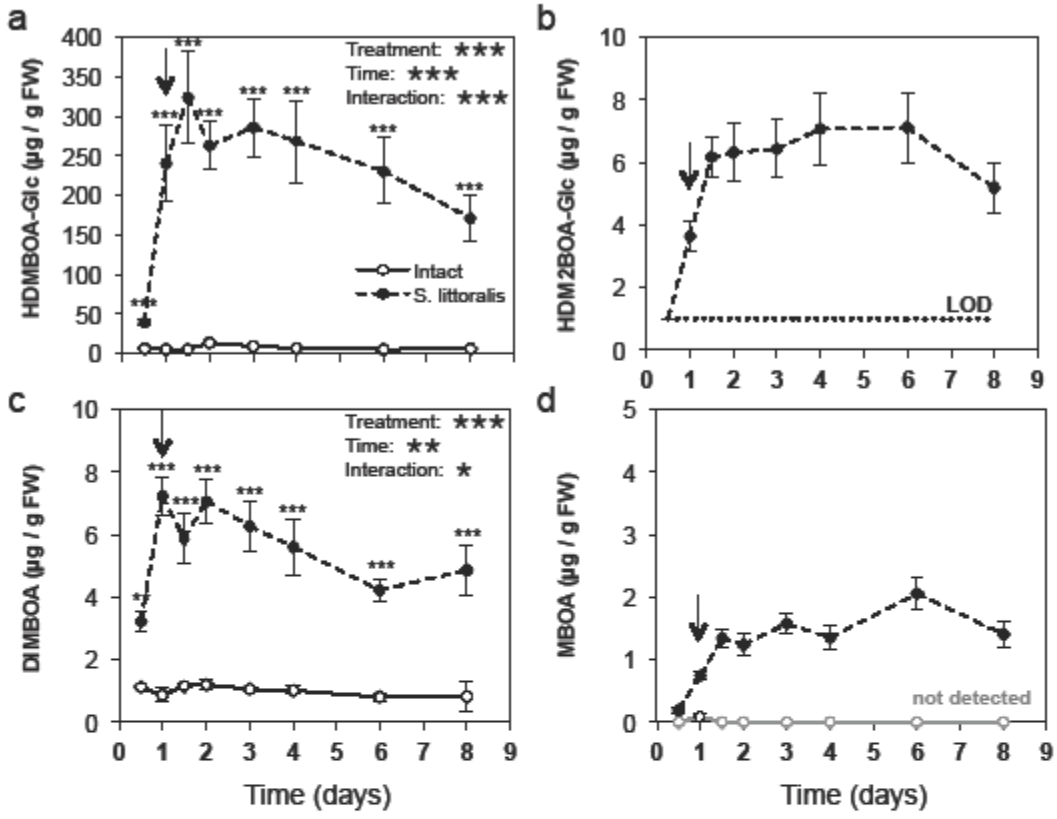

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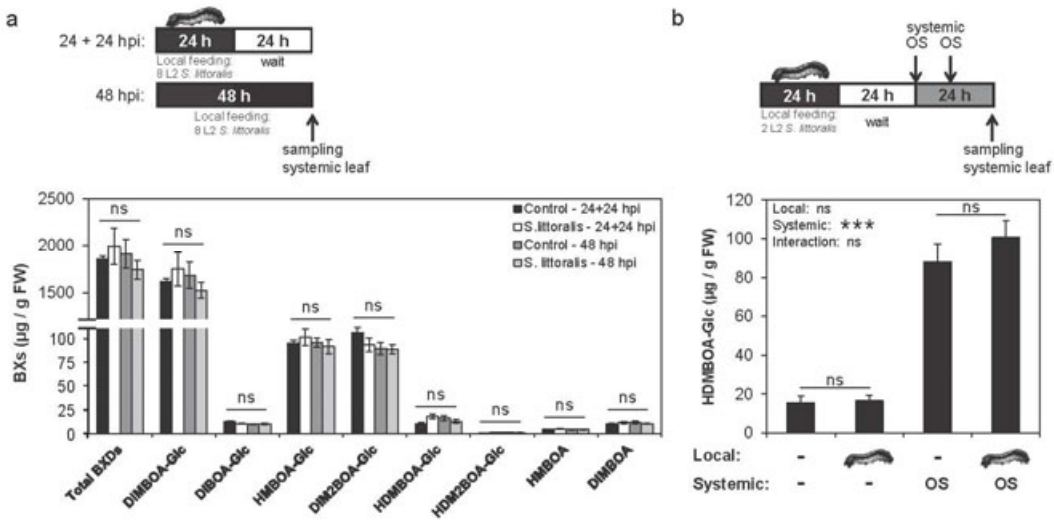

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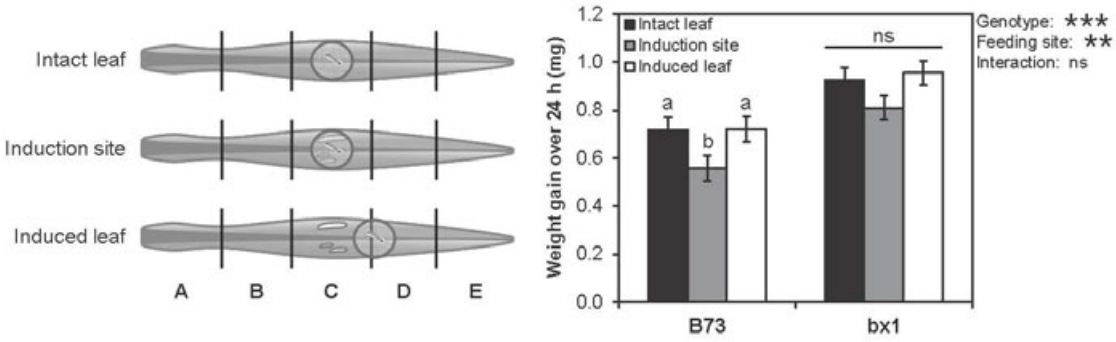

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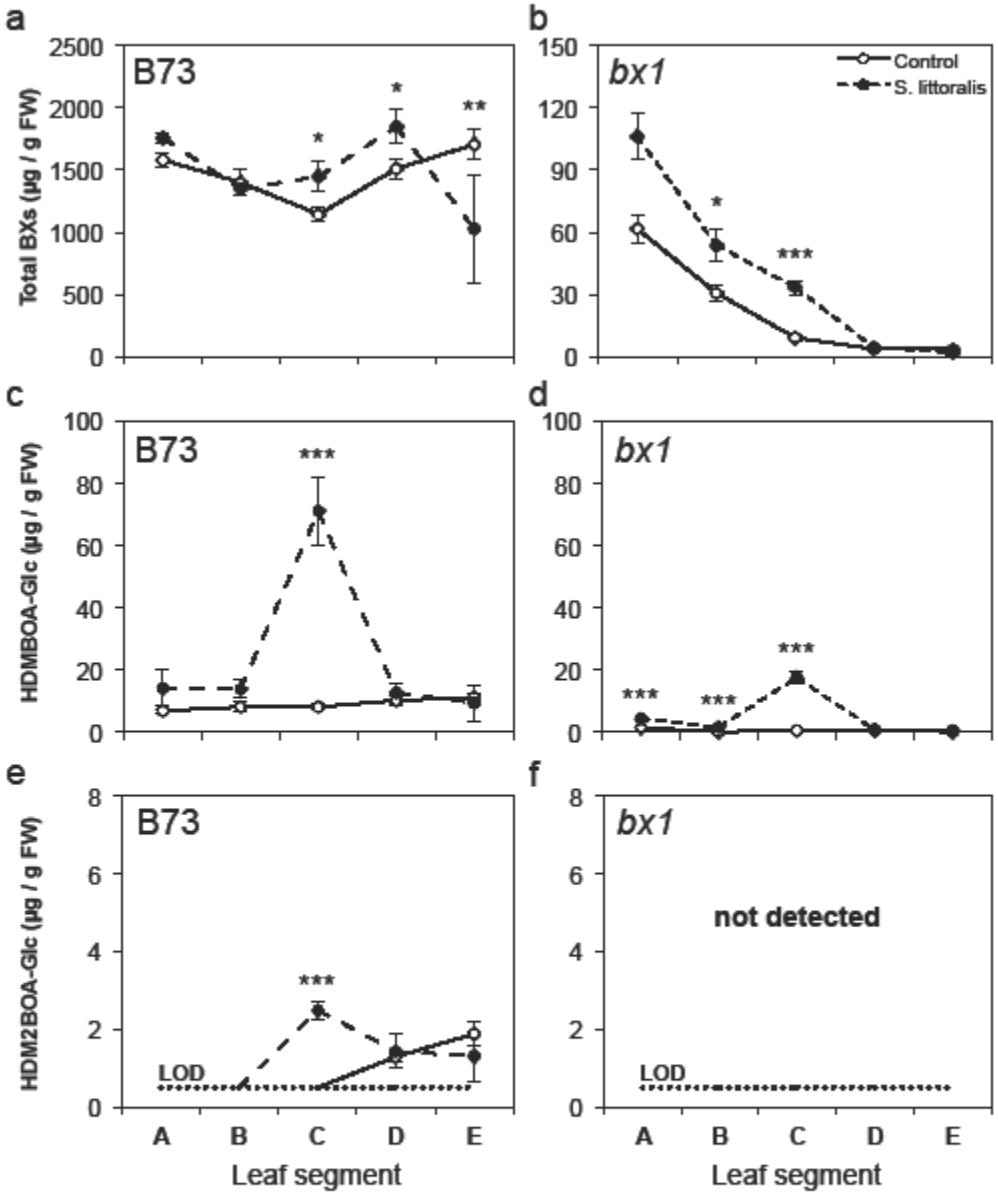

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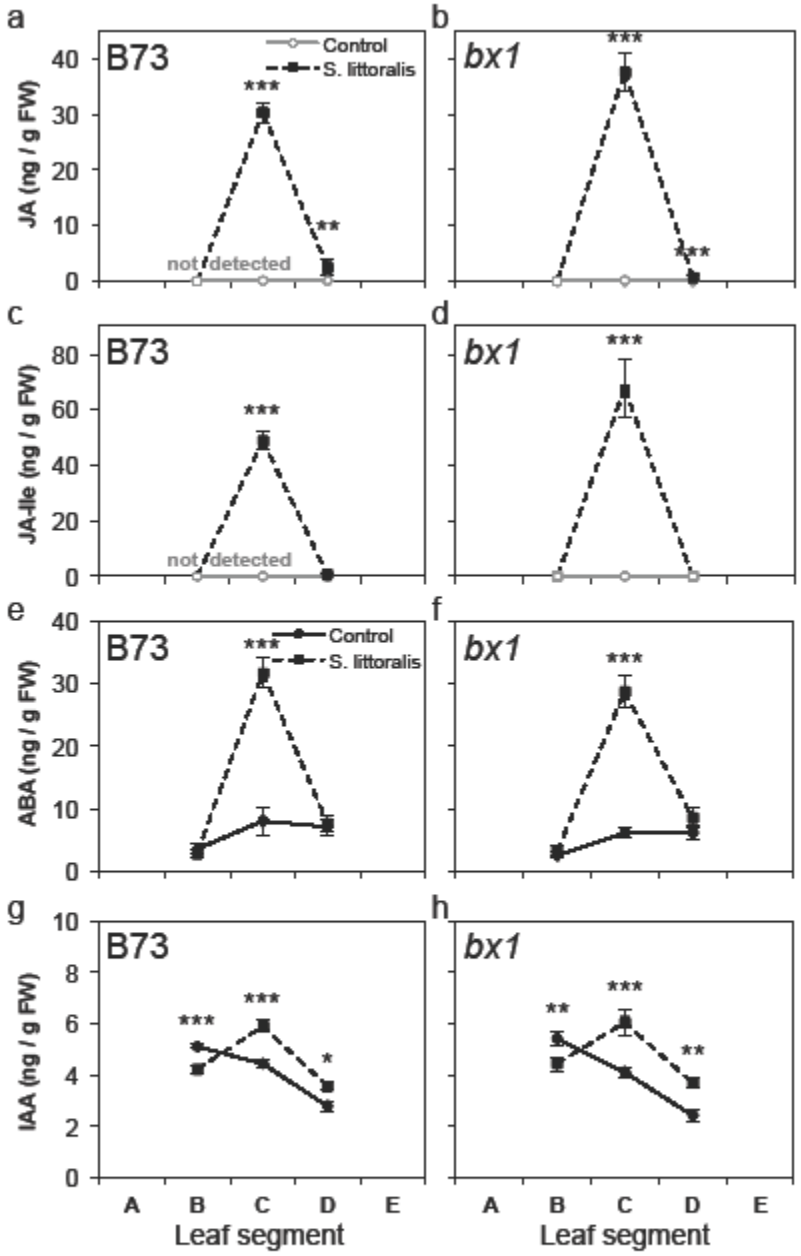

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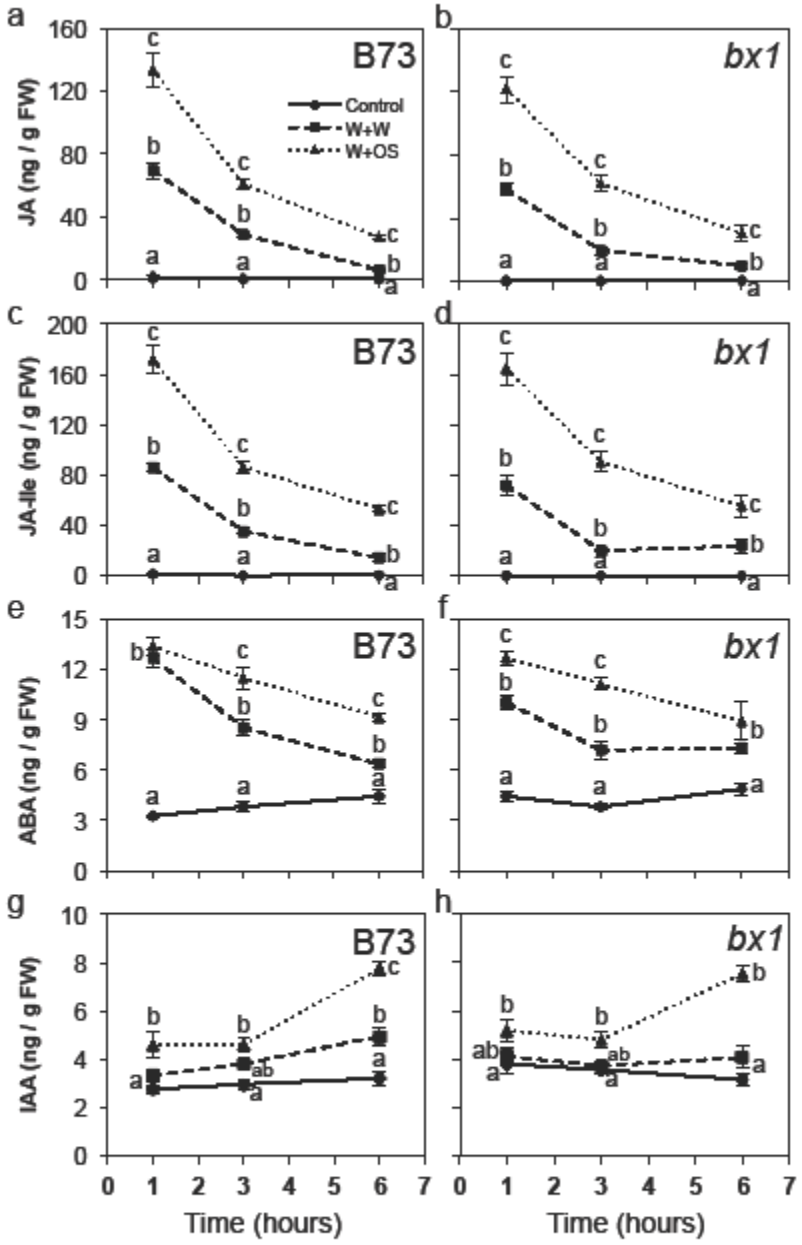

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