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Original Article

Oral secretions from *Mythimna separata* insects specifically induce defence responses in maize as revealed by high-dimensional biological data

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ABSTRACT

Attack from insect herbivores poses a major threat to plant survival, and accordingly, plants have evolved sophisticated defence systems. Maize is cultivated as a staple crop worldwide, and insect feeding causes large production losses. Despite its importance in agriculture, little is known about how maize reacts to insect herbivory. Taking advantage of advances in sequencing and mass spectrometry technology, we studied the response of maize to mechanical wounding and simulated *Mythimna separata* (a specialist insect) herbivory by applying its oral secretions (OS) to wounds. In comparison to the responses induced by mechanical wounding, OS elicited larger and longer-lasting changes in the maize transcriptome, proteome, metabolome and phytohormones. Specifically, many genes, proteins and metabolites were uniquely induced or repressed by OS. Nearly 290 transcription factor genes from 39 families were involved in OS-induced responses, and among these, more transcription factor genes were specifically regulated by OS than by wounding. This study provides a large-scale omics dataset for understanding maize response to chewing insects and highlights the essential role of OS in plant–insect interactions.

Key-words: metabolome; phytohormones; proteome; transcriptome.

INTRODUCTION

Insects have interacted with plants for more than 350 million years as pollinators, but also as herbivores. During ongoing coevolution, plants have gained sophisticated defence systems to perceive damage caused by insect feeding and respond accordingly to defend. Although still poorly understood, herbivore-associated molecular patterns (HAMPs) or herbivore-associated elicitors (Mithofer & Boland 2012; Bonaventure 2014), such as certain molecules in insect oral secretions (OS), can be recognized by plants and activate specific defence responses. Among the known HAMPs, fatty acid-amino acid conjugates (FACs) are widely distributed in the OS of lepidopteran insects and elicit specific responses in various plants; furthermore, H_2O_2, which is produced by glucose oxidase (GOX) in the OS, is also believed to take part in activating insect feeding-induced defence reactions (Wu & Baldwin 2010).

Among the early responses, accumulation of phytohormones, including jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), plays an important role in modulating defence (Howe & Jander 2008; Wu & Baldwin 2010). The function of JA in regulating plant defence against insects has been well studied. Knocking down or out genes important for JA biosynthesis or signalling decreases defensive metabolites and thus greatly compromises plant resistance to insects (Halitschke & Baldwin 2003; Zhou *et al.* 2009). Tomato (*Solanum lycopersicum*) plants impaired in ET signalling show decreased accumulation of proteinase inhibitors, which are important anti-herbivory metabolites (ODonnell *et al.* 1996); in the wild tobacco *Nicotiana attenuata*, compromising ET signalling down-regulated basal and herbivory-induced nicotine levels, probably by cross-talking with the JA pathway (von Dahl *et al.* 2007). SA plays a role in fine-tuning JA-induced responses, usually as an antagonist (Thaler *et al.* 2012).

Comparisons between mechanical wounding and real or simulated insect feeding revealed large-scale differences on the transcriptomic and proteomic levels in Arabidopsis and *N. attenuata*, respectively (Reymond *et al.* 2004; Giri *et al.* 2006; Gulati *et al.* 2013), demonstrating that plants respond specifically to insect herbivory. Among the transcriptionally regulated genes, transcription factors (TFs) have been intensively studied for their roles in controlling the biosynthesis of plant secondary metabolites. Arabidopsis *myc2 myc3 myc4* triple mutants have greatly decreased glucosinolate levels and are

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susceptible to insects (Schweizer *et al.* 2013). In *N. attenuata*, *MYB8* regulates the accumulation of phenylpropanoid-polyamine conjugates, which contribute to resistance against *Manduca sexta* and *Spodoptera littoralis* (Kaur *et al.* 2010).

Maize is among the most important cereal crops that is grown widely throughout the world, reaching more than 1 billion tons of grain yield in 2013 (http://faostat3.fao.org/browse/Q/QC/E). As for all crop plants, insects cause large losses in maize production; however, very little is known about how maize responds to insect herbivory. The first HAMP, volicitin, was isolated from the OS of *Spodoptera exigua* feeding on maize seedlings, and it was found that volicitin induces volatile compounds that attract the females of the parasitic wasp *Cotesia marginiventris* (Turlings *et al.* 1993; Alborn *et al.* 1997). Maize plants having mutations in *OPR7* and *OPR8*, important JA biosynthetic genes, show severely decreased JA levels; these plants exhibit male flower feminization, initiation of female reproductive buds at each node, extreme elongation of ear shanks and highly decreased resistance to insects and fungi (Yan *et al.* 2012). Thus, JA signalling in maize is largely similar to that in eudicots, such as Arabidopsis and tobacco, but has a specific function in maize flower sex determination.

A number of specialized metabolites involved in herbivore resistance have been identified in maize. For example, a 33 kD cysteine proteinase has a strong inhibitory effect on *Spodoptera frugiperda* growth, and its abundance was dramatically induced 1 h after insect feeding (Pechan *et al.* 2000); similarly, a maize proteinase inhibitor protein was also induced by insect (*Spodoptera littoralis*) feeding and showed anti-insect activity (Tamayo *et al.* 2000). A phenolic compound, silk maysin, is detrimental to the corn ear worm *Helicoverpa zea* (Cocciolone *et al.* 2005). The best studied group of defensive compounds are benzoxazinoids (Bxs), such as 2-4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2-ß-D-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA-Glc), playing an important role in maize resistance to piercing-sucking (*Rhopalosiphum padi* and *R. maidis*) and chewing insects (*S. frugiperda* and *S. littoralis*), respectively (Ahmad *et al.* 2011; Glauser *et al.* 2011; Meihls *et al.* 2013). In addition, maize-emitted volatiles also function as indirect defenses and/or priming agents; for example herbivory strongly induced the expression of a maize terpenes synthase (TPS) gene *TPS10*, which catalysed the production of (*E*)-*β*-farnesene, (*E*)-*α*-bergamotene and other sesquiterpenes, and these terpenes attracted the parasitic wasp *Cotesia marginiventris*, which is the natural enemy of the lepidopteran insects (Schnee *et al.* 2006). Exposure of insect-induced volatiles to maize plants enabled these plants to respond with stronger and/or earlier expression of defence-related genes to subsequent *Spodoptera littoralis* herbivory and improved direct and indirect resistance (Ton *et al.* 2007). Maize volatiles induced by the caterpillar *Mythimna separata* can prime neighbouring maize plants to defend against *M. separata* reinfestation, and this priming effect is associated with methylation of a trypsin inhibitor gene at the promoter region (Ramadan *et al.* 2011; Ali *et al.* 2013). Recently, insect feeding-induced indole was found to be able to function as a priming factor that elevates the adjacent maize plants' resistance (Erb *et al.* 2015).

The rapid development in microarray, next-generation sequencing and mass spectrometry (MS) technologies has enabled relatively high-throughput analyses of transcriptomes, proteomes and metabolomes, providing powerful tools for obtaining large-scale snapshot information on transcripts, proteins and metabolites. These omics and systems biology approaches have been applied to almost all areas of plant research (Yuan *et al.* 2008; Mochida & Shinozaki 2011; Stitt 2013). In maize, although omics data have also provided important insight into the mechanism of heterosis or regulation of gene expressions in hybrids (Swanson-Wagner *et al.* 2006; Hoecker *et al.* 2008; Paschold *et al.* 2014), only very few large-scale biological studies on maize-insect interactions have been reported. Metabolomic profiling of maize leaves, sap, roots and root exudates indicated that 32 metabolites were regulated by *Spodoptera littoralis* herbivory, including several Bxs (Marti *et al.* 2013). Transcriptomic and proteomic analysis indicated that European corn borer *Ostrinia nubilalis* feeding on maize stems induced over 1100 genes but only eight proteins had altered levels (Dafoe *et al.* 2013), and Yang *et al.* (2015) compared the transcriptomic data from methyl jasmonate-induced and Asian corn borer *O. furnacalis*-induced leaves and found that JA is the major defence signalling against *O. furnacalis*. Recently, Tzin *et al.* (2015) used RNA-seq and metabolome analysis to study the response of maize leaves to corn leaf aphids (*Rhopalosiphum madis*) at various intervals over 96 h, and hundreds of differentially regulated genes were found at each time point after aphid feeding; among these, four Bx biosynthesis genes and four *TPS*s were up-regulated, and using maize mutant lines, some of these genes' anti-aphid function was confirmed. These studies illustrate the power of omics approaches in rapidly identifying defence-related genes and metabolites and in creating frameworks for understanding the dynamic intertwined regulatory networks important for maize resistance to insects.

The oriental armyworm *Mythimna separata* (Lepidoptera: Noctuidae) is a specialist insect that feeds mainly on maize, sorghum and rice, causing large economic losses. Given that maize recognizes insect-produced volicitin and activates specific defence reactions (Alborn *et al.* 1997; Schmelz *et al.* 2009), we hypothesized that feeding of *M. separata* induces a signalling network that leads to changes on transcription, protein and metabolite level. Based on multiple-omics analyses, we show that compared with the responses induced by mechanical wounding, *M. separata* OS elicited stronger and longer-lasting changes in the maize transcriptome, proteome, metabolome and phytohormones. We generated a large-scale dataset that provides insight into the responses of maize to insect herbivory, and these data could be used to generate new hypotheses and select candidate genes for further functional studies.

MATERIAL AND METHODS

Plants, herbivores and plant treatments

Maize inbred line A188 was grown in a greenhouse under controlled conditions (20–28 °C, day length 16 h), and *M. separata* was obtained from the Institute of Zoology, Chinese Academy

of Sciences. OS were collected from around 100 larvae of *M. separata* (third to fifth instar) reared on A188, and OS were kept on ice during collection and were aliquoted to small amounts and subsequently frozen in liquid nitrogen and stored at −80 °C until use. W + W and W + OS treatments were performed as described previously by gently rubbing 20 μL of water or *M. separata* OS to wounds generated by rolling a pattern wheel along the midvein generating two rolls of wounds on each side on the third fully expanded leaves of V3 stage plants. Treatments were all performed at the indicated times prior to harvest, in order to avoid diurnal effect. Together with non-treated controls (Con), samples were harvested at the same time and immediately frozen in liquid nitrogen and were stored at −80 °C until use.

Transcriptome data acquisition and data analysis

Libraries from the maize leaf total RNA (3 μg) were sequenced on an Illumina HiSeq 2000 system using the TruSeq PE Cluster Kit v3-cBot-HS (Illumia). The resulting sequences were trimmed based on quality scores and mapped to the maize B73 reference genome sequence V2 and maize working gene set V5a with Tophat2 (Kim *et al.* 2013) using the following modifications from default parameters: maximum intron size, 100 000; minimum intron size, 20; up to two mismatches allowed (Trapnell *et al.* 2010). The expression levels of genes were estimated using Cufflink and were normalized using the numbers of reads per kb of exon sequence in a gene per million mapped reads. Differential expression analysis was performed using Cuffdiff (Trapnell *et al.* 2010) with a cutoff of fourfold change relative to Con levels.

iTRAQ-based proteome determination and data analysis

Total protein extraction and purification was performed according to a method described previously (Lan *et al.* 2011). Protein digestion was performed according to the FASP procedure described by Wisniewski *et al.* (2009), and the resulting peptide mixture was labelled using the 4-plex/8-plex iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems). The three biological replicates were labelled as Control −113, W 1.5 h −114, OS 1.5 h −115, W 6 h −116 and OS 6 h −117, multiplexed, and vacuum dried. Liquid chromatography (LC)–electrospray ionization (ESI) tandem MS (MS/MS) analysis was performed on a Q Exactive mass spectrometer that was coupled to an Easy nLC (Thermo Fisher Scientific). MS data were acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Determination of the target value is based on predictive Automatic Gain Control. MS/MS spectra were compared with the Uniprot *Zea may* database using the MASCOT engine (Matrix Science) embedded into Proteome Discoverer 1.3 (Thermo Electron). A conservative estimate of differential expression was used: a protein had to be quantified with at least $P < 0.05$, and a ratio fold change of at least 1.2.

Phytohormone quantification

Phytohormone determination was performed on an HPLC-MS/MS (LCMS-8040 system, Shimadzu) according to a method described previously (Wu *et al.* 2007).

Non-volatile metabolite data acquisition and analysis

Samples were extracted with 80% methanol and analysed on an Agilent 1200 Rapid Resolution Liquid Chromatography: Agilent, Santa Clara, CA, US system equipped with a ZORBAX SB-Aq column: Agilent, Santa Clara, CA, US (2.1 × 100 mm, 1.8 μm) couple with an Agilent 6510 Q-TOF: Agilent, Santa Clara, CA, US performed in positive ionization mode. The column temperature was set at 50 °C, and the flow rate was 0.3 mL min^{-1}. Water with 0.1% formic acid and acetonitrile were used as mobile phases A and B, respectively. Mass spectrometric analysis was performed with nitrogen as the nebulizer gas at 45 psi and as drying gas at 350 °C with a flow rate of 9 l min^{-1}. The ESI spray voltage was 4000 V, and the voltage of the fragmentor was 175 V. Raw data files were exported in netCDF format and were processed as described by Kim and colleagues (2011), including peak detection, retention time correction and annotation of isotope and adduct ions using the bioconductor XCMS and CAMERA packages: Bioconductor, Seattle, WA, US. The peak areas were normalized to the total peak area, and peaks that were present in less than 80% samples were discarded from the total peak list to minimize the number of missing values. Peaks with CV values > 20% in quality control samples were deleted to ensure the reliability of the data. The known features were then used for the following analysis, and $P < 0.05$ was the standard for differential regulation.

Untargeted analysis of leaf headspace samples

The headspace of maize leaves was sampled on clean polydimethylsiloxane tubes and analysed using a TD-20 thermal desorption unit (Shimadzu) connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu) and equipped with an Rtx-5MS column: Restek, Bellefonte, PA, US as previously described (Kallenbach *et al.* 2014). Total ion current files exported from the GCMSsolutions software (version 2.72, Shimadzu) were processed using XCMS/CAMERA with modifications to peak parameters for GC analysis. In Excel (Microsoft), contaminants (retention time > 31 min) were removed, and the most abundant m/z feature, which could originate from a plant volatile, was selected to represent each pc group (m/z value < 300, not 73 which is typically from silica). Because of missing samples in some treatment groups, randomly chosen replicates were removed so that all groups had $n = 4$. Background and inconsistently present features were removed as described previously (Kallenbach *et al.* 2014). As a result 50 (0–0.5 h), 58 (0.5–8 h) or 56 features (24–32 h) were analysed in MetaboAnalyst (http://www.metaboanalyst.ca/). All features analysed were also checked in sample chromatograms and identified based on comparison to mass spectral libraries (Wiley, NIST), to compounds with known retention times, and when possible to pure standards run with the same method (Supporting Information Table S12).

FAC analysis and GOX activity of *M. separata* OS assay

Both FAC analysis and GOX activity assay have been described previously (Diezel *et al*. 2009).

Accession numbers

Transcriptome datasets can be retrieved from the NCBI SRA database under the project ID PRJNA299127.

RESULTS

Transcriptomic analysis of maize response to mechanical wounding and simulated *M. separata* herbivory

Given the important role of the insect-produced elicitors FACs and GOX in the OS (Diezel *et al*. 2009; Wu & Baldwin 2010), we determined the FAC contents and GOX activity in *M. separata* OS: OS was rich in OH-C18:3-Gln (volicitin), OH-C18:2-Gln and C18:3-Gln, which were around 100 ng μL^{-1} (Supporting Information Fig. S1a), a concentration that is around the same level of as the FACs in the OS of *Spodoptera* spp. (Pohnert *et al*. 1999), and is lower than those in the OS of *M. separata* feeding on rice (Mori *et al*. 2003). GOX in *M. separata* OS was 0.094 U mg^{-1} protein (Supporting Information Fig. S1b), and this was much lower than that in the OS of *Manduca sexta* (~0.8 U mg^{-1} protein) and *Spodoptera exigua* (~2.8 U mg^{-1} protein), when they fed on *Nicotiana attenuata* (Diezel *et al*. 2009).

In order to investigate the role of OS in eliciting maize defence response, we wounded the third fully expanded leaf of V3-stage maize with a pattern wheel and applied *M. separata* OS to the wounds to simulate *M. separata* feeding (hereafter W + OS); samples in which water was applied to wounds (hereafter W + W) were used to compare OS-specific responses, and non-treated samples served as Con. To obtain a global transcriptome map of genes regulated by W + OS and W + W treatment, samples were harvested 1.5 and 6 h after the start of treatment with three replicates for each group. These times were selected, because we found that at 1.5 h, JA, SA and ABA (abscisic acid) levels were highly induced by W + W or W + OS (refer to the '*Phytohormones induced by W + W and W + OS treatment*' section for details); given the important role of these hormones in modulating plant defence against insects and that 1.5 h is a reasonable early time for transcriptional regulation, 1.5 h was chosen. We reasoned that 6 h post treatment is suitable for determining genes and proteins that play a role in modulating the enzymes for biosynthesis of defence -related metabolites, and it is also when most of these enzymes should have at least moderately increased levels of transcripts.

In total, 52012 genes were detected in all samples combined (Supporting Information Table S1). We selected genes whose transcript levels were up-regulated or down-regulated by at least fourfold with statistical significance ($q < 0.05$) compared with those in Con, and in total 4406 genes were found to be differentially expressed in all samples combined (Supporting Information Table S2).

Samples at 1.5 and 6 h after W + W or W + OS treatment exhibited 1774 (1.5 h after W + W; 1.5 W, for simplicity), 1893 (1.5 h after W + OS; 1.5 OS), 866 (6 h after W + W; 6 W) and 1428 (6 h after W + OS; 6 OS) up-regulated genes, respectively, and 572 up-regulated genes were in common in all samples (Fig. 1, Supporting Information Table S3). Notably, W + OS treatment induced more transcriptomic changes than did W + W (Fig. 1a, b): 1.5 W and 1.5 OS induced 176 and 295 genes specifically; only 33 genes were specifically induced by 6 W, whereas 595 genes were specifically induced at 6 OS (Fig. 1a,b). The top 20 of specifically induced genes by 1.5 W, 1.5 OS, 6 W, and 6 OS after treatments are shown in Supporting Information Table S4, and among these, the highest induced was an *INDOLE-3-ACETIC ACID-AMIDO SYNTHETASE* (GRMZM2G378106) after 1.5 OS (37.6-fold). We also analysed OS-further induced (FI) genes (expression levels significantly up-regulated at least 1-fold by W + OS compared with the levels induced by W + W) and found that 76 and 370 genes were amplified by OS 1.5 and 6 h after treatment, respectively, and there were only 20 genes in common (Fig. 1d, Supporting Information Table S3).

The top 60 up-regulated genes by W + OS treatment (30 each after 1.5 OS and 6 OS treatment) were chosen to show their average expression levels after log10 transformations relative to Con. These included *LIPOXYGENASE* (GRMZM2G156861), *WOUND-INDUCED SERINE PROTEASE INHIBITOR* (GRMZM2G156632), *TERPENE SYNTHASE* (GRMZM2G179092), *CHITINASE* (GRMZM2G005633, GRMZM2G051943), *ALLENE OXIDE SYNTHASE* (GRMZM2G002178), *CYTOCHROME P450* (GRMZM2G024331, GRMZM2G075461), *O-METHYLTRANSFERASE* (GRMZM2G099297 and GRMZM2G336824) and *PEROXIDASE* (GRMZM2G427815), whose homologs are involved in plant resistance to insects in other plant species (Fig. 1e, Supporting Information Table S3). It should be noted that a number of the highly up-regulated genes had no annotations, such as GRMZM2G470882, which showed the highest induced expression levels after 1.5 OS and was also highly elevated at other time points, implying that many maize-specific genes/pathways are involved in maize-insect interactions and their functions should be explored.

We further analysed transcripts, which were down-regulated at least threefold. We found that 766, 1152, 122 and 495 genes were down-regulated by 1.5 W, 1.5 OS and 6 W, and 6 OS, respectively (Fig. 2a,b; Supporting Information Table S5), and only 39 genes were found in common among all samples (Fig. 2c). The top 20 of specifically reduced genes by 1.5 W, 1.5 OS and 6 W, and 6 OS are show in Supporting Information Table S6, and most of proteins had not been annotated. Relatively, W+OS-treated samples had a larger portion of down-regulated genes than did W + W-treated samples; for instance, 400 genes were specifically suppressed by 6 OS, whereas only 27 genes were specifically down-regulated by 6 W (Fig. 2 a,b). There were 119 and 83 genes 1.5 and 6 h after treatments whose transcript levels after W + W treatment were further down-regulated by W + OS, and 7 genes were commonly regulated at both times (Fig. 2d, Supporting Information Table S5).

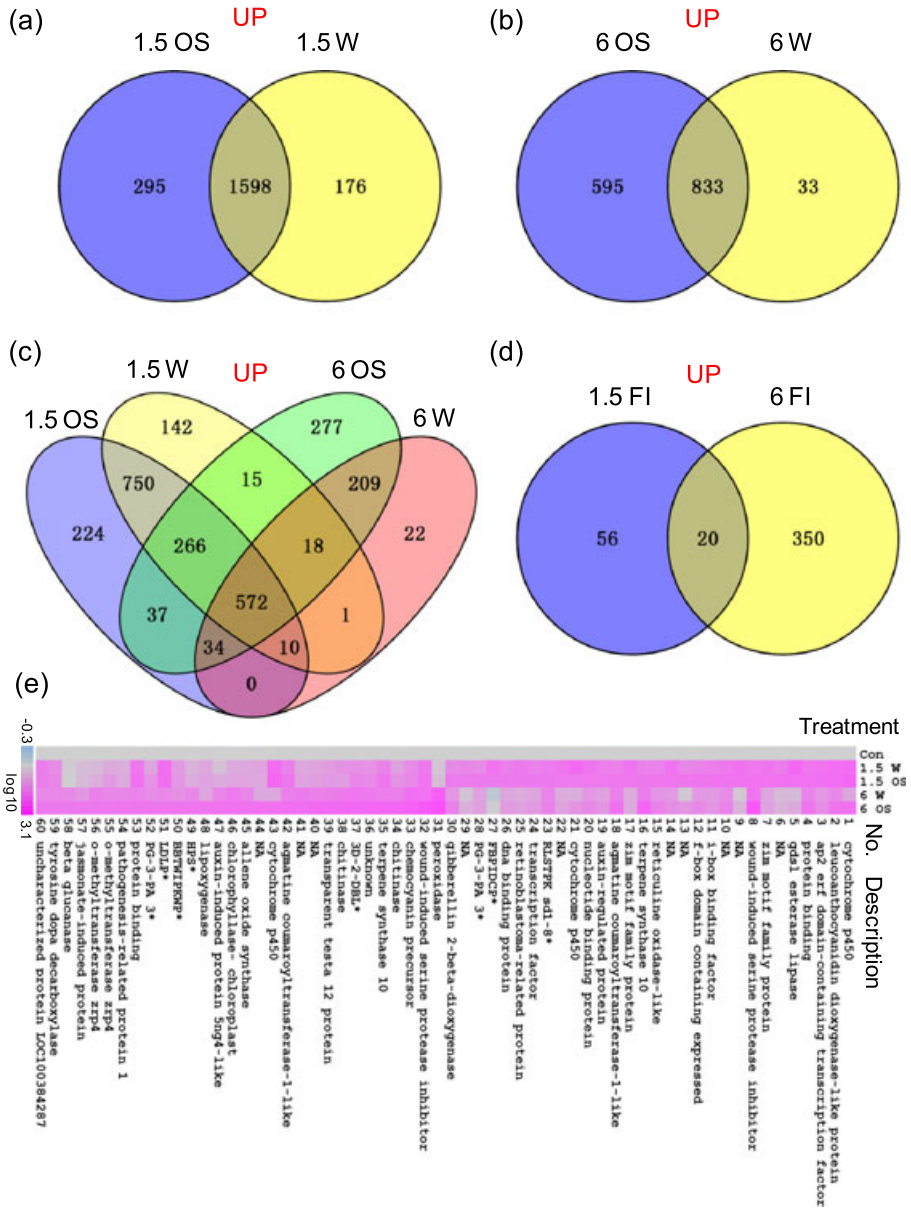

Figure 1. The profile of up-regulated maize transcripts. Maize leaves were treated with W + W or W + OS, and samples were collected at 1.5 and 6 h (for simplicity, named 1.5 W, 1.5 OS and 6 W, 6 OS); non-treated ones severed as controls (Con). Venn diagrams of the numbers of genes up-regulated by treatment of (a) 1.5 W and 1.5 OS and (b) 6 W and 6 OS. (c) Overall up-regulated numbers of genes after W + W and W + OS treatment. (d) Venn diagram of the gene numbers further induced (FI) 1.5 and 6 h after W + OS treatment compared with W + W treatment. (e) Heatmap of the relative expression levels (fold change after log10 transformation) of the 60 most up-regulated genes at 1.5 and 6 h (30 genes for each time point) induced by W + OS treatment (detailed descriptions of these genes can be found in Supporting Information Table S3; NA = not annotated; *: name abbreviated because of length).

The top 60 down-regulated genes by 1.5 OS or 6 OS included *CYTOCHROME P450* (GRMZM2G034471), *JASMONATE-INDUCED GENE* (GRMZM2G020423), *CYTOKININ-O-GLUCOSYLTRANSFERASE 1* (GRMZM2G074631), and various genes without annotations (Fig. 2e, Supporting Information Table S5). Compared with the up-regulated genes, more genes involved in growth rather than defence were found to be down-regulated, such as *VACUOLAR PROCESSING ENZYME* (GRMZM2G093032) and *CATALYTIC HYDROLASE* (GRMZM2G066636).

Comparison between the transcriptome changes induced by simulated *M. separata* herbivory and mechanical wounding demonstrated that W + OS has a stronger and longer-lasting effect on maize than does W + W, indicating that maize specifically recognizes *M. separata* OS and initiates a tailored transcriptome response.

Proteomic profiling of maize responses to simulated *M. separata* feeding and mechanical wounding

To obtain the global changes of proteins regulated by W + W or W + OS treatment, the proteomes of the same leaf samples described earlier (three biological replicates in each group) were

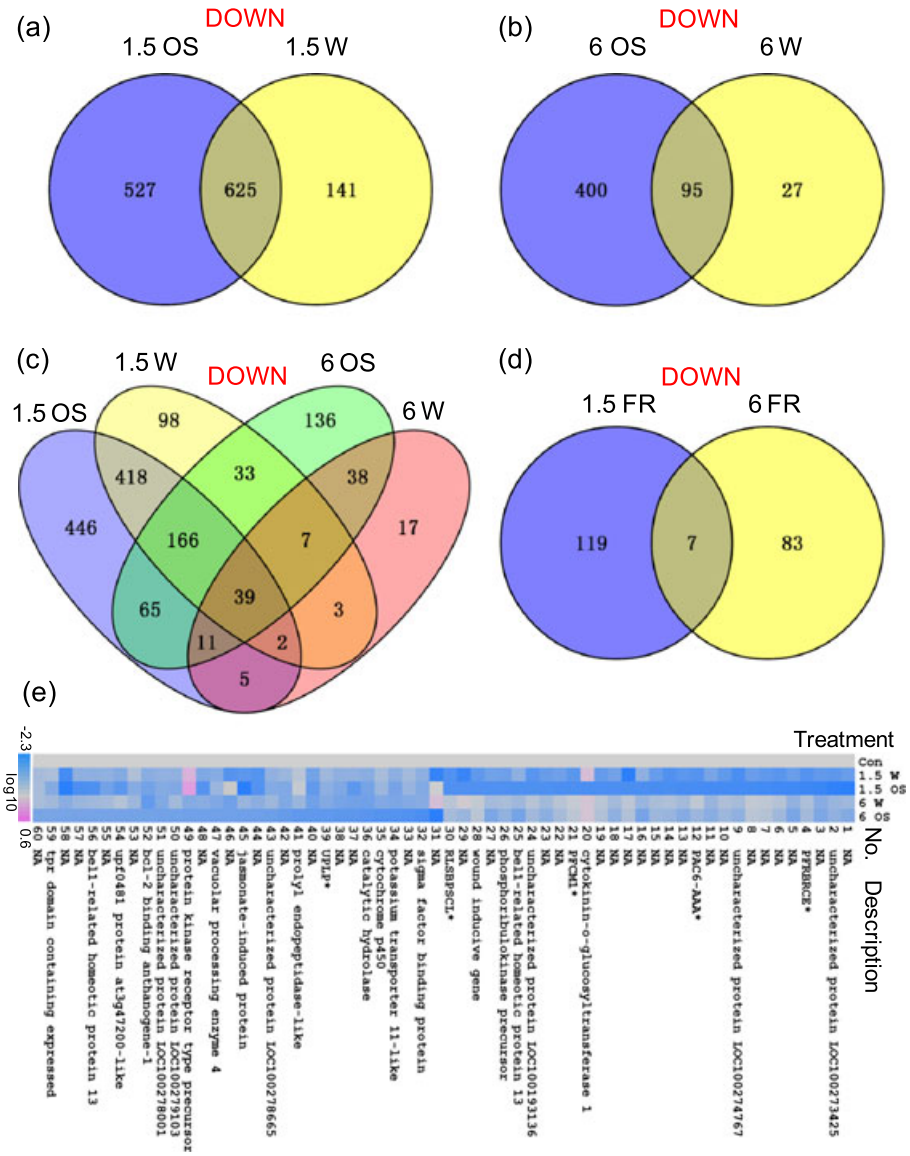

Figure 2. The profile of down-regulated maize transcripts. Maize leaves were treated with W + W or W + OS, and samples were collected at 1.5 and 6 h (for simplicity, named 1.5 W, 1.5 OS and 6 W, and 6 OS); non-treated ones severed as Con. Venn diagrams of the numbers of genes down-regulated by treatment of (a) 1.5 W and 1.5 OS (a) and (b) 6 W and 6 OS. (c) Overall down-regulated gene numbers after W + W and W + OS treatment. (d) Venn diagram of the gene numbers further repressed (FR) 1.5 and 6 h after W + OS treatment compared with W + W treatment. (e) Heat map of the relative expression levels (fold change after log10 transformation) of the 60 most down-regulated genes at 1.5 and 6 h (30 genes for each time point) after W + OS treatment (detailed descriptions of these genes can be found in Supporting Information Table S5; NA = not annotated; *: name abbreviated because of length).

analysed using iTRAQ technology. In total, 2350 proteins were identified (Supporting Information Table S7), and among these, 294 proteins showed altered levels either between treatment groups and Con, or between W + W and W + OS treatments.

Compared with those in Con samples, proteins whose levels changed by at least 20% with significance ($p < 0.05$) were selected. Fifteen (1.5 W), 114 (1.5 OS), 33 (6 W) and 49 (6 OS) proteins were up-regulated, and only 2 common proteins were induced in all treatment groups (Fig. 3, Supporting Information Table S7). Seven and 106 proteins were specifically induced by 1.5 W and 1.5 OS treatment, respectively (Fig. 3a,c, Supporting Information Table S7), and only 8 proteins were in common. Thus, *M. separata* OS specifically and rapidly reconfigures maize proteome (as early as 1.5 h). Six hours after treatment, only 4 proteins were specifically induced by W + W, but 20 proteins were specifically induced by W + OS (Fig. 3b, Supporting Information Table S7). The top 20 specifically induced proteins by W + W and W + OS 1.5 and 6 h after treatment are show in Supporting Information Table S8. We further analysed OS-FI proteins (protein levels up-regulated at least 20% by W + OS compared with those induced by W + W) and found that 86 and 5 proteins were FI by 1.5 OS and 6 OS, and there was only 1 protein in common (Fig. 3d, Supporting Information Table S7).

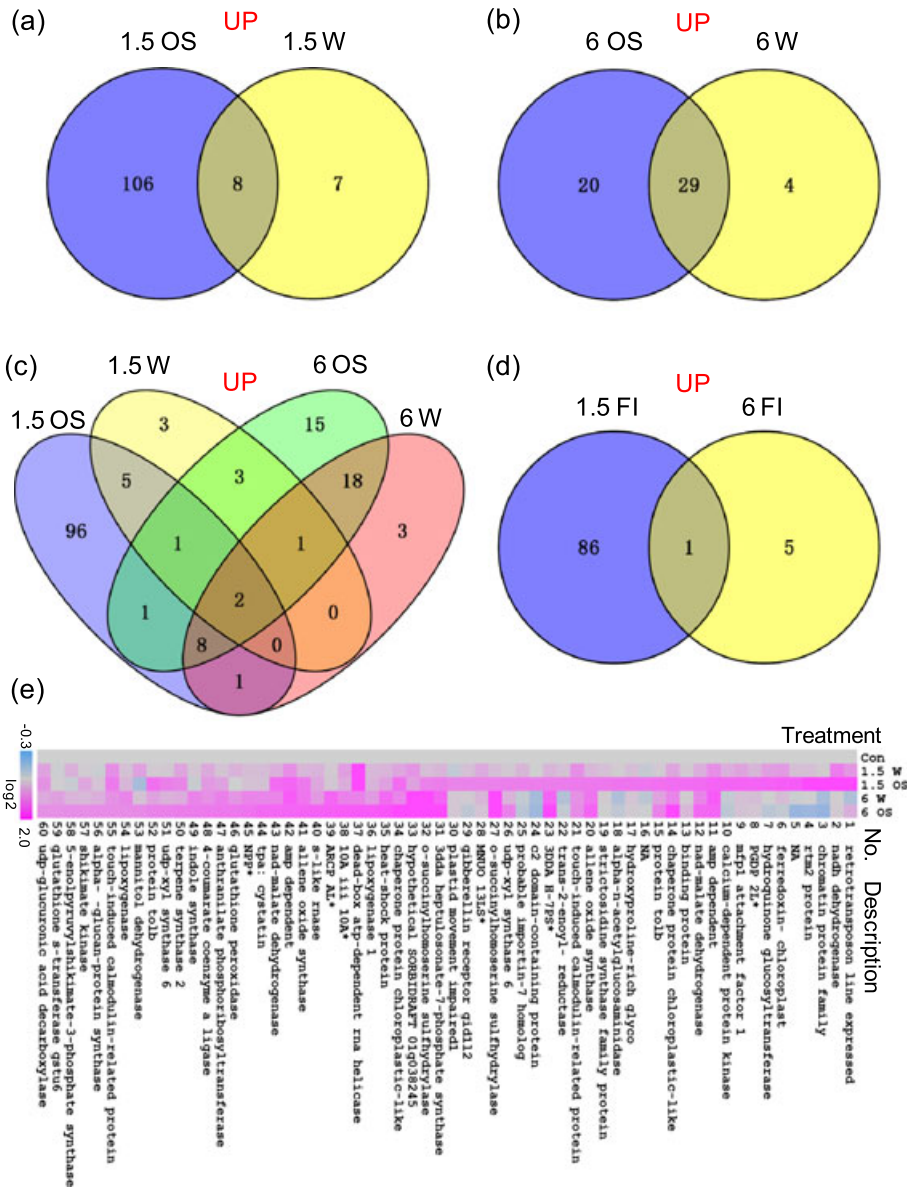

Figure 3. Up-regulated proteins in response to W + W and W + OS treatment. Maize leaves were treated with W + W or W + OS, and samples were collected at 1.5 and 6 h (for simplicity, named 1.5 W, 1.5 OS and 6 W, 6 OS); leaves from untreated plants severed as Con. Venn diagrams indicate the numbers of proteins up-regulated by (a) 1.5 W and 1.5 OS and (b) 6 W and 6 OS. (c) Overall up-regulated protein numbers. (d) Venn diagram of the numbers of proteins further induced (FI) 1.5 and 6 h after W + OS treatment compared with W + W treatment. (e) Relative protein levels (fold change after log2 transformation) of the 60 most up-regulated genes at 1.5 and 6 h (30 proteins for each time point) induced by W + OS treatment (detailed descriptions of these proteins can be found in Supporting Information Table S7; NA = not annotated; *: name abbreviated because of length).

The top 60 proteins up-regulated by W + OS treatment (30 each after 1.5 and 6 h treatment) were chosen to show their average expression levels by log2 transformations relative to Con (Fig. 3e). These included NADPH DEHYDROGENASE (GRMZM2G149414), CALCIUM-DEPENDENT PROTEIN KINASE (GRMZM2G112057), NAD-MALATE DEHYDROGENASE (GRMZM2G161245), ALLENE OXIDE SYNTHASE (GRMZM2G033098), LIPOXYGENASE (GRMZM2G109130, GRMZM2G040095), INDOLE SYNTHASE (GRMZM2G085381), TERPENE SYNTHASE (GRMZM2G085381, GRMZM2G046615) and TOUCH-INDUCED CALMODULIN-RELATED PROTEIN (GRMZM2G097900) (Fig. 3e, Supporting Information Table S7), which are involved in JA signalling, calcium signalling, herbivory-induced plant volatile biosynthesis and redox regulation pathways.

We further analysed the down-regulated proteins applying the standard that the ratios of protein levels between Con and treatment groups be at least 1.2 and found that 8, 75, 9 and 12 proteins were down-regulated by 1.5 W, 1.5 OS and 6 W, and 6 OS, respectively (Fig. 4a,b; Supporting Information Table S7), and only 1 protein was commonly regulated in all samples (Fig. 4c). There were fewer down-regulated than up-regulated proteins at both times in the same samples. Similar

to the pattern among up-regulated proteins, W+OS-treated samples had a larger number of down-regulated proteins than did W+W-treated samples. The top 20 (for samples having less than 20 specifically regulated proteins, all proteins were included) specifically down-regulated proteins by W + W and W + OS 1.5 and 6 h after treatment are listed in Supporting Information Table S9. Proteins whose levels after W + W treatment were further repressed by OS were found to be 45 and 1 proteins, 1.5 and 6 h after treatments, respectively, and 1 protein was shared in both times (Fig. 4d, Supporting Information Table S7).

The top 60 down-regulated proteins by 1.5 OS and 6 OS were also analysed, and these included GLUTATHIONE-TRANSFERASE (GRMZM2G028821), ATP-DEPENDENT CLP PROTEASE PROTEOLYTIC SUBUNIT (GRMZM2G056373), PHOTOSYSTEM I REACTION CENTER SUBUNIT V (GRMZM2G329047, GRMZM2G377855) and 50S RIBOSOMAL PROTEIN (GRMZM2G162369) (Fig. 4e, Supporting Information Table S7). These proteins are related to growth regulation rather than defence, supporting the idea that maize regulates signalling cascades favouring defence over growth in response to *M. separata* infestation.

The correlation between transcriptome and proteome

Among the 4406 regulated genes, 4142 showed no corresponding proteins in the proteome data, probably because of the

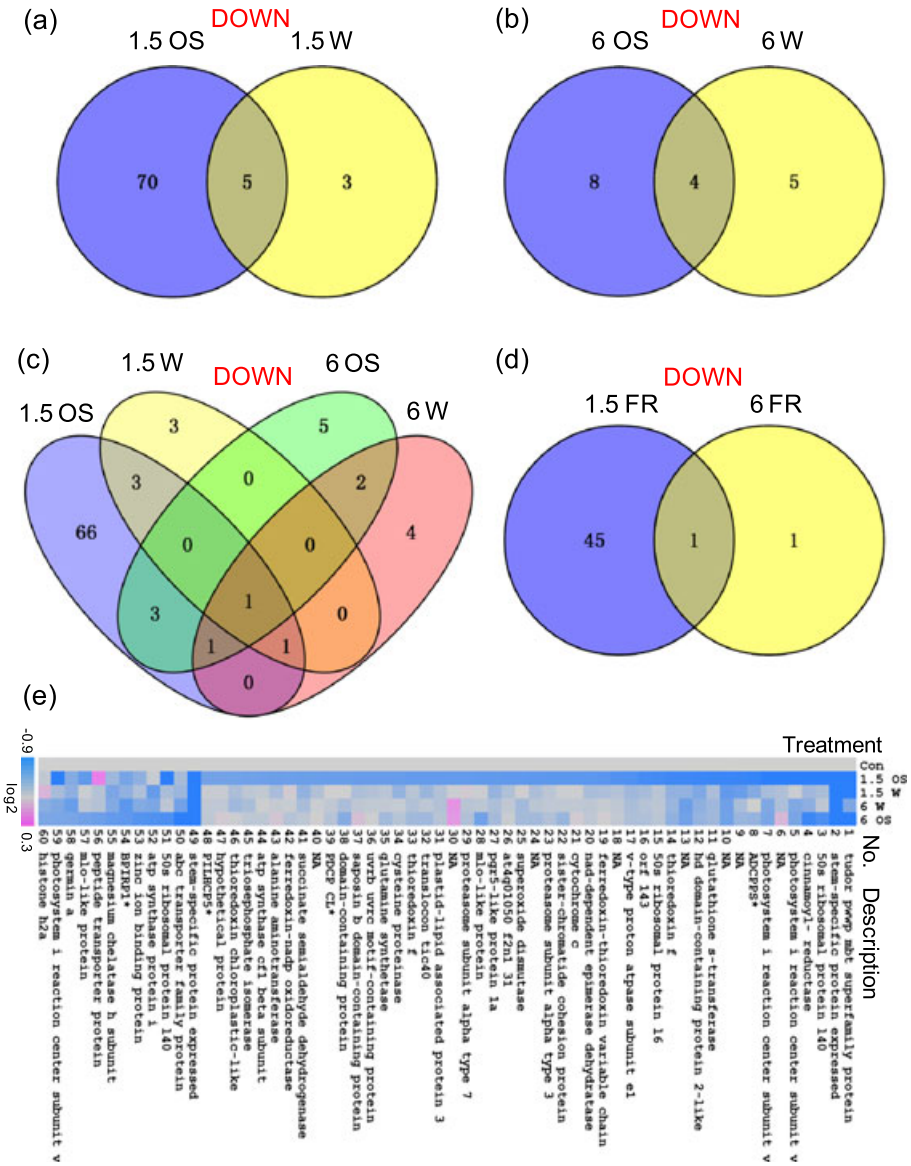

Figure 4. Down-regulated proteins in response to W + W and W + OS treatment. Maize leaves were treated with W + W or W + OS, and samples were collected at 1.5 and 6 h (for simplicity, named 1.5 W, 1.5 OS and 6 W, 6 OS); leaves from untreated plants severed as Con. Venn diagram of the numbers of proteins down-regulated by (a) 1.5 W and 1.5 OS and (b) 6 W and 6 OS. (c) Overall, down-regulated protein numbers. (d) Venn diagram of the numbers of proteins further repressed (FR) 1.5 and 6 h after W + OS treatment compared with W + W treatment. (e) Relative protein levels (fold change after log2 transformation) of the 60 most down-regulated genes at 1.5 and 6 h (49 proteins for 1.5 h and 11 proteins for 6 h) by W + OS treatment (detailed descriptions of these proteins can be found in Supporting Information Table S7; NA = not annotated; *: name abbreviated because of length).

relatively low sensitivity of proteome detection, and 200 regulated genes' protein products showed no changes (Fig. 5a). In the proteome data, 1757 proteins were found to have no changes, and these proteins consistently had unaltered transcript levels (Fig. 5a). Among the differentially regulated 294 proteins, most (214 proteins) had no changes of transcript levels; the transcripts of 64 regulated proteins were also regulated, and 16 proteins did not have corresponding transcripts in the transcriptome (Fig. 5a).

Within the 200 regulated transcripts whose protein levels were unchanged, we found that more genes showed increased levels than those down-regulated after any treatment, except that 1.5 OS treatment induced more down-regulated genes than up-regulated ones (Fig. 5b, Supporting Information Table S10). In detail, the majority of the down-regulation events happened after 1.5 W and 1.5 OS: 29 genes were down-regulated by both 1.5 W and 1.5 OS treatments, such as GLYCOLATE OXIDASE (GRMZM2G129246); 49 genes were specifically down-regulated by 1.5 OS treatment, such as SUCROSE SYNTHASE (GRMZM2G152908), and 6 OS treatment only specifically down-regulated 9 genes (Supporting Information Table S10). The up-regulated genes were evenly distributed between two time points and 14 of them were up-regulated by all treatments, including ALLENE OXIDE CYCLASE (GRMZM2G334181), a JA biosynthetic gene. Twenty-two genes were specifically up-regulated by 6 OS, and none by 6 W treatment (Supporting Information Table S10).

The 214 regulated proteins whose transcripts were unaltered were categorized according to their increased or decreased levels and treatments (Fig. 5c). It was found that samples treated with 1.5 OS showed the largest number of regulated proteins (Fig. 5c), including the upregulated NADH DEHYDROGENASE (GRMZM2G149414) and ASCORBATE PEROXIDASE (GRMZM2G014397), and the down-regulated 50S RIBOSOMAL PROTEIN (GRMZM2G162369, GRMZM2G170870) and SUPEROXIDE DISMUTASE (GRMZM2G124455) (Supporting Information Table S10). It is likely that changes in these protein levels are regulated on a post-transcriptional level.

We further examined the 64 IDs, which exhibited changes at both transcript and protein levels (Fig. 5a). Among the IDs whose transcripts level were up-regulated, the majority of their corresponding protein levels were also up-regulated, and W + OS treatment induced more proteins than did W + W treatment (Fig. 5d), such as ALLENE OXIDE SYNTHASE (GRMZM2G033098, GRMZM2G067225), 12-OXOPHYTODIENOIC ACID REDUCTASE (GRMZM2G000236), TERPENE SYNTHASE (GRMZM2G046615) and LIPOXYGENASE (GRMZM2G109130) (Supporting Information Table S10); 5 IDs showed decreased protein levels, such as CYTOCHROME C (GRMZM2G070199) (Fig. 5d, Supporting Information Table S10). Briefly, those 64 IDs can be sorted into two groups: (1) 55 IDs showed consistent regulation patterns and 9 IDs showed opposite regulation patterns (Supporting Information Table S10). Among the 55 consistent IDs, 48 were up-regulated by at least one treatment at both transcript and protein levels (Supporting Information Table S10), which indicated up-regulation rather than down-

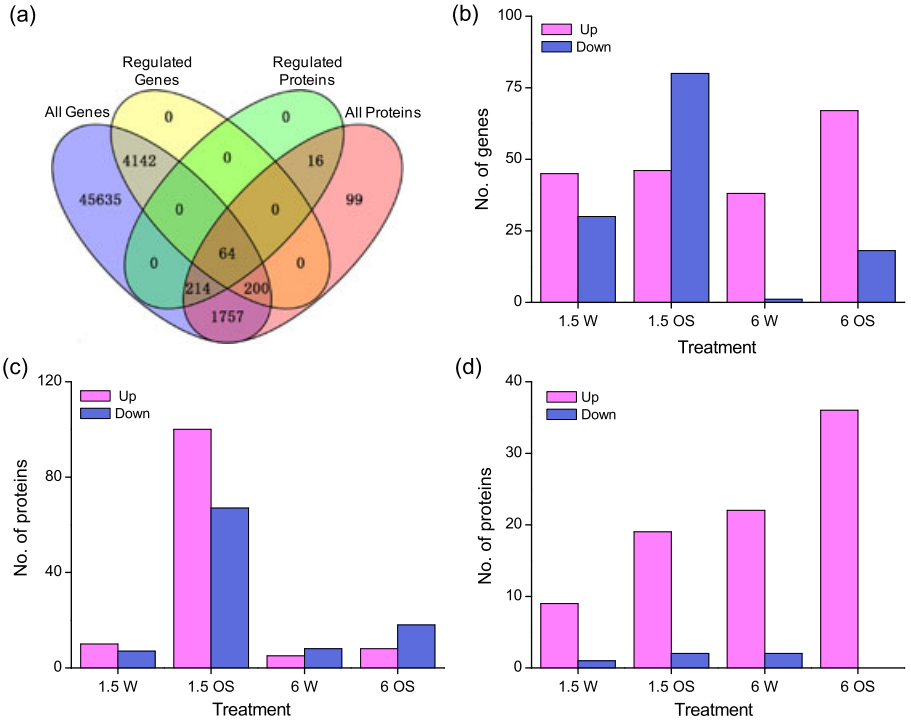

Figure 5. Correlations between of proteins and transcripts. (a) Venn diagram of the numbers of all detected genes, regulated genes, all detected proteins and regulated proteins. Numbers of up- and down-regulated genes having no changes in their protein levels (b), numbers of regulated proteins, whose transcript levels were unchanged after indicated treatments (c), and numbers of proteins up- or down-regulated, whose transcript levels increased after indicated treatments (d).

regulation is the major response of maize to defence against *M. separata.*

Phytohormones induced by W + W and W + OS treatment

Phytohormones play a key role in orchestrating plant resistance against insects (Stam *et al.* 2013). Thus, we analysed the levels of JA, JA-Ile (JA-isoleucine conjugate), SA, ABA and ET induced by W + W and W + OS in samples collected at different times after treatments (five biological replicates for each group).

The levels of JA and JA-Ile were rapidly up-regulated by both W + W and W + OS, and the peak values induced by W + OS were more than onefold greater than those induced by W + W (Fig. 6a,b). Notably, SA levels were highly up-regulated by W + OS treatment (4.4-fold increased 0.5 h after W + OS); in contrast, SA levels in the W + W-treated maize leaves increased less than onefold (Fig. 6c). W + OS treatment also had a longer-lasting, and stronger effect on ABA levels: ABA contents were more than 2 times higher in W + OS-treated samples than in W + W-treated samples, at 1.5 h (Fig. 6d). W + W treatment did not induce increase of ET levels, while W + OS-treated maize leaves emitted almost 4 times more ET than did Con and W + W samples (Fig. 6e). Therefore, *M. separata* herbivory strongly alters the levels of stress-related phytohormones.

We further analysed the genes that are important for the biosynthesis of previously mentioned phytohormones. In total, we found 29 related genes to be differently expressed between different samples (Fig. 6f, Supporting Information Table S10, except for *LOX10* and *OPR8*). Genes involved in JA (*LOX*, *AOS*, *AOC*, and *OPR*), SA (*PAL*), ET (*ACS*) and ABA (*NCED*, *AO*, and *SDR*) biosynthesis (Fig. 6f, Supporting Information Table S10) were up-regulated by W + W and/or W + OS treatment. Among these *OPR7* (GRMZM2G148281) and *LOX8*/TASSEL SEED 1 (GRMZM2G104843) (Fig. 6f) have been confirmed to be important for JA biosynthesis (Yan *et al.* 2012; Christensen *et al.* 2013); consistent with the expression profile of *OPR8* (GRMZM2G082087; another OPR gene important for JA biosynthesis; Yan *et al.* 2012) in maize B73, *OPR8* did not show significant changes after any treatment (Fig. 6f, Supporting Information Table S10); we also did not detect changes of *LOX10*, which is critical for the production of herbivory-induced plant volatiles (Christensen *et al.* 2013). *ICS* (GRMZM2G022837), a SA biosynthesis-related gene, was reduced. This implies that the *PAL* pathway may play a more important role in *M. separata* herbivory-induced SA biosynthesis than does the *ICS* pathway. Proteins related to the biosynthesis of the detected phytohormones were identified in the proteome data, and five which are responsible for JA biosynthesis (LOX: GRMZM2G109130, GRMZM2G040095, AOS: GRMZM2G067225, GRMZM2G067225 and OPR: GRMZM2G000236) were found to be up-regulated after W + W or W + OS (Fig. 6f, Supporting Information Table S10). Notably, JA levels remained high at 6 h, while JA-Ile highly decreased. Thus, JAR, the enzyme for JA-Ile conjugation from JA and Ile, was examined in the transcriptomic and proteomics data. Blast analysis indicated that GRMZM2G091276 had the highest similarity the Arabidopsis *JAR1* (Staswick & Tiryaki 2004). This putative maize *JAR* gene expression elevated 1.1, 3.3, 3.3 and 4 times after 1.5 W, 1.5 OS and 6 W and 6 OS, respectively (Supporting Information Table S2). Therefore, it is likely the discrepancy between the pattern of JA and JA-Ile dynamics was resulted from increased activity of JA-Ile catabolism enzymes (Koo *et al.* 2011; Widemann *et al.* 2013), instead of reduced JAR activity.

Changes in metabolites

Specialized metabolites are generally the active chemicals mediating resistance to herbivores. Hence, we analysed the changes in various non-volatile metabolites 48 h after W + W and W + OS treatment, using an UPLC-Q-TOF MS system (five biological replicates for each group). The detected metabolites belong to four major groups, which are amino acids, Bxs, phenolics and flavonoids and lipids (Supporting Information Table S11).

Consistent with the transcriptome and metabolome data, W + OS treatment showed a stronger effect on secondary metabolites than did W + W: W + W did not specifically induce or repress any of these secondary metabolites, while W + OS treatment specifically induced seven and repressed eight metabolites (Fig. 7a,b; Supporting Information Table S11). The concentrations of amino acids including valine, threonine, leucine, phenylalanine, tyrosine and arginine were elevated by W + OS treatment; among these, valine, threonine and phenylalanine increased 2.8-, 3.0- and 3.4-fold, respectively, and the rest increased less than 1.3-fold (Supporting Information Fig. S2a, Table S11). The amino acid most induced by W + OS was phenylalanine, the precursor of SA, and this was consistent with the SA levels induced by W + OS and supports that the PAL pathway might be more important than is the ICS pathway for herbivory-induced SA production (Supporting Information Fig. S2a, Fig. 6c). In contrast, the levels of serine and glutamic acid were suppressed 40% and 38% by W + OS treatment (Supporting Information Fig. S2a, Table S11).

The concentrations of most phenolics and flavonoids changed less than onefold after W + W or W + OS treatment, except for neochlorogenic acid which was strongly induced by both (Supporting Information Fig. S2a, Table S11). Most lipids did not respond to either treatment (Supporting Information Table S11), and the most induced and repressed by W + OS treatments were MGDG16:4/18:2 (monogalactosyldiacylglycerols; 6.7-fold increase) and sulfoquinovosyl diacylglycerol (82% decreased), respectively (Supporting Information Fig. S2b, Table S11). Notably, linolenic acid, the precursor of JA, increased 43% by W + OS, and 36% by W + W (Supporting Information Fig. S2b, Table S11). Given that these samples were harvested 48 h after treatment, we conclude that *M. separata* feeding has a long-term impact on membrane lipid composition.

Bxs are thought to be among the most important anti-herbivore compounds in maize found so far and are up-regulated by feeding of different herbivore species (Glauser

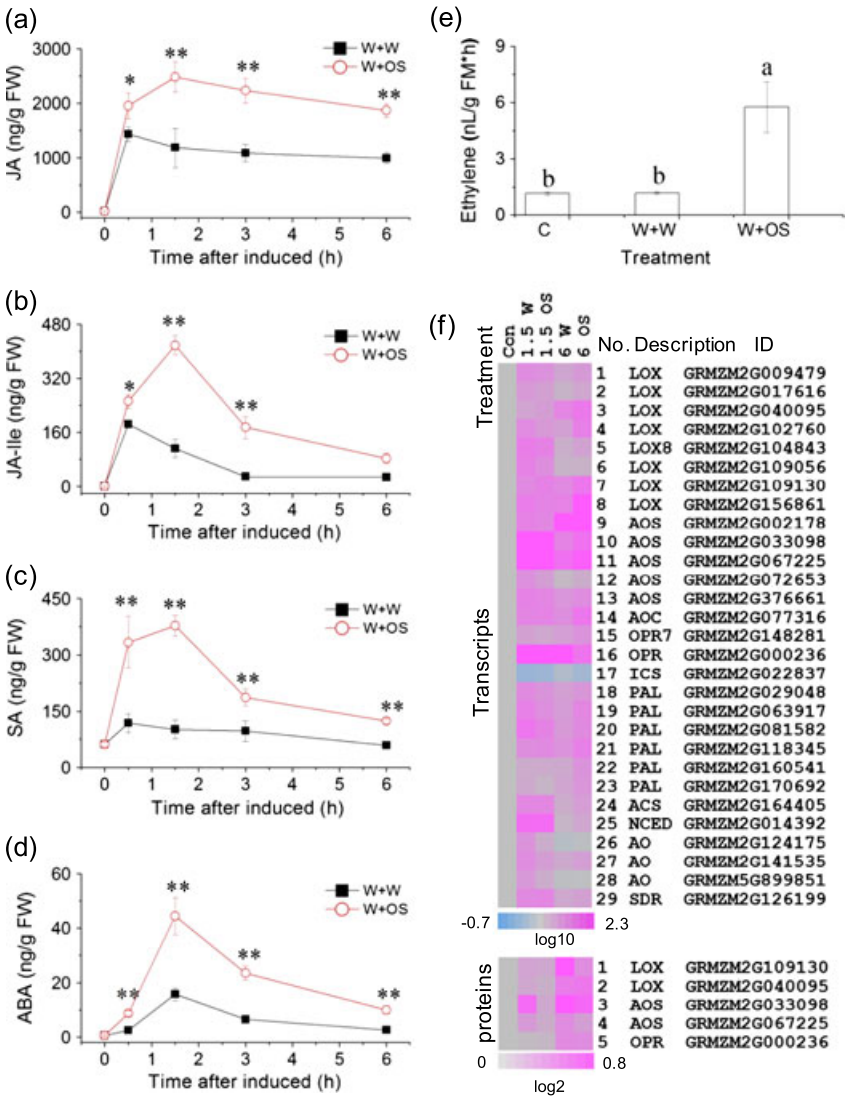

Figure 6. Changes of phytohormones and phytohormone biosynthesis-related gene expressions and proteins. Mean levels (± SE, $n = 5$) of JA (a), JA-Ile (b), SA (c), ABA (d) and ethylene (e) induced by W + W and W + OS treatment (Con = control). Asterisks represent significant differences between the levels in samples treated with W + OS and W + W at the indicated times (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$). 'a' and 'b' indicate significant differences of ET levels (one-way ANOVA, Duncan's multiple range test). (F) Relative levels (fold change after log10 transformation) of hormone biosynthesis-related genes for JA (*LOX*, *AOS*, *AOC* and *OPR*), SA (*ICS* and *PAL*), ET (*ACS*) and ABA (*NCED*, *AO* and *SDR*) and levels (fold change after log2 transformation) of JA biosynthesis-related proteins LOX, AOS, and OPR detected in the transcriptome and proteome data (detailed descriptions of these genes and proteins can be found in Supporting Information Table S10).

et al. 2011). Some Bxs have identical molecular masses (HMBOA and M_2BOA, for example), and these compounds were not distinguished. Compared with W + W treatment, W + OS treatment showed a stronger effect on Bxs (Fig. 7c): the most up-regulated compound was HMBOA/M_2BOA-1, with a 38.1-fold increase after W + W and 86.9-fold after W + OS treatment, and the most down-regulated was DIBOA/DHBOA with an ~65% decrease after either treatment (Fig. 7c, Supporting Information Table S11). All the detected Bx biosynthesis-related genes were up-regulated by W + W and W + OS with *Bx10* showing the highest induction level (130.4- and 504.5-fold, respectively, at 6 h), and most of the Bx biosynthesis-related genes were also up-regulated 6 h after treatment (Fig. 7d). Proteins in the Bx biosynthesis pathway, BX1, BX4 and BX5 were also up-regulated 6 h after W + W and W + OS treatment, but with only 26–45% increases (Fig. 7d).

Herbivory often induces volatile emissions from plants, and these volatile compounds can function as indirect defenses by attracting herbivore predators or parasites (Dicke 2009). Thus, headspace samples were taken from maize leaves 0–0.5, 0.5–8 and 24–32 h after a morning treatment with W + OS, W + W, or no treatment (Con). Most differences among treatment groups were observed in the 0.5–8 h samples (Supporting Information Table S12): in particular, headspace samples from W + OS-treated leaves contained significantly higher amounts of linalool, benzyl acetate, phenylethyl acetate(putative), geranyl acetate, cubedol (putative), an unidentified putative

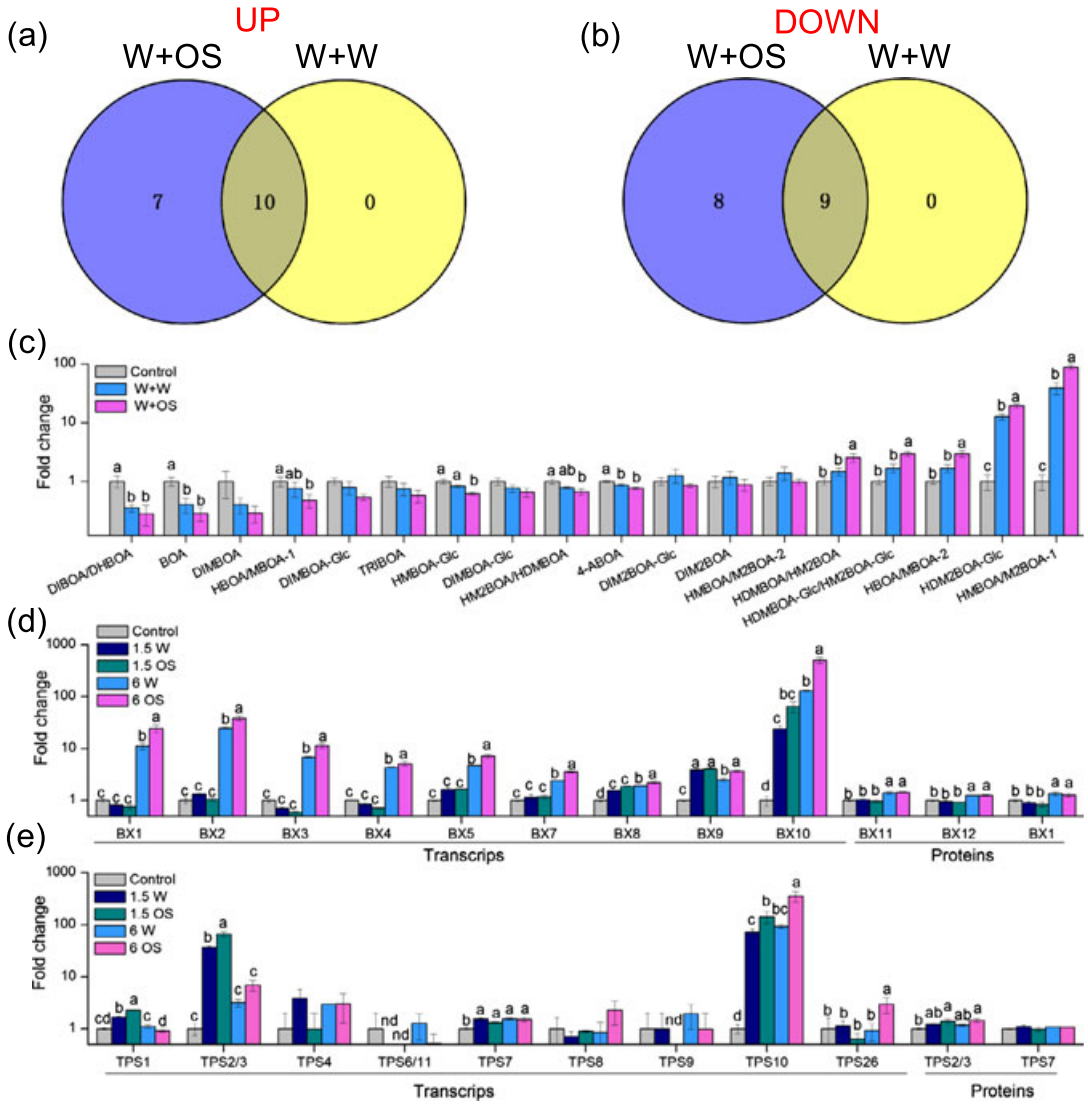

Figure 7. Changes of maize metabolites and Bx and terpene biosynthesis-related genes and proteins after W + W and W + OS treatments. Maize leaves were treated with W + W or W + OS, and samples were collected at 48 h; non-treated leaves severed as Con ($n = 5$); Venn diagram of the number of induced (a) or repressed (b) non-volatile metabolites; (c) relative changes of Bxs ($n = 5$); transcript (average ± SE; n = 3) and protein (average ± SE; $n = 3$) levels of the Bx biosynthesis-related (d) or terpene biosynthesis-related (e) genes or proteins in W + W- and W + OS -treated samples (Con levels in plot c-e were normalized to 1, and the data were retrieved from the metabolites, transcriptome and proteome data, respectively. Note that the *y*-axis has been log10 transformed. Bxs having the same molecular masses and cannot be distinguished by MS are indicated by suffixes '−1' and '−2' after the names of the two Bxs separated by a slash). Letters indicate significant differences between different treatment groups (one-way ANOVA and Duncan's multiple range test). Detail IDs of Bx biosynthesis-related and TPS biosynthesis-related genes can be found in Supporting Information Table S10.

sesquiterpene and (*3E*,*7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene than either W + W-treated or Con samples. Linalool was also more abundant in W + W-treated samples than in Con samples. Thus, all plant volatiles distinguishing W + OS-treated samples from other samples were detected over the longer collection time during the first photoperiod after treatment.

In maize, TPS1, TPS23, TPS2/3 and TPS10 are important for herbivory-induced production of terpenes (Schnee *et al.* 2002; Schnee *et al.* 2006; Kollner *et al.* 2008; Tzin *et al.* 2015) . Thus, we analysed all the maize genome-annotated *TPS*s (Fig. 7e) in the transcriptomic data. Among these, TPS2/3 and TPS10 were all highly induced by W + W and W + OS, and W + OS induced greater levels than did W + W either at 1.5 or 6 h. Both TPS2/3 and TPS7 protein were detected in the proteomic data, and TPS2 showed significantly increased levels (44% increase) after W + OS treatment at 6 h (Fig. 7e).

Transcription factor genes involved in W + W- and W + OS-induced responses

Given the important regulatory function of TFs, maize transcripts were searched against the Plant Transcription Factor

Database (http://planttfdb.cbi.pku.edu.cn/), and we found that 289 TFs distributed in 39 families showed altered expression levels after W + W and/or W + OS treatment (Supporting Information Fig. S3a, Table S13). ERFs (ethylene responsive element binding factors) were the most enriched TFs, which made up 20.3% of all differently expressed TFs and 26.7% of the top 60 most up-regulated TFs (Supporting Information Table S13), and together with bHLH (basic helix-loop-helix, 11.1%), MYB (9.2%) and WRKY (8.8%), these TFs were 49.3% of all the differently expressed TFs (Supporting Information Fig. S3a, Table S13).

Although a relatively large number of TFs were commonly induced or repressed by W + W and W + OS treatment, W + OS influenced more TFs than did W + W: 1.5 OS, 1.5 W, 6 OS and 6 W treatment specifically up-regulated 30, 14, 35 and 0 TFs and down-regulated 16, 3, 8 and 0 TFs, respectively (Fig. 8a–d, Supporting Information Table S13). Compared with W + W, at 1.5 and 6 h, W + OS FI 7 and 50 TFs and further repressed 12 and 1 TFs specifically (Supporting Information Fig. S3, Table S13). The top 60 up-regulated TFs induced by 1.5 OS and 6 OS (30 each) treatment were chosen to show their average expression levels by log10 transformations relative to their Con levels (Fig. 8e), and the highest reached 217-fold induction (ERF, GRMZM2G544539). Only 31 and 9 TFs were down-regulated 1.5 and 6 h after W + OS treatment, respectively (Fig. 8c,d,f), and a TALE-type TF (GRMZM2G076272) was the most strongly repressed (96.2%). We also searched the proteome data for regulated TFs, and only 1 TF protein was found (KNOTTED-LIKE TRANSCRIPTION FACTOR, GRMZM2G370332), whose protein levels were only up-

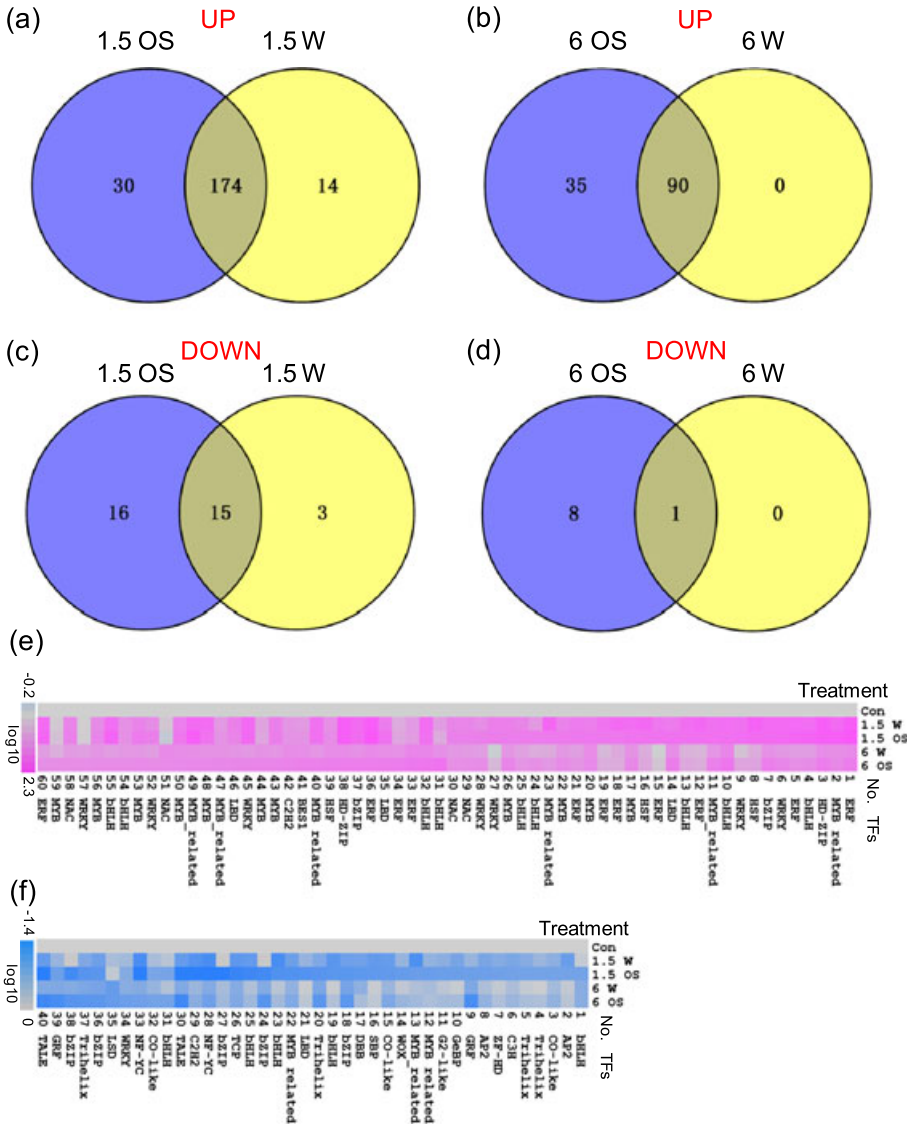

Figure 8. Responses of maize transcription factors to wounding and simulated *M. separata* feeding. Maize leaves were treated with W + W or W + OS, and samples were collected at 1.5 and 6 h (named 1.5 W, 1.5 OS and 6 W, 6 OS); non-treated leaves severed as Con. Venn diagram of the numbers of up-regulated TF genes at 1.5 h (a) and 6 h (b), and the numbers of down-regulated TF genes at 1.5 (c) and 6 h (d). (E, F) Relative expression levels (log10 transformed) of the 60 most up-regulated TFs (30 genes at each time point) (e) and all the down-regulated TFs (F) at 1.5 and 6 h after W + OS treatment. Detailed descriptions of these TFs can be found in Supporting Information Table S12).

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regulated 33% by 1.5 OS treatment (Supporting Information Table S7).

Recently, an AP2/ERF type TF, *EREB58* (GRMZM 2G381441), was shown to bind to the promoter of *TPS10* and mediate jasmonate-induced biosynthesis of sesquiterpenes (Li *et al*. 2015). We found that *EREB58* was specifically induced by W + OS (~7-fold increase at 1.5 or 6 h), but not W + W (Supporting Information Table S1).

DISCUSSION

Using multi-dimensional omics approaches, we revealed the defence responses of maize to a specialist insect *M. separata* and found that compared with mechanical wounding, simulated *M. separata* feeding more strongly and specifically activates maize defence reactions, highlighting the critical role of OS in the interface of plant–insect interactions. Because of the high cost and the intention of focusing on relatively early responses, transcriptomic and proteomic response at 1.5 and 6 h and metabolomic changes after 2 days were analysed. Samples collected in a long timeframe and short intervals will provide more detailed information for understanding the changes and regulation of maize physiology in response to insect herbivory.

Our analyses indicate a general pattern that compared with mechanical wounding, simulated *M. separata* herbivory resulted in (1) greater changes on phytohormonal, transcriptomic, proteomic and metabolomic level and (2) many more up-regulated genes, proteins and metabolites than those down-regulated. Thus, *M. separata* OS induce a stronger and longer-lasting transcriptome and proteome rearrangement than does mechanical wounding, and in turn, maize highly increases its defensive metabolites. Furthermore, after maize perceives chewing insect herbivory, the significant changes in the regulatory network mainly occur in a rapid fashion, including transcriptome, proteome and phytohormones.

We propose that FACs play a critical role in activating *M. separata* OS-induced responses on transcriptomic, proteomic and metabolomic levels, although other components, such as proteins in the OS, cannot be ruled out to have a synergistic or antagonistic effect (Diezel *et al*. 2009; Erb *et al*. 2015). Schmelz *et al*. (2009) applied different insect-derived elicitors to maize wounds and found that compared with mechanical wounding, applying FACs (volicitin and *N*-linolenoyl-Gln) to maize wounds induced greater levels of ET and JA, but not SA. In contrast, *M. separata* OS elicited at least onefold higher concentrations of JA, JA-Ile, SA, ABA and ET, than did wounding (Fig. 6a–e), indicating that not only FACs but also other components in the *M. separata* OS are responsible for the induced responses. Similarly, beat armyworm (*Spodoptera exigua*) feeding on maize inhibited the expression of some volicitin-induced genes, suggesting certain components also function as elicitors (Lawrence & Novak 2004). Therefore, multiple types of receptors might exist in maize, and they are responsible for perceiving insect-produced elicitors, such as FACs and yet-to-be-identified proteins or other molecules (Fig. 9). Moreover, although relatively low, GOX activity was detected in the OS; it is possible that the H_2O_2 produced by GOX was also involved in eliciting OS-induced responses as a signalling molecule (Fig. 9).

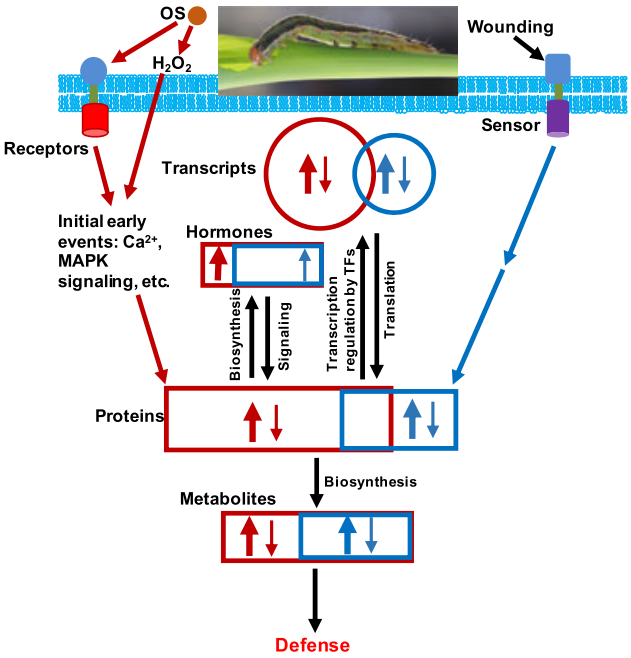

Figure 9. A working model summarizing the responses of maize to *M. separata* feeding. The components of the OS of *M. separata* are perceived by unknown receptors, and H_2O_2 is produced by GOX activity in the OS. In turn, initial early signalling events, such as Ca^{2+} influxes and MAPK activation are triggered, leading to the earliest proteome reconfigurations, including activation of certain transcription factors (TFs) and protein activity changes resulted from posttranslational modifications. Changes in the levels of transcripts and proteins follow rapidly, resulting in reconfiguration of the transcriptome, proteome and phytohormone levels. The crosstalk among transcriptome, proteome, and phytohormones continue to reshape each other and finally lead to augmented levels of defence metabolite biosynthesis enzymes and in turn accumulation of defence metabolites, such as Bxs and terpenes, counteracting *M. separata* feeding. Although mechanical wounding generates similar responses, it generally induces much less changes on the transcriptomic, proteomic, metabolomic and phytohormone levels. Red and blue arrows, circles and rectangles represent OS- and wounding-elicited responses, respectively. Up and down arrows in the rectangles or circles indicate up-regulation or down-regulation, and the thickness of these arrows represent the number of genes, proteins, metabolites or hormones.

It is likely that the earliest responses in maize include Ca^{2+} influxes and activation of MAPK signalling (Maffei *et al*. 2004; Wu *et al*. 2007), and these are induced within minutes after elicitations by OS or wounding (Fig. 9); thereafter, certain TFs are activated by post-translational modifications, such as phosphorylation by MAPKs (Mao *et al*. 2011; Meng *et al*. 2013), and rapidly initiate transcriptional regulation of downstream targets (Kim & Zhang 2004), including TF genes, resulting in further changes in maize transcriptome. Alternation in transcriptome and post-translational regulation of proteins in turn reshape maize proteome. Importantly, increase in the activity of JA, JA-Ile, SA, ABA and ET biosynthesis enzymes lead to accumulation of these phytohormones, and activation of their signalling pathways (Fig. 9).

JA, SA, ET and ABA signalling are important in regulating defence against insects (Paschold *et al.* 2007; Koornneef *et al.* 2008; Vos *et al.* 2013). The function of JA in dicotyledonous plants, such as wild tobacco (*N. attenuata*), Arabidopsis and tomato, has been intensively studied (Wu & Baldwin 2010), and much evidence has indicated that JA is also critical for the resistance of rice and maize to insects (Qi *et al.* 2011; Christensen *et al.* 2013). The highly induced ABA contents after simulated *M. separata* herbivory are particularly intriguing: although wounds may cause slight leaf dehydration and in turn increased ABA levels, *M. separata* OS seems to activate a signalling pathway which leads to augmented ABA levels. The function of ABA in plant resistance to insects has been demonstrated in tomato (Thaler & Bostock 2004) and Arabidopsis (Vos *et al.* 2013), and in rice, applying ABA to roots did not change the performance of two root herbivores – the generalist cucumber beetle (*Diabrotica balteata*) and the more specialized rice water weevil (*Lissorhoptrus oryzophilus*) (Lu *et al.* 2015). The function of SA and ABA in the defence of maize against insects deserves further in-depth studies. Moreover, we found that the highest W + OS-induced gene was an *INDOLE-3-ACETIC ACID-AMIDO SYNTHETASE* (*IAA-AS*), which putatively belongs to the GH3 gene family. Maize infected by European corn borer (*Ostrinia nubilalis*) also show highly increased IAA-AS levels (Dafoe *et al.* 2013). In rice, bacterial infection increases IAA (indole-3-acetic acid) content, and *GH3-8* (an *IAA-AS*) was found to conjugate IAA with amino acids and thus influence IAA homeostasis and immunity to bacteria (Ding *et al.* 2008). An *CYTOKININ-O-GLUCOSYLTRANSFERASE 1* was highly and specifically suppressed by W + OS at 1.5 h (Fig. 2e), and this gene might be involved in conjugating cytokinins with glucose (Veach *et al.* 2003). Strong down-regulation of this gene implies that cytokinin levels might increase during *M. separata* feeding, and this is consistent with the finding that in the wild tobacco *Nicotiana attenuata*, simulated *Manduca sexta* feeding increased cytokinin concentrations (Schafer *et al.* 2015). Whether IAA and cytokinin levels are affected by insect herbivory and the function of these hormones in maize resistance to insects deserve further study.

Compared with the transcriptomic changes, much less detected proteins showed up-regulation or down-regulation after either W + OS or W + W treatment, and the correlation between proteome and transcriptome was poor. This is consistent with several other studies in which transcriptomes and proteomes did not match well (Taniguchi *et al.* 2010; Vogel & Marcotte 2012; Walley *et al.* 2013). For example, in developing maize seeds, Walley *et al.* (2013) found poor associations between proteomes and transcriptomes, and three possible explanations were proposed: (1) transport of proteins between tissues; (2) diurnal, out-of-phase accumulation of mRNAs and cognate proteins; and (3) differential lifetimes of mRNAs compared with proteins. It is very likely that these possibilities also account for the discrepancies of our transcriptomic and proteomic data.

We found that *M. separata* OS strongly induced HDMBOA/HM2BOA, HDMBOA-Glc/HM2BOA-Glc, HBOA/MBOA-2, HDM2BOA-Glc and HMBOA/M2BOA-1. Among these, HDMBOA has been shown to be toxic to both *S. littoralis* and *S. frugiperda* (Glauser *et al.* 2011). *S. littoralis* feeding induces HDM_2BOA-Glc and HDMBOA-Glc (Glauser *et al.* 2011), and similarly, simulated *M. separata* herbivory also induced these two Bxs; however, DIMBOA-Glc and HMBOA-Glc were specifically repressed by *S. littoralis* feeding and *M. separata* OS, respectively (Marti *et al.* 2013) (Fig. 7c). It is likely that maize is able to recognize different OS and in turn produces herbivore species-specific defensive metabolites (Glauser *et al.* 2011).

Maize *TPS2/3* are induced by aphid feeding, and mutant analysis indicated that they are involved in producing 6 terpenes and are important for maize defence against aphids (Tzin *et al.* 2015), and *TPS2/3* were also found to be up-regulated in our W+OS-induced maize transcriptome data. *TPS10*, which catalyses the production of (*E*)-*α*-bergamotene and (*E*)-*β*-farnesene (Schnee *et al.* 2006; Kollner *et al.* 2009), was up-regulated more than 300 times 6 h after W + OS treatment (Fig. 7e). However, none of the 8 terpenes catalysed by *TPS2/3* and *TPS10* were detected. and whether this was because of limitations of our volatile detection system or cultivar-specific differences and if *TPS2/3* and *TPS10* also have a function in maize defence against *M. separata* should be examined. Moreover, maize terpene synthases, *TPS1* and *TPS23* (GRMZM2G127336), catalyse the formation of certain terpenes that are involved in maize indirect defence against herbivores; however, *TPS1* and *TPS23* are induced by lepidopteran caterpillar feeding in certain cultivars but not in others (Schnee *et al.* 2002; Schnee *et al.* 2006; Kollner *et al.* 2008). In our maize line, A188, *TPS1* was not induced after wounding or simulated *M. separata* feeding and *TPS23* showed very low expression level and was detected only in 1 sample among the 15 samples (Supporting Information Table S1), corroborating the diversity of volatile productions in maize varieties.

Thus far, very little is known about the regulation of Bx enzymes and TPSs in maize, although many of the biosynthetic genes have been cloned (Niemeyer 2009). *EREB58*, is important for JA-induced elevation of *TPS10* transcripts (Li *et al.* 2015). Given that wounding and insect feeding induce JA signalling, *EREB58* gene was expected to be upregulated after both treatments. However, either because W + W-induced JA levels were not as high as those induced by W + OS, or certain factors induced by W + W suppressed its expression, only W + OS, but not W + W treatment, induced *EREB58*. In contrast, the target gene of *EREB58*, *TPS10*, was also highly induced by wounding treatment, suggesting that other TFs that are specifically downstream of W + W regulate the transcript level of *TPS10*. The function of *EREB58* in regulation of wounding- and insect feeding-induced *TPS10* and terpene production should be studied further. Transcriptome analysis revealed 289 TFs that were up-regulated or down-regulated, and these are potential transcriptional regulators of Bx or terpene biosynthesis. Genetic studies using maize varieties with variations in Bx or terpene levels and mutant/RNAi lines of these TFs will shed light on the regulatory mechanism of the biosynthesis and degradation of these important defensive metabolites.

Our multi-dimensional analysis provides large-scale datasets that reveal the physiological responses of maize against *M. separata* herbivory on multiple dimensions and highlights the

critical role of recognition of OS in maize resistance to chewing insects (Fig. 9). These data provide a framework for further genetic studies on maize resistance to insects, including genome-wide association studies on diverse cultivars, and also lay the groundwork for breeding new maize varieties with greater resistance to insect herbivores.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Compositions of the oral secretions (OS) from *M. separata*.
Figure S2. Relative concentrations of other non-volatile metabolites induced by W + W or W + OS treatment.
Figure S3. Profile of the maize transcriptional factors that transcriptionally responded to W + OS and W + W treatment.
Table S1. Genes detected in all samples.
Table S2. All differentially expressed genes with a cutoff of 4 times change in relative to control levels.
Table S3. All genes up-regulated by W + W or W + OS treatment and genes further induced by W + OS treatment than by W + W treatment.
Table S4. List of the 20 most up-regulated genes specifically induced by W + W and W + OS treatment at 1.5 and 6 h.
Table S5. All genes down-regulated by W + W or W + OS treatment, and genes further repressed by W + OS treatment than by W + W treatment.
Table S6. List of the 20 most down-regulated genes specifically repressed by W + W and W + OS treatment at 1.5 and 6 h.
Table S7. All identified proteins, the up-regulated or down-regulated proteins by W + W or W + OS treatment, and proteins further induced or repressed by W + OS than W + W treatment.
Table S8. List of the 20 (or all, if less than 20) most up-regulated proteins specifically induced by W + W and W + OS at 1.5 and 6 h.
Table S9. List of the 20 (or all, if less than 20) most down-regulated proteins specifically repressed by W + W and W + OS at 1.5 and 6 h
Table S10. The levels of regulated transcripts and proteins and their corresponding proteins and transcripts.
Table S11. Non-volatile metabolites induced by W + W and W + OS treatment.
Table S12. Volatiles identified in the headspace of W + OS -, W + W -, or untreated maize leaves at 0–0.5, 0.5–8 or 24–32 h after treatment.
Table S13. All transcriptional factors up-regulated or down-regulated by W + W or W + OS treatment and those further induced or repressed by W + OS treatment.