The Asian corn borer *Ostrinia furnacalis* feeding increases the direct and indirect defence of mid-whorl stage commercial maize in the field

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**Summary**

The Asian corn borer (*Ostrinia furnacalis* Guenée) is a destructive pest of maize (*Zea mays* L.). Despite large-scale commercial maize production, little is known about the defensive responses of field-grown commercial maize to *O. furnacalis* herbivory, and how these responses result in direct and indirect defence against this pest. To elucidate the maize transcriptome response to *O. furnacalis* feeding, leaves of maize hybrid Jingke968 were infested with *O. furnacalis* for 0, 2, 4, 12 and 24 h. *Ostrinia furnacalis* feeding elicited stronger and more rapid changes in the defence-related gene expression (i.e. after 2 h), and more differentially expressed genes (DEGs) were up-regulated than down-regulated at all times post-induction (i.e. 2, 4, 12 and 24 h) in the *O. furnacalis* pre-infested maize plants. KEGG pathway analysis indicated that the DEGs in the *O. furnacalis* pre-infested maize are involved in benzoxazinoids, phytohormones, volatiles, and other metabolic pathways related to maize resistance to herbivores. In addition, the maize leaves previously infested by *O. furnacalis* for 24 h showed an obvious inhibition of the subsequent *O. furnacalis* performance, and maize volatiles induced by *O. furnacalis* feeding for 24 and 48 h attracted the parasitic wasp, *Macrocentrus cingulum* Brischke. The increased direct and indirect defences induced by *O. furnacalis* feeding were correlated with *O. furnacalis*-induced phytohormones, benzoxazinoids, and volatiles. Together, our findings provide new insights into how commercial maize orchestrates its transcriptome and metabolome to directly and indirectly defend against *O. furnacalis* at the mid-whorl stage in the field.

**Introduction**

Plants have evolved many defence systems to combat insect attack (Chuang et al., 2014; Wu and Baldwin, 2010). Certain systems, termed ‘direct defences’, involve the production of antifeedant or toxic compounds that inhibit insect performance (Chen, 2008; Howe and Jander, 2008). Other ‘indirect defences’ involve the emission of herbivore-induced plant volatiles (HIPVs) that attract the pest’s natural enemies (Turlings and Erb, 2018; Turlings et al., 1990). Plant defences are mediated by phytohormones, including jasmonic acid (JA), abscisic acid (ABA) and salicylic acid (SA) (Ankala et al., 2009; Howe and Jander, 2008; Kessler and Baldwin, 2002; Rehrig et al., 2014; Schweiger et al., 2014; Thaler et al., 2012; Tzin et al., 2015; Yan et al., 2012), and the accumulation of JA and SA plays an important role in regulating plant-induced resistance to insects (Kawazu et al., 2012; Kerchev et al., 2012; Nahar et al., 2011; Wang and Wu, 2013). JA-mediated signalling is generally activated by feeding insects (Stam et al., 2014), and directly and indirectly functions in the activation of both local and systemic defences in plants (Bozorov et al., 2017; Kaur et al., 2010). Insect attack generally causes release of several interacting phytohormones (Caarls et al., 2015; Erb et al., 2012; Pieterse et al., 2012; Vos et al., 2013), allowing plants to make fast and specific responses to the complex biotic and abiotic environment (Stam et al., 2014).

Maize (*Zea mays* L.) is widely planted globally (FAOSTAT, 2017), and is used in food, fodder and industrial products. During the lifetime of the plant, different parts are inevitably subject to attack from various groups of insects (Meihls et al., 2012). In China, the Asian corn borer (*Ostrinia furnacalis* Guenée) is considered one of the most destructive insect pests of maize. Despite numerous control measures, this pest causes an estimated 6–9 million tons loss of yield annually (He et al., 2003). Current control of *O. furnacalis* primarily relies on pesticides. However, undesirable consequences of pesticide use, including environmental pollution, threats to human health, development of resistance in pests and secondary pest outbreaks, cannot be ignored (Bruce, 2010; Mitchell et al., 2016). A sustainable, cost-effective and environmentally friendly option in the control of *O. furnacalis* is to increase the natural resistance of maize to this pest.

Well-documented examples of maize defensive compounds against *O. furnacalis* are benzoxazinoids, a major class of indole-derived plant metabolites with a wide range of insecticidal, antifeedant, antimicrobial and allelopathic activities (Niemeyer,
Response to  To identify the global transcriptomic changes that occurred in feeding (2009). Benzoxazinoid responses to insect herbivory have been well documented at the V3 stage in the maize inbred line B73 (Maag et al., 2016; Tzin et al., 2015, 2017). Other important defensive chemicals are HIPVs [terpenes, indoles and green leaf volatiles (GLVs)]. They recruit natural enemies of the pest, such as Cotesia marginiventris Cresson (Turlings et al., 1990), or serve as signals to trigger maize defences (Erb et al., 2015). Maize exhibits high genetic variability in its inducible volatile emissions (Degen et al., 2006). For example, most American maize lines fail to release (E)-β-caryophyllene in response to pest attack (Köllner et al., 2008; Rasmann et al., 2005; Tamiru et al., 2011), indicating some chemical defences are possibly lost during crop domestication (Chen et al., 2015). However, whether these defences are lost in Chinese commercial maize, and if not, how they respond to O. furnacalis attack at the vulnerable mid-whorl stage, is still unknown. Maize genotypes exhibit tremendous diversity (Buckler et al., 2006), for example only 32% of the maize line K11 and 39% of W22 could be mapped to a B73 RefGen_v4 reference (Jiao et al., 2017). Genetic variation in maize leads to variation in transcriptome and metabolite responses to pest attack. Song et al. (2017) demonstrated that maize line B73 and Mo17 showed extensive differences in their responses in transcriptome and metabolite to Rhopalosiphum padi L. feeding. There is, therefore, an urgent need to study the transcriptomic and the metabolic responses of commercial maize to O. furnacalis attack at the mid-whorl stage, which could be of great practical significance in maize cultivation.

In this study, we investigated direct and indirect defences of maize to O. furnacalis attack through performance of the pest and behaviour of its dominant endoparasitoid Macrocentrus cingulum Brischke. The integration of maize transcriptional dynamics with profiles of phytohormones, benzoxazinoids and volatiles, allowed us to get more insight into the molecular and biochemical defences against O. furnacalis.

Results

Transcriptomic analysis of maize responses to O. furnacalis feeding

To identify the global transcriptomic changes that occurred in response to O. furnacalis attack, transcriptome data from maize leaves pre-infested by O. furnacalis for 0, 2, 4, 12 and 24 h post-infestation were collected. Detailed information on RNA sequencing and mapping is summarized in Table S1. Gene expression levels for each replicate were assessed using principal component analysis (PCA) (Figure 1a). Samples from 2-, 4-, 12- and 24-h clustered far from the 0-h (control) samples, indicating that O. furnacalis feeding induced changes in gene expression. A total of 41 009 transcripts were detected across all samples (Data S1), and genes with a false discovery rate (FDR) <0.05 and absolute value of log2ratio ≥ 1 were selected as differentially expressed genes (DEGs) for further analysis. Samples at 2-, 4-, 12- and 24 h exhibited 7463 (4547 up and 3096 down), 9037 (5120 up and 3917 down), 10 190 (5706 up and 4484 down) and 10 033 (5638 up and 4395 down) DEGs, respectively (Figures 1b, S1, Data S2 and S3). The expression patterns of four selected DEGs (5638 up and 4395 down) DEGs, respectively (Figures 1b, S1, 3917 down), 10 190 (5706 up and 4484 down) and 10 033 genes (DEGs) for further analysis. Samples at 2-, 4-, 12- and 24 h post-infestation were assigned to 31, 28, 34 and 33 significant KEGG pathways involved (Data S4). The DEGs at 2, 4, 12 and 24 h post-infestation were assigned to 31, 28, 34 and 33 significant KEGG pathways respectively (P < 0.05), and the top 10 pathways for each time point are listed in Figure 1d. Of these significant pathways, metabolism of phenylalanine and alpha-linolenic acid, as well as the biosynthesis of phenylalanine, tyrosine, tryptophan, benzoxazinoids, phenylpropanoids and flavonoids was involved in maize responses to O. furnacalis at all time points post-infestation (Figure 1e, Table S2).

Dynamic transcriptome responses to O. furnacalis attack

In order to understand the dynamics of the maize transcriptome in response to O. furnacalis herbivory, we performed Short Time-series Expression Miner analysis (Ernst and Bar-Joseph, 2006) on the total DEGs. Illustrated are nine important temporal gene expression profiles (Figure 2, Data S5). Profile 66 contained the most transcripts (3034), and many transcripts in this profile controlled the biosynthesis of flavonoids, phenylpropanoids, monoterpeneoids and benzoxazinoids, as well as other metabolic pathways associated with plant defense. These transcripts were immediately up-regulated at 2 h and had increased by the same amount at each of the later time points sampled, suggesting that these defence pathways are continuously induced by O. furnacalis herbivory. Most of the genes in profiles 75, 78 and 79 were involved in primary metabolism such as the metabolism of carbohydrates, lipids and amino acids, and these genes were dynamically up-regulated with O. furnacalis herbivory. The genes related to the control of circadian rhythm were only strongly up-regulated after 24 h following onset of O. furnacalis feeding (profile 40). Profiles 0, 2, 4 and 11 contained genes that were down-regulated from 2 to 24 h following onset of herbivory. These down-regulated genes were mainly involved in the metabolism of amino acids, nucleotides, carbohydrates, and lipids, as well as energy production and other primary metabolism. The up- and down-regulation of expression of genes involved in maize primary metabolism may be caused by the readjustment of plant primary metabolism in response to insect attack (Zhou et al., 2015a).

Plant hormone-related genes and metabolites induced by O. furnacalis feeding

To determine phytohormone changes in response to O. furnacalis feeding, we analysed the concentrations of JA, JA-isoleucine conjugate (JA-Ile), SA and ABA in maize leaves at 0, 2, 4, 8 and 12 h following infestation by O. furnacalis. Accumulation of both JA and JA-Ile was strongly induced and both reached a peak value following 2 h of herbivory (Figure 3a,b). Levels of SA were not increased by O. furnacalis feeding at any time (Figure 3a), while levels of ABA increased significantly compared with the control at all time points (Figure S3b). JA, JA-Ile, SA and ABA biosynthesis-related genes were also significantly induced by O. furnacalis feeding. Among all the genes involved in JA pathway (Figure 3c), except for LOX7, LOX8, LOX12, OPR6 and JAR2, all the other transcripts of lipoxigenases (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), o xo-phytodienoate reductase (OPR)
Figure 1 Overview of a time course of maize transcriptome responses to *Ostrinia furnacalis* attack. (a) PCA plots of transcripts identified by RNA-seq of maize leaves attacked by *O. furnacalis* at 2, 4, 12 and 24 h post-infestation. (b) Number of individual transcripts significantly up- or down-regulated at each time point. (c) Venn diagram illustrating the number of transcripts up- or down-regulated by *O. furnacalis* feeding over the time course. (d) KEGG pathway enrichment analysis of DEGs in the maize transcriptome induced by *O. furnacalis* infestation for 2, 4, 12 and 24 h. Data were visualized using a scatter diagram with q-value levels indicated by '-'$\log_{10}$(q-value)' and an enrichment factor indicative of individual pathways. Values in parentheses represent the number of components in each pathway present in the DEG dataset. (e) Venn diagram showing the overlap of common and unique pathways present in the transcriptome following 2, 4, 12 and 24 h post *O. furnacalis* infestation.
Figure 2 Time-series transcriptomic analysis of significant DEGs induced in maize by *Ostrinia furnacalis* herbivory. The top five KEGG pathways for each profile are listed on the right. Enrichment scores are shown as $-\log_{10}(q)$.

| Gene cluster | Enriched KEGG pathways |
|--------------|-------------------------|
| profile 66   | Flavonoid biosynthesis  |
|              | Phenylpropanoid biosynthesis |
|              | Stillbenoid, diarylethanol and gibererol biosynthesis |
|              | alpha-Linolenic acid metabolism |
|              | Amino sugar and nucleotide sugar metabolism |
| profile 75   | Fatty acid degradation  |
|              | Pyruvate metabolism     |
|              | Propanoate metabolism   |
|              | Fatty acid metabolism   |
|              | Tyrosine metabolism     |
| profile 78   | Peroxisome              |
|              | Valine, leucine and isoleucine degradation |
|              | Glycine, serine and threonine metabolism |
|              | Lysine degradation      |
|              | Glutathione metabolism  |
| profile 79   | Glutathione metabolism  |
|              | Amino acid metabolism   |
|              | Starch and sucrose metabolism |
|              | Steroid biosynthesis    |
|              | Tyrosine metabolism     |
| profile 40   | Circadian rhythm - plant |
|              | Carotenoid biosynthesis |
|              | Fatty acid elongation   |
|              | Cysteine and methionine metabolism |
|              | Riboflavin metabolism  |
| profile 0    | Biotin metabolism       |
|              | Protein export          |
|              | Ribosome                |
|              | Aminoacyl-tRNA biosynthesis |
|              | Fatty acid biosynthesis |
| profile 2    | Photosynthesis          |
|              | Carbon metabolism       |
|              | Oxidative phosphorylation |
|              | Glycine, serine and threonine metabolism |
|              | Porphyrin and chlorophyll metabolism |
| profile 4    | anched dibasic acid metabolism |
|              | Biosynthesis of amino acids |
|              | Pentose phosphate pathway |
|              | Oxocarboxylic acid metabolism |
|              | Carbon metabolism       |
| profile 11   | Photosynthesis - antenna proteins |
|              | Photosynthesis           |
|              | Nitrogen metabolism      |
|              | Carbon metabolism        |
|              | Glyoxylate and dicarboxylate metabolism |

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and jasmonate resistant (JAR) were significantly up-regulated following O. furnacalis herbivory (Figure 3d). Isochorismate synthase (ICS) and phenylalanine ammonia lyase (PAL) are the two major genes involved in ICS and PAL pathways respectively for SA biosynthesis (Dempsey et al., 2011). Following O. furnacalis infestation, all PAL genes were up-regulated, but ICS expression was reduced. 9-Cis-epoxy carotenoid dioxygenase (NCED), short chain dehydrogenase/reductase (SDR) and one transcript of aldehyde oxidase (AO, GRMZM2G141535) involved in ABA biosynthesis were all significantly up-regulated after O. furnacalis feeding; however, a second AO transcript (GRMZM5G899851) remained at control levels (Figure 5c).

Benzoazinoids induced by O. furnacalis feeding and their roles in plant defences following subsequent O. furnacalis herbivory

We investigated the metabolites and gene expression of benzoazinoid pathway (Figure 4a) in maize in response to O. furnacalis attack. PCA revealed an obvious separation in the relative abundances of benzoazinoids following herbivory for different lengths of time. The benzoazinoid profiles in maize leaves induced by O. furnacalis feeding for 48 and 72 h were clearly separated from that of the control (0 h) along the first PC axis, and the separation was also observed between 24 h and control along the first and second PC axes. Variable loadings in the first two PCs revealed that DIMBOA-Glc, DIMBOA and HDM2BOA-Glc were the strongest contributors to the difference among the benzoazinoid profiles at 0, 24, 48 and 72 h post-infectionation (Figure 4b). Ostrinia furnacalis feeding had a strong effect on benzoazinoid abundance. The most up-regulated benzoazinoid was HDM2BOA-Glc, with a peak level of 291-fold at 48 h. HDMBOA-Glc, HM2BOA-Glc, DIMBOA-Glc and MBOA were also significantly up-regulated and showed the highest induction level at 24 h with 24.9–54.6-fold increase. DIMBOA, DIMBOA-Glc and DIBOA-Glc reduced significantly with continuing O. furnacalis feeding (Figure 4c). Ostrinia furnacalis feeding also caused significant changes in benzoazinoid gene expression. All BX genes except for BX1, BX5, BX7 and BX8 were significantly up-regulated during O. furnacalis feeding (Figure 4d).

Next, we evaluated the performance of O. furnacalis when it was feeding on O. furnacalis pre-infested (24 h) and uninfested (control) maize leaves to determine whether leaf feeding by O. furnacalis could produce direct defences. Ostrinia furnacalis pre-infested maize leaves inhibited 2nd instar O. furnacalis performance with a significantly lower relative growth rate (RGR) and a significantly higher relative consumption rate (RCR) (Figure 5a). Food processing efficiencies, measured as efficiency of conversion of ingested food (ECI) and efficiency of conversion of digested food (ECD), were 33% and 27% less respectively on the 5th instar, the larval and pupal stage, and the total pre-oviposition period of the larvae was also inhibited (P < 0.0001 for all the observed values) (Table S3). Negative effects of O. furnacalis pre-infested maize leaves were also observed on O. furnacalis population parameters, net reproductive rate (r), intrinsic rate of increase (P), finite rate of increase (r) and mean generation time (T) (P < 0.0001) (Figure 5c). All the data indicate that O. furnacalis infestation increased maize direct defences against this pest.

Ostrinia furnacalis-induced maize volatile emission and their roles in attracting M. cingulum

A total of 26 volatile compounds were collected across all plants following infestation with O. furnacalis after 0 (control), 2, 4, 12, 24, 48 and 72 h using a dynamic headspace system (Figure 6a). Partial least squares projection to latent structures-discriminant analysis (PLS-DA) showed a clear separation between the control and O. furnacalis-induced maize volatiles (12, 24, 48 and 72 h) along the first axis, and the O. furnacalis-induced maize volatiles at all time points were clearly separated from control along the second axis (Figure 6b). For this model, the volatile compounds like β-farnesene, α-cubebene and germacrene D with VIP values ≥1.0 contributed most to the separation between the control and O. furnacalis-induced maize volatiles (Figure 6c,d).

The concentrations of key volatile compounds β-caryophyllene, bergamolone, linalool, α-cubebene and β-farnesene were significantly increased after O. furnacalis feeding and reached the peak value at 24 or 48 h of O. furnacalis feeding (Figure 6e). In addition, the terpene biosynthesis-related genes, TP51, TP52, TP56, TP58, TP10, TP23 and TP26 were strongly up-regulated at all time points compared with control (Figure 6f). These results indicated that maize undergoes drastic reprogramming of the volatile profile and gene expression in response to O. furnacalis feeding.

Next, we identified M. cingulum preferences to O. furnacalis-induced maize volatiles in a Y-tube olfactometer bioassay (Figure 7a). Macrocentrus cingulum females showed an obvious preference to the maize volatiles following 4, 12, 24, 48 and 72 h of O. furnacalis feeding (P < 0.0001), and M. cingulum males only showed an obvious preferences to the volatiles of 24 and 48 h (P < 0.0001) (Figure 7b). However, neither males nor females showed a preference for the volatiles following 2 h (male, χ² = 0.14, P = 0.7103; female, χ² = 0.32, P = 0.5471).

Discussion

Ostrinia furnacalis is a major insect pest of maize, and usually causes most damage to leaves at the mid-whorl stage (Zhang et al., 2016). Current knowledge of induced defences in maize in response to O. furnacalis attack remains limited. In this study, we analysed the transcriptome dynamics of field-grown maize, Jingke968 following O. furnacalis attack. Unlike prior studies, which reported transcriptome responses of V3-stage maize (Wang et al., 2017a; Yang et al., 2015), our analysis focused on the mid-whorl stage, which is more vulnerable to early-season O. furnacalis larvae in the field. Our analysis showed that large-scale reprogramming of the transcriptome occurred following O. furnacalis infestation, and more abundant up-DEGs than down-DEGs were found at each induction time point. We propose that O. furnacalis feeding drove more induction of gene expression than suppression, which is similar to the maize transcriptome responses to herbivory by Rhopalosiphum maidis Fitch (Tzin et al., 2015) and Spodoptera exigua Hübner (Tzin et al., 2017). Maize at the mid-whorl stage is known to be more vulnerable to O. furnacalis than at the V3 stage due to the decrease in defensive compounds like DIMBOA (Maag et al., 2015). The maize plants at the mid-whorl stage have more
potential than plants at the V3 stage to induce genes, and produce more DEGs, when subjected to *O. furnacalis* herbivory according to the hypothesis of trade-off between constitutive and inducible resistance traits in plants (Morris et al., 2006).

Plant transcription response to insect attack is a dynamic and complex reprogramming process for signalling and synthesis, and varies with the timing of attack (Kant et al., 2004). Previous studies of the maize transcriptome responses to *O. furnacalis* herbivory have focused on only one induction time point (12 or 8 h) (Wang et al., 2017a; Yang et al., 2015). However, a single sampling time will give only a snapshot of the transcriptional changes and to determine the full extent of temporal patterns of gene activity during plant–insect interactions; it is important to investigate different times after insect infestation. In this study, a dynamic transcriptome analysis showed that a considerable number of genes (3102 up- and 1844 down-DEGs) were continuously induced by *O. furnacalis* feeding, indicating that some biological processes, such as the biosynthesis of primary and secondary metabolites, were continuously induced during *O. furnacalis* feeding.

Genes involved in plant primary and secondary metabolite biosynthesis are also regulated by plant circadian clock (Covington et al., 2008; Kim et al., 2011). In this study, some circadian clock-associated genes were significantly up-regulated after 24 h of *O. furnacalis* feeding. We speculated that maize defences following *O. furnacalis* attack may be orchestrated by the maize circadian clock. The circadian clock is intrinsically linked to the plant redox rhythm (i.e. NADP/H oscillation) through...
nonexpression of the pathogenesis-related gene 1 (NPR1) (Zhou et al., 2015b), which plays a central role in scheduling and gating plant defence responses in the daytime, while preventing conflict with growth at night (Karapetyan and Dong, 2017). Further study is essential to research the interplay between redox rhythms and the circadian clock balancing maize defence and growth.

Many studies have focused on the maize transcriptome responses to insect attack under controlled conditions, including

Figure 4 Effects of Ostrinia furnacalis feeding on benzoxazinoid-related genes and metabolites. (a) Overview of benzoxazinoid biosynthesis in maize (modified from Tzin et al., 2017). (b) PCA plot of benzoxazinoids induced in maize by O. furnacalis feeding for 0 h (red circles), 24 h (blue triangle), 48 h (green asterisk) and 72 h (black rhombus) (n = 5). (c) Concentrations (µg/g FW) of benzoxazinoid-related metabolites and (d) Relative expression changes of the genes involved in the benzoxazinoid biosynthesis pathway induced in maize by O. furnacalis feeding for different periods of time. Gene expressions (mean ± SE, n = 3) are presented as fold change relative to control (0 h). Different letters above the bars indicate significant differences, P < 0.05, ANOVA followed by Tukey’s HSD test. Notation is the same as for Figure 6.
Benzoxazinoids are key secondary metabolites in the direct defence against Lepidoptera (Ahmad et al., 2011). We found, besides DHBOA-Glc, expression of the other benzoxazinoids measured was dramatically changed after O. furnacalis attack, indicating that O. furnacalis attack can induce the accumulation of these compounds. DIMBOA, DIMBOA-Glc, MBOA and HDMBOA-Glc are the major benzoxazinoids involved in defence against Lepidoptera (Ahmad et al., 2011; Glauser et al., 2011; Niemeyer, 2009). Our results show that O. furnacalis attack induces a significant decrease in DIMBOA-Glc and a significant increase in HDMBOA-Glc, consistent with the findings of Dafoe et al. (2011) and Meihls et al. (2013). It is also thought that low DIMBOA-Glc/high HDMBOA-Glc levels are more resistant to insects than high DIMBOA-Glc/low HDMBOA-Glc in maize leaves (Meihls et al., 2013), because DIMBOA can be detoxified by specialist insects via glycosylation, but HDMBOA cannot (Glauser et al., 2011). In maize leaves pre-infested by O. furnacalis for 24 h, accumulation of DIMBOA-Glc/HDMBOA-Glc and MBOA reached a peak, while the 2nd instar larvae consumed more leaves (increased RCR), had lower food processing efficiencies (reduced ECD and ECI), and grew more slowly (reduced RGR) on these treated leaves compared with those on un-preinfested leaves (Figure 5a). This inhibitory effect was further confirmed by the prolonged developmental stage and adverse population parameters of O. furnacalis, which describe the reproducing ability, rate of increase and the whole life time of the population. These results are congruent with the finding that herbivore-induced plant defences can reduce the fitness of subsequently feeding insects (Karban, 2011), as is reported for Diabrotica virgifera virgifera LeConte in maize (Erb et al., 2011a). Therefore, we propose that the low DIMBOA-Glc/high HDMBOA-Glc and high MBOA levels in maize leaves induced by O. furnacalis feeding for 24 h may result in the subsequent poor performance of O. furnacalis.

However, benzoxazinoids provide only an incomplete picture of the maize defence responses to O. furnacalis, and other defences like proteinase inhibitors and toxic proteins induced by insect herbivory may also inhibit insect performance (Howe and Jander, 2008; Karban et al., 1997; Vila et al., 2005). Additionally, primary metabolites are considered to play as important a role in growth chambers or greenhouses (Lawrence et al., 2012; Tzin et al., 2015, 2017; Wang et al., 2017a), in which determination of the drivers of gene expression patterns in maize following insect attack may be more straightforward. In cultivation, however, maize is grown in the complex fluctuating natural environment, so the transcriptome responses are not only governed by the biotic factors like plant age and insect damage, but also influenced by abiotic factors, such as wind, humidity, air temperature and solar radiation (Nagano et al., 2012). A recent study by Qi et al. (2018) showed that ultraviolet-B (UV-B) treatment can enhance maize resistance to Spodoptera litura Fabricius by elevating the levels of JA-ile and defence-related secondary metabolites. However, controlled conditions may fail to reflect the influence of real biotic and abiotic stresses on gene expression, and we believe that our research, in which we attempt to decipher some of the maize transcriptome dynamics following O. furnacalis herbivory in the field, provides an important next step. In this study, many genes involved in plant resistance-related pathways were strongly up-regulated following O. furnacalis herbivory, consistent with the results collected under controlled conditions (Qi et al., 2016; Tzin et al., 2015), implying that maize also mobilizes defences in response to O. furnacalis herbivory in the field.

Benzoazinoids are key secondary metabolites in the direct defence of maize against insects (Ahmad et al., 2011; Meihls et al., 2013; Niemeyer, 2009; Wouters et al., 2016). We found, besides DHBOA-Glc, expression of the other benzoxazinoids measured was dramatically changed after O. furnacalis attack, indicating that O. furnacalis attack can induce the accumulation of these compounds. DIMBOA, DIMBOA-Glc, MBOA and HDMBOA-Glc are the major benzoxazinoids involved in defence against Lepidoptera (Ahmad et al., 2011; Glauser et al., 2011; Niemeyer, 2009). Our results show that O. furnacalis attack induces a significant decrease in DIMBOA-Glc and a significant increase in HDMBOA-Glc, consistent with the findings of Dafoe et al. (2011) and Meihls et al. (2013). It is also thought that low DIMBOA-Glc/high HDMBOA-Glc levels are more resistant to insects than high DIMBOA-Glc/low HDMBOA-Glc in maize leaves (Meihls et al., 2013), because DIMBOA can be detoxified by specialist insects via glycosylation, but HDMBOA cannot (Glauser et al., 2011). In maize leaves pre-infested by O. furnacalis for 24 h, accumulation of DIMBOA-Glc/HDMBOA-Glc and MBOA reached a peak, while the 2nd instar larvae consumed more leaves (increased RCR), had lower food processing efficiencies (reduced ECD and ECI), and grew more slowly (reduced RGR) on these treated leaves compared with those on un-preinfested leaves (Figure 5a). This inhibitory effect was further confirmed by the prolonged developmental stage and adverse population parameters of O. furnacalis, which describe the reproducing ability, rate of increase and the whole life time of the population. These results are congruent with the finding that herbivore-induced plant defences can reduce the fitness of subsequently feeding insects (Karban, 2011), as is reported for Diabrotica virgifera virgifera LeConte in maize (Erb et al., 2011a). Therefore, we propose that the low DIMBOA-Glc/high HDMBOA-Glc and high MBOA levels in maize leaves induced by O. furnacalis feeding for 24 h may result in the subsequent poor performance of O. furnacalis.

However, benzoxazinoids provide only an incomplete picture of the maize defence responses to O. furnacalis, and other defences like proteinase inhibitors and toxic proteins induced by insect herbivory may also inhibit insect performance (Howe and Jander, 2008; Karban et al., 1997; Vila et al., 2005). Additionally, primary metabolites are considered to play as important a role in

Figure 5 Performance of Ostrinia furnacalis reared on the maize leaves that have been exposed to O. furnacalis for 24 h. (a) The nutritional indices of the 2nd and 3rd instar O. furnacalis larvae. ECD, efficiency of conversion of ingested food; ECI, efficiency of conversion of digested food; AD, approximate digestibility; RCR, relative consumption rate, RGR, relative growth rate. Different letters above the bars indicate significant differences at P < 0.05 (LS means, ANCOVA) (n = 15). (b) Developmental time and (c) population parameters of O. furnacalis. L1, 1st instar; L2, 2nd instar; L5, 5th instar; TPOP, total pre-oviposition period; r0, net reproductive rate, r, intrinsic rate of increase; λ, finite rate of increase; T, mean generation time. Different letters above the bars indicate significant differences (P < 0.05) using the Tukey–Kramer procedure.
insect performance as secondary metabolites (Roeder and Behmer, 2015). A recent study showed that JA-dependent depletion of soluble sugars (glucose and fructose) weakened *Nicotiana attenuata* defence response to *Manduca sexta* L. (Machado et al., 2015). In our study, JA concentrations are significantly increased, and the genes involved in the metabolism of primary metabolites (e.g. amino sugar and nucleotide sugar metabolism) were strongly induced after *O. furnacalis* herbivory.

Figure 6 Effects of *Ostrinia furnacalis* feeding on the blend of volatile compounds collected in the headspace of maize plants. (a) The maize volatile collection method used in the field. [The schematic view was drawn according to the description by Degen et al. (2012).] (b) Separation of the headspace composition of maize volatiles induced by *O. furnacalis* infestation for different periods of time using PLS-DA, depicted as a two-dimensional score plot using the first two PLS components. The PLS-DA resulted in a model with two significant components: \( R^2_X = 0.41 \) \( R^2_Y = 0.15 \). (c) Bar plot of variable importance of each volatile compound to the first and second PLS components. (d) Loading plot indicating the contribution of each volatile compound to the separation between groups. The ellipse defines Hotelling’s \( T^2 \) confidence region with a 95% confidence interval for the samples. (e) Effects of *O. furnacalis* feeding on the value of key individual maize volatiles. Values are mean normalized quantities of volatiles. Values are mean normalized quantities of volatiles

\[
\text{Normalized quantity} = \frac{\text{Peak area of volatile compound}}{\text{Peak area of IS}} \pm \text{SE (n = 8)}.
\]

Data were log10\((x + 1)\) transformed before analysis. (f) Relative expression changes of the genes involved in the terpene biosynthesis pathway.
Insect herbivory can also induce plant indirect defences by releasing HIPVs attractive to natural enemies of the pest (Aljbory and Chen, 2017). Many previous studies have focused on maize indirect defences in North American and European maize lines (Aljbory and Chen, 2017; D’Alessandro et al., 2009; Fontana et al., 2011; Schnee et al., 2006; Turlings et al., 1990). Certain North American lines have been reported to have lost their ability to release (E)-β-caryophyllene during domestication. (E)-β-caryophyllene is a signal that can strongly attract an entomopathogenic nematode in response to D. virgifera virgifera attack (Köllner et al., 2008; Rasmann et al., 2005). In this study, we found the levels of (E)-β-caryophyllene and its biosynthesis-related gene TPS23, were significantly elevated after O. furnacalis herbivory. Moreover, O. furnacalis-induced volatiles could attract M. cingulum, particularly at 24 and 48 h following onset of herbivory. These results suggested that the Chinese commercial maize still possesses this indirect defence mechanism against O. furnacalis herbivory. As well as (E)-β-caryophyllene, levels of other terpenes such as linalool, β-farnesene, bergamotene and α-cubebene all showed significant increases following O. furnacalis herbivory. However, little is known about the effects of different concentrations of these key odour molecules, or the importance of their relative concentrations in M. cingulum attraction. Further work in this area will include screening for the key odours involved in M. cingulum attraction, and using mutants and transgenic maize with different levels of volatile emissions to investigate the biosynthesis, metabolic engineering and function of O. furnacalis-induced maize volatiles. Moreover, field studies are needed in order to reveal the role of plant volatiles in determining the population dynamics of pests and their natural enemies, because the responses of natural enemies to HIPVs are more complex in real agricultural settings than in the laboratory (Salamanca et al., 2017). It is known that there is a significant variation both in individual odour concentrations and volatile diversity between different

Figure 7 Preference of female and male Macrocentrus cingulum towards the maize volatiles with or without Ostrinia furnacalis attack. (a) Experimental setup for M. cingulum behaviour. [The schematic view was drawn according to the description by Takemoto and Takabayashi (2015).] (b) Behaviour response (%) of female and male M. cingulum to O. furnacalis versus uninduced maize volatiles. Numbers in orange bars represent the number of M. cingulum responding to O. furnacalis-induced maize volatiles and numbers in green bars represent the number of M. cingulum responding to control within 5 min. The asterisks are based on χ² analysis, ***P < 0.001; **P < 0.01; *P < 0.05; ns, P > 0.05.

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research systems. The differences mainly stem from the fact that each research group uses their own, and in some cases unique, research system to explore the effects of herbivore attack on maize volatiles. Maize genotypes (Degen et al., 2004), plant age (Wason and Hunter, 2014), insect feeding guilds (De Boer et al., 2005), duration of induction, and biotic and abiotic environments (Gouinguené and Turlings, 2002), volatile-collection methods and time, as well as volatile analysis platform used all vary greatly between different experiments.

Plant direct and indirect defences to insects are both mediated by phytohormones such as JA, SA and ABA (Hove, 2004; Stam et al., 2014). JA is well known for its predominant role in mediating plant defence to chewing insect attack (Bodenhausen and Reymond, 2007; Lortzing and Steppuhn, 2016; Lu et al., 2015; Yan and Xie, 2015). In this investigation, we found a temporal change in JA response to O. furnacalis herbivory, which reached a peak soon after onset O. furnacalis feeding (2 h), and then gradually declined, suggesting the JA signalling pathway is an early signal transduction in maize defences to O. furnacalis. However, most of the JA biosynthesis-related genes were strongly induced between 2 and 24 h of O. furnacalis feeding. Therefore, we speculated that O. furnacalis feeding may after a certain time suppress JA production by the antagonistic action of other phytohormones or post-transcriptional modification, or shifting the JA biosynthesis to other long-term defence responses. It is worth noting that JA concentration is usually very low before receiving stimulation from biotic and abiotic factors, indeed, the JA constitutive concentration in the inbred maize line A188 is close to zero (Qi et al., 2016). However, the basal level of JA in our study was approximately 1000 ng/g FW, which was markedly higher than JA levels of maize plants grown in growth chamber and greenhouse conditions (Qi et al., 2016; Tzin et al., 2015, 2017). This obvious discrepancy in JA levels may be likely due to the difference in growing conditions. In contrast to the greenhouse-based experiments which JA concentration in maize is only influenced by one attacking pest, JA concentration of field-grown maize can be governed by multiple external biotic and abiotic stress (Creechm and Mullent, 1995). Addition to the high level of JA constitutive concentration, we also found many of the genes involved in the biosynthesis of JA, terpenes and benzoxazinoids showed stronger induction as early as 2 h following O. furnacalis infestation when compared with similar studies on other herbivores in maize (Qi et al., 2016; Tzin et al., 2017). In this case, we speculate that Jingke968 may prepare for O. furnacalis attack before it actually experiences O. furnacalis, a phenomenon known as defence priming (Group et al., 2006; Martinez-Medina et al., 2016). Plants with primed defence can respond quickly, strongly and over long periods to biotic and abiotic stress (Douma et al., 2017; Mauch-mani et al., 2017), which can reduce the damage inflicted over the time it takes to produce induced defences (Frost et al., 2008). Although we could not exclude the external factors from field conditions including GLVs from other plants, insects body odour and abiotic factors that may exert a priming effect on these maize plants (Frost et al., 2008), we speculate that Jingke968 may be naturally primed to combat O. furnacalis, because it not only had high levels of JA, but also had high levels of DIMBOA and DIMBOA-Glc before being subjected to O. furnacalis attack, and high levels (424.20 μg/g FW) of HDMBOA-Glc after O. furnacalis infestation.

Unlike JA, SA concentration showed minor and insignificant changes following O. furnacalis herbivory, indicating O. furnacalis feeding did not induce the SA-dependent signalling pathway. The ABA-dependent signalling pathway is also involved in maize defences against O. furnacalis herbivory, consistent with previous studies of maize resistance to D. virgifera virgifera (Erb et al., 2009, 2011b) and M. separata (Qi et al., 2016) herbivory.

In this study, we have evaluated the direct and indirect defences of field-grown commercial maize at the mid-whorl stage in response to O. furnacalis attack. We examined the transcriptome, phytohormones, benzoxazinoids, volatiles, O. furnacalis performance and the behaviour of a parasitic wasp. The dynamic transcriptome analysis showed a rapid, strong transcriptome response in the first 2 h following O. furnacalis infestation, and continued striking changes until 24 h. Integrative analysis of transcriptomic and metabolomic data revealed that phytohormones, benzoxazinoids and volatiles were involved in maize resistance to the pest at this stage. The poor performance of O. furnacalis on pre-infested maize leaves and the preference of M. cingulum to O. furnacalis-induced maize volatiles directly proved that these defensive compounds have a function in maize direct and indirect defences. This work not only provides several insights into the molecular and biochemical mechanisms of commercial maize resistance to O. furnacalis, but may be of significance in the uncovering of as yet unknown defensive genes with high levels of expression in natural environments, benefiting the breeding of maize cultivars with enhanced and eco-friendly resistance to insects in the field.

**Experimental procedures**

**Plant growth and insect rearing**

Maize genotype Jingke968 was grown in the field at Langfang Experimental Station of Plant Protection, Chinese Academy of Agricultural Sciences (IPP, CAAS), Hebei province (39°30’N, 116°36’E) in 2016, with 1 m between rows and 0.35 m between individual plants. Plants for different treatments were spaced 2 m apart to hinder communication between individuals. Each plant was enclosed in an individual nylon cage with 60 mesh. Field management proceeded according to the agricultural practices used in local farming. All the maize plants used for the experiments were developmentally similar and healthy. The meteorological parameters during the growth period were recorded in detail (Table S4).

*Ostrinia furnacalis* and the parasitoid wasp *M. cingulum* were obtained from a laboratory colony from the IPP, CAAS, Beijing. *Ostrinia furnacalis* larvae were reared on modified diets (Zhou et al., 1980) for 3–4 generations in a controlled incubator with 27 ± 1 °C, 70%–80% relative humidity (RH) and a photoperiod of 16 h. *M. cingulum* colony was maintained following Wang et al. (2017b).

**Plant treatments**

When the plants were at the mid-whorl stage (approximately 30–35 days after germination), 20 3rd instar *O. furnacalis* larvae were placed in each maize whorl and allowed to feed freely. Damaged leaves from approximately 2 cm surrounding the initial O. furnacalis feeding sites were taken from the leaves with a knife. Leaf samples were harvested for gene expression at 0, 2, 4, 12 and 24 h after initial *O. furnacalis* infestation. For phytohormone analysis, leaf samples were harvested at 0, 2, 4, 8 and 12 h post-inestation, and for benzoxazinoid analysis, leaf samples were harvested at 0, 24, 48 and 72 h. The plants at 0 h post-inestation were used as control plants and leaf sections were

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taken as before. Leaves from four or five plants per time point were pooled for each biological replicate. Leaf samples were harvested, immediately frozen in liquid nitrogen, and stored in a −80 °C freezer until use. Three replicates were collected for transcriptome analysis, and five replicates each for phytohormone and benzoxazinoid analysis were collected. The infestations of *O. furnacalis* were staggered to ensure all the samples were collected at the same time. The meteorological parameters of the collection date were given in Table S4.

**RNA isolation, cDNA library preparation, transcriptome sequencing, RNA sequencing data analysis and quantitative real-time PCR analysis**

Total RNA from each leaf sample was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA library preparation and transcriptome sequencing follow Liu et al. (2015) and Zhong et al. (2011). Clean reads were mapped to the maize reference genome (B73 RefGen_v3.31) (Schnable et al., 2009) using TopHat2 software (Kim et al., 2013), and only unique mapping reads were retained for calculating gene expression. RNA-seq data analysis was performed according to previously published protocols (Trapnell et al., 2010, 2012). DEGs were identified by the edge package (http://www.r-project.org/) with FDR <0.05 and absolute value of log2ratio ≥1. To validate the accuracy of the RNA-seq data, qRT-PCR analyses were performed on Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystem, Foster City, CA) using SYBR Premix Ex Taq (Tli RNaseH Plus) master mix (Takara-Bio, Shiga, Japan) following the manufacturer’s instructions. The PCR amplification program was 95 °C (15 s), followed by 40 cycles at 60 °C (60 s) and 95 °C (30 s). Fold changes of gene expression level were calculated using the 2−ΔΔCT method (Livak and Schmittgen, 2001). The actin gene was used as a candidate reference gene (Manoli et al., 2012). The primers used in this study are given in Table S5.

**Quantification of phytohormone and benzoxazinoids**

JA, JA-ile, ABA and SA concentrations in leaves were analysed using HPLC-MS/MS (LCMS-8040 system, Shimadzu, Kyoto, Japan) following Wu et al. (2007). Benzoxazinoids were extracted and analysed following Glauser et al. (2011).

**Collection and quantification of volatiles**

A dynamic headspace system detailed described by Degen et al. (2012) and Huang et al. (2014) was used to collect the volatiles from the plants at 0, 2, 4, 12, 24, 48 and 72 h following infestation with *O. furnacalis*. The leaves and stems of each sampling plants were covered by a transparent polyethylene terephthalate bag. A stream of charcoal filtered air was pumped into the bag at a flow rate of 500 mL/min with a vacuum pump (Beijing Institute of Labor Instrument, Beijing, China). The outgoing air passed through a 8-mm diameter glass outlet tube containing 60 mg Tenax TA (60/80 mesh; Sigma-Aldrich, Oakville, ON, Canada) to retain volatiles. For each treatment, eight replicates were analysed using the TWOSEX-MSChart program (Chi, 2015). *M. cingulum* preferences were analysed using a χ² test with the null hypothesis of 50% probability of making each choice. PCA of benzoxazinoids was conducted and plotted using *‘pca’* function in the mixOmics package 6.30 in R (v.3.4.2; R Development Core Team, 2017). PLSDA of maize volatiles was achieved using *‘cim’*, *‘plotIndiv’* and *‘plotVar’* functions in the mixOmics package 6.30 in R (v.3.4.2; R Development Core Team, 2017). Statistical comparisons of metabolite concentration were made using SAS statistics package version 9.2 (SAS Institute, 2009) and a significance level of *P* < 0.05 was applied.

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**Conflict of interest**

The authors declare no conflict of interest.
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Figure S1 Volcano plots of differential expression genes (DEGs) in maize induced by *O. furnacalis* attack for 2, 4, 12 and 24 h compared with control.

Figure S2 Correlations between RNA-seq and qRT-PCR gene expression data.

Figure S3 Effects of *O. furnacalis* feeding on salicylic acid (SA) and abscisic acid (ABA) biosynthesis.

Table S1 Summary of RNA sequencing and mapping using the maize genome as the reference.

Table S2 The common pathways of DEGs in the transcriptome of maize induced by *O. furnacalis* infestation for different periods of time.

Table S3 The developmental time and fecundity of *O. furnacalis* reared on the maize leaves previously infested by *O. furnacalis* for 0 and 24 h.

Table S4 The meteorological parameters during the maize growth period.

Table S5 Primers used for qRT-PCR.

Data S1 Genes detected in all samples.

Data S2 All up-regulated DEGs in maize leaves induced by *O. furnacalis* feeding for 2, 4, 12 and 24 h with a cut-off of twofold change relative to the control.

Data S3 All down-regulated DEGs in maize leaves induced by *O. furnacalis* infestation for 2, 4, 12 and 24 h with a cut-off of twofold change relative to the control.

Data S4 KEGG pathway enrichment analysis of DEGs in the transcriptome of maize induced by *O. furnacalis* infestation for different periods of time.

Data S5 Overrepresentation analysis of each profile using the Short Time-series Expression Miner (STEM) analysis tool to identify metabolic pathways that are being regulated.