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Analyzing Molecular Basis of Heat-Induced Loss of Wheat Resistance to Hessian Fly (Diptera: Cecidomyiidae) Infestation Using RNA-Sequencing

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ABSTRACT

Heat stress compromises wheat resistance to Hessian fly (HF, *Mayetiola destructor*) infestation. The objective of this research is to analyze the molecular basis of heat-induced loss of wheat resistance to HF infestation using RNA Sequencing (RNA-seq). To this end, two resistant wheat cultivars 'Molly' and 'Caldwell' containing the resistance genes *H13* and *H6*, respectively were infested with an avirulent HF biotype *GP* and treated with different temperatures to examine the impact of heat stress on their resistance phenotypes. Tissue samples collected from HF feeding sites in Molly plants were subjected to RNA-seq analysis to determine the effect of heat stress on transcript expression of genes in wheat plants. Our results indicate that resistance to HF infestation in Caldwell is more sensitive to heat stress than that in Molly, and that heat stress down-regulates most genes involved in primary metabolism and biosynthesis of lignin and cuticular wax, but up-regulate most or all genes involved in auxin and 12-oxo-phytodienoic acid (OPDA) signaling pathways. Our results and previous reports suggest that heat stress may impair the processes in wheat plants that produce and mobilize chemical resources needed for synthesizing defensive compounds, weaken cell wall and cuticle defense, decrease OPDA signaling, but increase auxin signaling, leading to the suppressed resistance and activation of susceptibility.

KEYWORDS Wheat, Hessian fly, RNA-Sequencing, transcript expression

Elevated temperature (heat stress) is one of the most important environmental factors that affect the resistance of plants to parasites. In many plant-parasite interactions, heat stress compromises plants defenses mediated by *R* proteins (Tyler and Hatchett 1983, Buntin et al. 1990, Wang et al. 2009a, Chen et al. 2014, Tang et al. 2018). For example, *R* gene mediated resistance to bacterial pathogen *Pseudomonas syringae* in *Arabidopsis thaliana* and *Nicotiana benthamiana* was inhibited under a moderately high temperature (Wang et al. 2009a), and the resistance efficiency of wheat plants to Hessian fly (HF, *Mayetiola destructor*) was reduced at 'higher than normal' temperatures (Tyler and Hatchett 1983, Buntin et al. 1990, Chen et al. 2014, Currie et al. 2014a, Tang et al. 2018).

HF is one of the most destructive pests of wheat (*Triticum aestivum*) in North America and North Africa (Berzonsky et al. 2003). The battle between wheat to HF is a matter of life and death. In corresponding to avirulence or virulence of a HF biotype, a single wheat plant can be either resistant or susceptible. In an incompatible interaction, a resistant plant containing an effective *R* gene will kill the invading avirulent HF larvae and develop normally after some initial growth deficits (Shukle et al. 1990), while in a compatible interaction, HF larvae will induce nutritive tissues at their feeding sites in plants, resulting in eventual death of the plants (Harris et al. 2006). Higher temperature, however, can make a resistant plant with an effective *R* gene susceptible to the otherwise avirulent HF larvae (Tyler and Hatchett 1983, Buntin et al. 1990, Liu et al. 2013, Chen et al. 2014, Currie et al. 2014a, Cheng et al. 2018). The dramatic switch of wheat resistance to susceptibility under heat stress makes wheat-HF interaction an excellent system for investigating the impact of heat stress on plant resistance and the molecular basis of heat-induced loss of host plant resistance to parasites.

Logically, the loss of wheat resistance to HF infestation caused by heat stress can be attributed to the disruptive effect of heat stress on wheat plants, HF, and the dynamics of wheat-HF interaction. Heat stress may suppress resistance responses and/or activate susceptible responses of wheat plants to HF infestation (Liu et al. 2013). Regardless, both resistance and susceptible responses of wheat plants involve significant changes in primary, secondary, and phytohormone metabolisms (Liu et al. 2007, Zhu et al. 2008, Zhu et al. 2012). Based on previous studies, resistance responses of wheat to HF in the incompatible interaction relies on rapid mobilization of plants' chemical and energy resources including increase in degradation of lipid, sugar, and amino acid to provide substances and energy needed for synthesizing defense compounds, which leads to increased synthesis and accumulation of defensive compounds such as phenylpropanoids, flavonoids, and wax (Kosma et al. 2010, Zhu et al. 2012, Khajuria et al. 2013). In the susceptible plants, however, the expression level of genes related to resource mobilization and synthesis of defensive secondary metabolisms were reduced, especially at early time points of wheat-HF interaction (Khajuria et al. 2013). Phytohormone accumulation is also distinctively different between resistant and susceptible plants. High levels of 12-oxo-phytodienoic acid (OPDA) accumulation have been revealed in HF feeding tissues of resistant wheat plants during the incompatible interaction, but high levels of auxin (IAA) have been found in susceptible plants during the compatible interaction (Zhu et al. 2010a). While extensive studies have been conducted to disclose the molecular responses associated with wheat resistance and susceptibility to HF infestation (Williams et al. 2002, Giovanini et al. 2007, Liu et al. 2007, Zhu et al. 2008), little is known, at molecular level, about how heat stress causes the loss of wheat resistance to HF.

Previously, we analyzed the changes in profiles of polar lipids and phytohormones at HF feeding sites of wheat plants under heat stress to investigate the molecular processes underlying heat-induced loss of wheat resistance to HF infestation (Currie et al. 2014a, Currie et al. 2014b). Differential accumulation of polar lipids and phytohormones were found between resistant and susceptible wheat plants and between wheat plants with and without heat treatment (Currie et al. 2014a, Currie et al. 2014b), suggesting that the impact of heat stress on metabolism of polar lipids and phytohormones may contribute to the compromised wheat resistance to HF infestation under heat conditions. The objective of the current study was to determine the impact of heat stress on primary, secondary, and phytohormone metabolisms of wheat plants to reveal the molecular basis of heat-induced loss of wheat resistance to HF infestation. To this end, we infested two resistant wheat cultivars, Molly and Caldwell which contain *H13* and *H6 R* genes, respectively, with an avirulent HF biotype *GP*, and investigated the changes in their resistance phenotype under temperature treatment of different degrees and durations. Furthermore, we analyzed transcript expression of genes in Molly plants treated with heat stress at 35ºC for six hours (6-h) using RNA-Sequencing (RNA-Seq). Our results suggest that the sensitivity of Molly and Caldwell to heat stress in their resistance to HF are different, and that the impact of heat stress on primary, secondary, and phytohormone metabolisms may contribute to the heat-induced loss of wheat resistance to HF. The findings from this study will improve our understanding of plant resistance to parasites under stressed environmental conditions.

Materials and Methods

Plant preparation and infestation

Two wheat cultivars Molly and Caldwell and a HF population named 'White eyes' were used in this study. Molly possesses the *R* gene *H13*, and Caldwell possesses the *R* gene *H6* (Patterson et al. 1994, Tang et al. 2018). The HF population White eyes consists chiefly of avirulent biotype *GP*. Molly and Caldwell are resistant to the biotype *GP* HF at room temperature or below (Ratcliffe et al. 1997, Cheng et al. 2018). To prepare the plants, 20 germinated seeds were planted in each pot of 10-cm in diameter filled with Potting mix (Scotts Miracle-Gro Company, Marysville, OH). The plants were placed in a growth chamber set at 18ºC and 14:10 (day: night) photoperiod until most plants grew into seedlings at 1.5 leaf stage. To infest, eight newly emerged female flies and two male flies were released onto plants confined within a cage covered with a piece of cheese cloth on the top. Female HFs laid eggs on leaves of wheat seedlings. The eggs developed into larvae, which crawled down to the base of plants, living between leaf sheaths and attacking the inner leaf sheath. To determine the time when larvae began to attack plants, some infested plants were dissected and observed hourly under a dissection microscope beginning at 96 h following the release of adult HF onto plants. The time when HF larvae were first seen at the base of a plant was taken as the time for initial HF larval attack (time 0).

Experimental design, treatments, and statistical analysis for phenotyping

Molly and Caldwell wheat seedlings infested with Hessian fly biotype GP were subjected to different temperature treatments at 23, 25, 27, 30, and 35°C for 24 h, respectively, starting at time 0 (initial larval attack time). To execute the experiment, wheat plants were grown and infested in a growth chamber set at 18°C with 14/10 h (L:D) cycle. At time 0, plants designated for higher temperature treatment were moved to a second growth chamber set at 23, 25, 27, 30,

or 35°C, respectively, and returned to 18°C after the treatments. Control plants were maintained at 18°C until the end of each experiment. The experiments were designed as Randomized Complete Block Design (RCBD) with three biological replicates (one pot for each replicate of each treatment). Resistance and susceptibility of each plant was determined 7 d after time 0 as described previously (Currie et al. 2014b). A plant was rated as resistant if it contained only dead larvae and susceptible if it contained at least one live larva. For each replicate of each treatment, the numbers of resistant and susceptible plants were counted, and the percentage of susceptible plants was calculated. Mean percentages of susceptible plants of each treatment and their standard deviation were calculated using Excel 2016, and the significance of differences among treatments of each cultivar was determined by least significant difference (LSD; $\alpha = 0.01$) using PROC GLM (SAS 1999).

RNA-seq analysis

Molly was used for RNA-seq analysis. Four treatments were applied in this experiment:1) control plants without heat treatment and HF infestation (CK), 2) plants heat-stressed at 35°C for 6 h without infestation (Heat), 3) HF-infested plants under normal temperature (Infest), and 4) HF-infested plants under heat stress at 35°C for 6 h (Heat+Infest). The experiment was conducted in two Percival growth chambers (Perry, IA 50220) following Randomized Complete Block Design (RCBD) with three biological replicates. Plants without heat treatment grew at 18ºC. The plants receiving heat treatment grew at 18ºC before and after the heat treatment. The heat stress was applied as described in the above section. Sampling began right after the completion of the heat treatment. At the time of sampling, 2-5 HF larvae were found at the feeding site of each infested plant. To collect samples, each wheat seedling was cut from its base.

The second leaf sheath was carefully peeled off, and a10-mm section of each second leaf sheath was collected into a 2-ml Eppendorf tube filled with RNAlater (Thermal Fisher Scientific, Waltham, MA). For plants infested with HF, the second leaf sheath were rinsed in water to remove larvae from infested plants and dried on paper towel to remove excessive water before collecting the sample. These rinse and dry steps were also applied to samples collected from plants without infestation to maintain consistency in sampling. Samples were stored at −20°C before shipping to a commercial sequencing facility for RNA extraction, library construction, and sequencing.

RNA extraction, library construction, and sequencing

RNA was extracted using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA) following the manufacture's instruction. Libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA) following manufacturer's recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer 5X. First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was performed using DNA Polymerase I and RNase H followed by a round of purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection and PCR enrichment. Library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA) and then diluted to 1 ng/μl before checking insert size on an Agilent 2100 system (Agilent Technologies, Santa Clara, CA). Libraries were sequenced using an Illumina HiSeq-PE150 sequencing platform (Illumina, San Diego, CA, USA).

182

183 **Quality control, sequencing alignment, and quantification of transcript abundance**

184 Raw reads in fastq format were filtered to remove the reads containing adapters or reads of low
185 quality to produce clean reads. Paired-end clean reads were then mapped to the *Triticum*
186 *aestivum* genome sequence (ftp://ftp.ensemblgenomes.org/pub/plants/release-
187 39/gtf/triticum_aestivum/) using HISTAT (Wang et al. 2009b). Only uniquely mapped reads
188 were used for further read counting per gene, normalization of read counts and gene expression
189 analyses. HTSeq v0.6.1 was used to count the reads numbers mapped to each gene (Anders et al.
190 2015). And then FPKM, expected number of Fragments Per Kilobase of transcript sequence per
191 Millions base pairs sequenced of each gene, was calculated based on the length of the gene and
192 reads count mapped to the gene (Trapnell et al. 2009). Fold change in transcript abundance of a
193 gene between a treatment and control or two different treatments were expressed as normalized
194 read count of the gene.

195

196 **Differential expression analysis**

197 Differential expression analyses were performed on normalized read counts using DESeq R
198 package (Anders and Huber 2010). The resulting p-values were adjusted using the Benjamini and
199 Hochberg's approach for controlling the false discovery rate (Benjamini and Hochberg 1995).
200 Genes with an adjusted p-value (q value) < 0.05 were considered differentially expressed. All
201 treatments were normalized to the control, however, for the pairwise comparison of transcript
202 abundance between treatments Heat+Infest and Infest, Heat+Infest was normalized to Infest so
203 that the comparison would reveal the effect of heat stress on HF infested plants. Cluster analysis

of gene expression was based on the log10 FPKM value of all 12 samples pertaining to four experimental treatments.

MapMan analysis

MapMan was specifically designed to cover plant-specific pathways and processes (https://mapman.gabipd.org/mapman). MapMan pathway analyses were performed on the Log_2 fold change of differentially expressed genes (DEGs) between the treatments 'Heat+Infest' and 'Infest' (Usadel et al. 2009) to determine the effect of heat stress on transcript expression of genes in the HF infested plants. Custom specific mapping file for the MapMan based on the wheat sequencing output was created using the Mercator pipeline (Lohse et al. 2014) (http://mapman.gabipd.org/web/guest/mercator) in which TAIR, PPAP, KOG, CDD, ORYZA, and IPR, BLAST CUTOFF of 80, and ANNOTATE options were selected as parameters for the transcript annotation to obtain the hierarchical BIN (functional) categories. The functions or putative functions of the sequences were assigned by the BLAST-based search function of Mercator based on the reference proteins and functional domains of the sequences.

Results

Effect of higher temperature treatment on resistance phenotypes of Molly and Caldwell to HF infestation

Our results indicate that Molly and Caldwell, which contain *H13* and *H6*, respectively, respond to higher temperature treatment differently (Fig.1). Molly plants remained resistant in the control plants and plants subjected to 24 h higher temperature treatment at 23, 25, and 27ºC,

respectively, and became susceptible or mostly susceptible when the temperature was increased to 30 and 35ºC (Fig. 1a), respectively. The percentage of susceptible Molly plants was greater at 30ºC than that at 35ºC (Fig. 1a). Caldwell, however, is more sensitive to heat stress. 14.3% of Caldwell plants were susceptible in the control plants, and the mean percentage of susceptible plants increased significantly to 65.6-97.3% under higher temperature treatments at 23, 25, 27, 30, or 35 °C, respectively (Fig. 1b).

Effect of heat stress on transcript expression of genes in the HF infested plants

Overview of RNA-Seq analysis

A total of 12 cDNA libraries were sequenced. An average of 82.2 million of 2 x 150 paired-end raw reads per sample, ranging from 69.4 to 89.1 million, were obtained (Table S1). The data have been deposited with links to BioProject accession number PRJNA589693 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/). Read counts between the replicates are highly correlated ($R^2 \geq 92.9\%$; Table S2), indicating the high repeatability of the sequencing data. An average of 79.1 million (95.9%) clean reads were obtained after quality trimming. Overall, 90.0% clean reads were mapped to the *Triticum aestivum* genome sequence, and 84.2% were uniquely mapped (Table S1), from which 14,353 informative genes were identified. Based on the Log_{10}(FPKM+1) value of all informative genes, a cluster heat map was generated, showing a distinctive transcript expression patterns among four treatments. The heat stress, alone or in combination with HF infestation, resulted in distinctly different patterns of transcript levels between control plants and plants infested by HF (Fig. 2). Differential Expression Analysis of transcript abundance between the treatments 'Heat+Infest' and 'Infest' identified 11, 675 genes that were regulated by heat stress in the HF-infested plants. Among

them, 7476 were functionally annotated and assigned to 34 functional categories using MapMan platform (Table S3).

Heat stress on genes involved in primary metabolism

To understand the impact of heat stress on primary metabolism at HF feeding sites of wheat plants, we examined the regulation of genes involved in eight major primary metabolic pathways including photosynthesis (171 genes), lipid metabolism (293 genes), amino acid metabolism (177 genes), major carbohydrate metabolism (102 genes), nucleotide metabolism (99 genes), glycolysis (96 genes), tricarboxylic acid cycle (TCA) (45 genes) and cell wall metabolism (188 genes) (Tables 1). Our results indicated that heat stress downregulated most genes involved in these primary metabolic pathways (Tables 1 and S4). The top four pathways with the highest percentage of downregulated genes are TCA cycle (95.9%), photosynthesis (90.1%), nucleotide metabolism (88.9%), and glycolysis (88.5%). 77.5, 74, and 72.5% genes involved in the metabolisms of lipids, amino acids, and major carbohydrates were downregulated, respectively, and the percentages of downregulated genes involved in biosynthesis of these metabolites are considerably greater than the percentages of downregulated genes involved their degradation (Tables 1 and S4). 58% of genes involved in cell wall metabolism were downregulated, among which most genes involved in biosynthesis of cell wall components were downregulated, but most genes involved in degradation of cell wall were upregulated (Tables 1 and S4).

Heat stress on genes involved in secondary metabolism

A total of 330 genes involved in secondary metabolism were impacted by heat stress, including 138 (41.8%) upregulated genes and 192 (58.2%) downregulated genes. These genes encode

enzymes functioning/putatively functioning in metabolism of seven types of secondary compounds including isoprenoids (85 genes), phenylpropanoids (81 genes), alkaloids-like compounds (14 genes), sulfur-containing compounds (44 genes), wax (22 genes), flavonoids (90 genes) and simple phenols (2 genes) (Table 2). We further examined the regulation of genes involved in the metabolism of phenylpropanoids and wax because of their significance in wheat resistance to HF (Kosma et al. 2010, Khajuria et al. 2013). Forty-two genes involved in metabolism of phenylpropanoids encode enzymes catalyzing biosynthesis of lignin (Table 3), and 71.4% of these genes were downregulated by the heat stress (Table 3). Those down-regulated genes include all or majority of genes encoding phenylalanine ammonia-lyases (PAL), caffeic acid O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), 4-coumarate:CoA ligase (4CL), p-hydroxycinnamoyl-CoA:quinate/ shikimate p-hydroxycinnamoyltransferase (HCT), and caffeoyl-CoA O-methyltransferase (CCoAOMT) (Tables 3 and S5). Such results suggest that heat stress downregulates biosynthesis of lignin in the HF infested plants. Twenty-two genes involved in wax synthesis were impacted by heat stress, of which most genes involved in biosynthesis of very long chain fatty acid (VLCFA) were downregulated, but all genes encoding wax synthase were upregulated by heat stress (Tables 3 and S5).

Heat stress on genes involved in phytohormone metabolism

Among 234 genes involved in phytohormone metabolism that were impacted by heat stress, 34, 51, 50, 42, 38, 20, 21, and 11 of the genes are involved in the metabolism of abscisic acid (ABA), auxin (IAA), brassinosteroid (BA), ethylene (ET), cytokinin (CK), JA, salicylic acid (SA), gibberellin (GA), respectively (Table 4). Our results indicate that the majority of those

296 genes involved in the metabolism of IAA (72.5%), ET (61.9%), SA (71.4%), and JA (65%) were
297 upregulated, while the majority of those genes involved in the metabolism of BA (74%) were
298 downregulated under heat stress. Of these phytohormones, IAA and OPDA in the JA pathway
299 were found playing important roles in wheat's susceptibility and resistance to HF infestation,
300 respectively (Zhu et al. 2010b, Zhu et al. 2012, Cheng et al. 2018). Therefore, we further
301 examined the transcript levels of genes involved in IAA and JA metabolisms. Forty-two of those
302 genes involved in auxin metabolism are either auxin responsive or encode proteins with a
303 function in auxin signaling. The vast majority (76.2%) of these genes in auxin signaling and/or
304 responsive to auxin treatments were upregulated under heat stress (Tables 4 and S6). Eight of
305 those genes involved in JA metabolism encode enzymes with functions in the JA pathway (Table
306 4), including genes encoding five lipoxygenases (LOX), an allene oxidase synthase (AOS), and
307 two 12-oxophytodienoic acid reductases (OPR) (Fig. 3). All these eight JA-related genes were
308 up-regulated under heat stress, but the increases in the transcript abundance of the OPR genes
309 (9.1 and 3.1-fold, respectively) were much greater in magnitude than that of the LOX genes (2.7,
310 3.3, 2.9, 1.8, and 2.5-fold, respectively) and the AOS gene (1.7-fold) (Fig 3 and table S6).
311
312 **Discussion**
313
314 Heat stress compromises wheat resistance to HF infestation (Tyler and Hatchett 1983, Buntin et
315 al. 1990). In this study, we analyzed effect of higher temperature treatment on resistance
316 phenotypes of wheat cultivar Molly and Caldwell, whose resistance to HF is conveyed by *R*
317 genes *H13* and *H6,* respectively. We found that the resistance of Caldwell is more sensitive to
318 higher temperature than that of Molly (Fig. 1). Difference in temperature sensitivity of wheat

319 resistance to HF between Caldwell and Molly is expected because these two cultivars carry
320 different *R* genes in different genetic backgrounds (Tang et al. 2018). Previous study has
321 indicated that higher temperature exerts more significant impact on wheat resistance to HF
322 infestation, causing more susceptible plants (Cheng et al. 2018, Tang et al. 2018), however, in
323 the current study, the percentage of susceptible Molly plants heat stressed at 30℃ is significantly
324 higher than that at 35℃. The lower percentage of susceptible plants at 35°C observed in this
325 study is likely caused by the lower number of attacking larvae in the plants. RNA-seq analyses
326 revealed that heat stress downregulated the majority of genes involved in primary metabolism
327 (Table 1, S3, S4) and the biosynthesis of lignin and very-long-chain fatty acids (Table 3, S5), but
328 upregulated the vast majority of genes involved in auxin signaling and genes responsive to auxin
329 as well as all genes involved in JA biosynthesis (Fig. 3, Tables 4 and S6). Further analyses of our
330 current results and previous findings suggest that heat stress impair the processes that produce
331 and mobilize chemical resources and energy needed for synthesizing defensive compounds,
332 leading to weakened cell wall and cuticle defense in wheat plants. Additionally, disrupting
333 phytohormone metabolism by heat stress may also help HF overcome wheat resistance, resulting
334 in heat induced susceptibility.
335
336 **Down-regulation of primary metabolism by heat stress may lead to insufficient resources**
337 **and impaired mobilization of resources for defense**
338 To launch an effective defense against invading parasites, plants need to rapidly mobilize
339 sufficient chemical and energy resources to provide building blocks for the production of
340 defensive molecules such as oxylipins, toxic proteins, lectins, phenolics, and antifeedant cell wall
341 components (Zhu et al. 2012, Khajuria et al. 2013, Schultz et al. 2013, Zhou et al. 2015).

342 Generally speaking, production of resources involves photosynthesis and biosynthesis of large
343 molecules, such as lipid, carbohydrate, protein and amino acid etc., and mobilization and re-
344 utilization of resources involves degradation of those aforementioned molecules (Schultz et al.
345 2013, Zhou et al. 2015). In wheat-HF interaction, the mobilization and re-utilization of resources
346 for defense purpose are represented by the strong upregulation of genes encoding enzymes
347 responsible for degradation of lipids, catabolism of sugars, and degradation of amino acids in
348 resistant plants during incompatible interactions (Zhu et al. 2012, Khajuria et al. 2013). Our
349 results indicate that heat stress downregulated most genes involved in degradation of
350 carbohydrates, amino acids, nucleotides, and genes involved in the central catabolic pathways
351 glycolysis and TCA cycle (Table 1, S4). The down-regulation of these genes suggests that the
352 degradation of sugars, amino acid, nucleotides and other compounds, which are often elevated in
353 plants during incompatible interaction between wheat and HF (Zhu et al. 2012, Khajuria et al.
354 2013), was decelerated in heat-stressed plants. The decelerated degradation of those compounds
355 will likely lead to less production of the chemical and energy resources needed to synthesize
356 defensive compounds. Moreover, our results demonstrated that greater than 80% of those genes
357 involved in photosynthesis and synthesis of lipids and fatty acids, carbohydrates, amino acids,
358 and nucleotides were down-regulated, which could result in decreased production and
359 accumulation of available molecular resources that can be mobilized for defense in plants under
360 heat stress (Table 1, S4). With less resources available for mobilization and suppressed ability to
361 mobilize molecular resources, heat-stressed plants are likely unable to produce enough molecular
362 intermediates and energy to synthesize adequate defensive compounds for effective defense
363 against HF infestation.
364

Weakened cell wall and cuticle defense in the heat-stressed wheat plants

Fortification of cell wall and cuticle provide both physical barriers and chemical defense against HF infestation in wheat plants and is, therefore, crucial to wheat resistance to HF (Kosma et al. 2010, Khajuria et al. 2013). Our results provided two pieces of evidence to suggest that heat stress may weaken cell wall fortification. First, heat stress upregulated most of the genes involved in cell wall degradation, but downregulated most of the genes involved in synthesis of cell wall precursors and components (Table 1 and S4), suggesting the accelerated process for cell wall destruction but decelerated process for cell wall generation. Second, heat stress downregulated most of the genes involved in lignin synthesis (Table 3 and S5). Lignin, a phenolic heteropolymer, is one of the major components of plant cell wall. Lignin can be anti-nutritional because their role in strengthening cell walls against digestion of insects (Brodeur-Campbell et al. 2006). Studies have suggested that enhanced expression of genes responsible for lignin biosynthesis and high lignin accumulation in plants limited the invasion of piercing sucking insects (Santiago et al. 2013, An et al. 2019). In wheat-HF interactions, strong upregulation of genes encoding enzymes for lignin biosynthesis including phenylalanine ammonia-lyases (PAL), cinnamate 4-hydroxylases (C4H), cinnamyl alcohol dehydrogenases (CAD), and cinnamoyl-CoA reductases (CCR) was observed in the resistant plants after HF larval attack during incompatible interactions (Khajuria et al. 2013). That said, the downregulation of most genes involved in lignin synthesis by heat stress (Table 3) suggests that lignin biosynthesis was hindered and lignin accumulation was reduced at HF feeding site under heat stress, which will likely lead to the reduced level of cell wall fortification. Plant cuticles comprise a hydrophobic layer of waxes and cutins, which covers the outermost epidermal cell walls (Nawrath 2006, Samuels et al. 2008, Kosma et al. 2010). Cuticular wax is largely

388 composed of very-long-chain fatty acids (VLCFAs) and compounds derived from VLCFAs
389 which include aldehydes, alcohols, alkanes, ketones, and esters (Jetter et al. 2008). Changes in
390 cuticle wax coverage and composition are associated with increased epidermal permeability and
391 susceptibility of wheat plants to HF infestation (Kosma et al. 2010). Our result revealed that heat
392 stress downregulated most of the genes involved in synthesis of VLCFAs (Table 3 and S5). The
393 downregulation of these VLCFA synthesis genes suggests the decreased production and
394 accumulation of VLCFA at HF feeding sites in heat-stressed wheat plants. Because VLCFAs are
395 the basic components of plant cuticular wax, the decreased production and accumulation of
396 VLCFAs will likely lead to decreased production and changed composition of cuticular wax,
397 resulting in decreased level of cuticle fortification and increased permeability of cell wall that
398 may benefit HF feeding. Taken together, our results suggest that heat-induced changes in
399 expression of genes involved in the metabolism of cell wall and biosynthesis of lignin and
400 VLCFAs may lead to weakened cell wall and cuticle fortification that benefit HF survival in the
401 heat-stressed wheat plants.
402
403 **Alteration in OPDA and auxin signaling and heat-induced susceptibility**
404 Phytohormones play crucial roles in defense signaling. Different types of phytohormones interact
405 antagonistically or synergistically to regulate plants' responses to biotic and abiotic stress factors
406 (Takatsuji and Jiang 2014). Our study revealed that heat stress affected transcript levels of 234
407 genes involved in metabolism of eight major phytohormones (Table 4 and S3). The changed
408 transcript abundance of these genes will likely result in changed accumulation of phytohormones
409 and intensity of phytohormone signaling, shaping the outcome of wheat-HF interaction. OPDA,
410 as an intermediate in JA synthesis, is a key defense signaling molecule against insect in its own

411 right (Stintzi et al. 2001, Varsani et al. 2019). Our previous studies have found that high level
412 accumulation of OPDA may contribute to the expression of wheat resistance to HF during
413 incompatible interactions (Zhu et al. 2010b, Zhu et al. 2010a, Zhu et al. 2012, Cheng et al. 2018),
414 and that the reduction in OPDA accumulation caused by heat stress in wheat plants likely
415 contributed to reduced wheat resistance to HF (Currie et al. 2014b). Our current results indicate
416 that heat stress upregulated genes encoding three important rate-limiting enzymes (AOS, LOX,
417 and OPR) involved in OPDA metabolism in the JA pathway, but the magnitude of upregulation
418 of OPR genes are greater than that of LOX and AOS genes (Fig. 3). Because LOX and AOS
419 catalyze the synthesis of OPDA from linolenic acid, and OPRs catalyze the conversion of OPDA
420 to a cyclopentenone ring, which undergoes three cycles of oxidation in the peroxisome to
421 generate JA (Turner et al. 2002), the greater upregulation of OPR genes suggests that OPDA
422 degradation was accelerated and that OPDA accumulation might have been reduced in the heat-
423 stressed wheat plants. The upregulation of OPR genes observed in the current study is consistent
424 with our previous finding that heat stress causes decreased accumulation of OPDA at feeding site
425 tissue of Molly wheat (Currie et al. 2014b), suggesting that the decreased OPDA accumulation in
426 wheat plants under heat stress may be, at least in part, attributed to the upregulation of OPR
427 genes.
428
429 Our results also revealed that most auxin-signaling and/or -responsive genes are upregulated by
430 heat stress (Tables 4 and S6), which suggest the increased accumulation of auxin and auxin
431 signaling at the feeding sites of the heat-stressed wheat plants. Auxin is a phytohormone
432 associated with susceptibility of many plant species to pathogens and insects (Wang et al. 2007,
433 Zhu et al. 2010b, Tooker and De Moraes 2011, Kunkel and Harper 2017). Auxin stimulates

formation of galls in plants where gall-inducing pathogens and insects can manipulate physiology and biochemistry of plants to obtain nutrition (Liu et al. 1982, Tooker and De Moraes 2011, Tokuda et al. 2013). HF is a plant-feeding gall midge because a virulent HF larva can manipulate susceptible plants to create nutritive tissue, establish feeding sites, and complete its life cycle (Harris et al. 2006). Given the importance of auxin in gall formation and the high accumulation of auxin in susceptible wheat plants (Zhu et al. 2010a), coupled with the findings that high temperature increases auxin accumulation in plants (Gray et al. 1998, Sun et al. 2012), it is reasonable to speculate that the upregulation of auxin signaling and responsive genes observed in our current study may be a result of increased auxin accumulation caused by the heat stress, and that the heat-induced auxin accumulation is likely to stimulate the formation of nutritive tissues in the heat-stressed wheat plants, activating the susceptibility of wheat plants to HF infestation.

Conclusion

The results of our current study indicate that resistance of wheat cultivar Caldwell is more sensitive to heat stress than that of Molly. Our RNA-seq data together with previous findings suggest that heat stress may impair the processes in wheat plants that produce and mobilize chemical resources needed for synthesizing defensive compounds, weaken cell wall and cuticle defense, decrease OPDA signaling, but increase auxin signaling, leading to the suppressed resistance and activation of susceptibility.

Acknowledgement

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606 Table 1. Regulation of genes involved in major primary metabolism by heat stress in Molly
607 plants. All genes are differentially expressed between treatments “Heat+Infest” and “Infest”.
608 **Infest:** The HF infested, normal temperature plants; **Heat+Infest:** The plants under the
609 combination of HF infestation and heat treatment at 35°C for 6 h.

Pathways of primary metabolism	**Total No.**	**No. (%) of upregulated genes**	**No. (%) of downregulated genes**
Photosynthesis	**171**	**17 (9.9)**	**154 (90.1)**
Light reaction	126	8 (6.3)	118 (93.7)
Calvin cycle	27	6 (22.2)	21 (77.8)
Photorespiration	15	3 (20.0)	12 (80.0)
Lipid metabolism	**293**	**66 (22.5)**	**227 (77.5)**
Fatty acid synthesis elongation	114	15 (13.2)	99 (86.8)
Phospholipid synthesis	36	1 (2.8)	35 (97.2)
glycolipid synthesis	8	1 (12.5)	7 (87.5)
Lipid and fatty acid degradation	76	34 (44.7)	42 (55.3)
Amino acid metabolism	**177**	**46 (26)**	**131 (74.0)**
Amino acid synthesis	114	24 (21.1)	90 (78.9)
Amino acid degradation	62	22 (35.5)	40 (64.5)
Major carbohydrate metabolism	**102**	**28 (27.5)**	**74 (72.5)**
Starch synthesis	24	1 (4.2)	23 (95.8)
Starch degradation	25	7 (28)	18 (72)
Sucrose degradation	53	20 (37.7)	33 (62.3)

Nucleotide metabolism	**99**	**11 (11.1)**	**88 (88.9)**
Nucleotide synthesis	17	3 (17.6)	14 (82.4)
Nucleotide salvage	26	4 (15.4)	22 (84.6)
Nucleotide degradation	28	5 (17.9)	23 (82.1)
Glycolysis	**96**	**11 (11.5)**	**85 (88.5)**
Tricarboxylic acid (TCA) cycle	**45**	**1 (4.1)**	**44 (95.9)**
Cell wall metabolism	**188**	**79 (42)**	**109 (58)**
Synthesis of cell wall component	87	21 (24.1)	66 (75.9)
Cell wall degradation	39	28 (71.8)	11 (28.2)

610

611

612 Table 2. Regulation of genes involved in secondary metabolism by heat stress in Molly plants.
613 All genes are differentially expressed between treatments “Heat+Infest” and “Infest”. **Infest:** The
614 HF infested, normal temperature plants; **Heat+Infest:** The plants under the combination of HF
615 infestation and heat treatment at 35°C for 6 h.

Pathways of secondary metabolism	**Total No.**	**No. (%) upregulated genes**	**No. (%) of downregulated genes**
Isoprenoids	85	14 (16.5)	71 (83.5)
Phenylpropanoids	81	37 (45.7)	44 (54.3)
Alkaloid like compounds	14	8 (57.1)	6 (42.9)
Sulfur-containing metabolites	44	25 (56.8)	19 (43.2)
Wax	22	10 (45.5)	12 (54.5)
Flavonoids	90	46 (51.1)	44 (48.9)
Simple phenols	2	2 (100)	0 (0.0)

616

Table 3. Regulation of genes involved in biosynthesis of lignin and wax metabolism by heat stress in Molly plants. All genes are differentially expressed between treatments "Heat+Infest" and "Infest". **Infest:** The HF infested, normal temperature plants; **Heat+Infest:** The plants under the combination of HF infestation and heat treatment at 35°C for 6 h.

Pathway/Gene name [a]	**Total No.**	**No. (%) gene upregulated**	**No. (%) gene downregulated**
Lignin synthesis	**42**	**12 (28.6)**	**30 (71.4)**
PAL	2	0 (0)	2 (100)
4CL	5	1 (20)	4 (80)
HCT	4	1 (25)	3 (75)
CCoAOMT	10	4 (40)	6 (60)
CCR	7	4 (57.1)	3 (42.9)
COMT	3	0 (0)	3 (100)
CAD	11	2 (18.2)	9 (81.8)
Wax metabolism	**22**	**10 (45.5)**	**12 (54.5)**
VLCFA synthesis	13	3 (23.1)	10 (76.9)
Wax synthase	6	6 (100)	0 (0)

[a:] PAL: Phenylalanine ammonia-lyases, 4CL: 4-coumarate:CoA ligase), HCT: p-hydroxycinnamoyl-CoA:quinate/shikimate p-hydroxycinnamoyltransferase, CCoAOMT: caffeoyl-CoA O-methyltransferase, CCR: cinnamoyl-CoA reductase, COMT: caffeic acid O-methyltransferase, CAD: cinnamyl alcohol dehydrogenase, VLCFA: Very long chain fatty aci

Table 4. Regulation of genes involved in phytohormone metabolism by heat stress in Molly plants. All genes are differentially expressed between treatments "Heat+Infest" and "Infest". **Infest:** The HF infested, normal temperature plants; **Heat+Infest:** The plants under the combination of HF infestation and heat treatment at 35°C for 6 h.

	Total No.	**No. (%) upregulated genes**	**No. (%) of downregulated genes**
Abscisic acid (ABA)	34	17 (50.0)	17 (50.0)
Auxin (IAA)	51	37 (72.5)	14 (27.5)
Auxin signaling/responsive genes	42	32 (76.2)	10 (23.8)
Brassinosteroid (BA)	50	13 (26.0)	37 (74.0)
Ethylene (ET)	42	26 (61.9)	16 (38.1)
Cytokinin (CK)	38	20 (52.6)	18 (47.4)
Jasmonic acid (JA)	20	13 (65.0)	7 (35.0)
JA pathway	8	8 (100)	0 (0)
Salicylic acid (SA)	21	15 (71.4)	6 (28.6)
Gibberellic acid (GA)	11	5 (45.5)	6 (54.5)

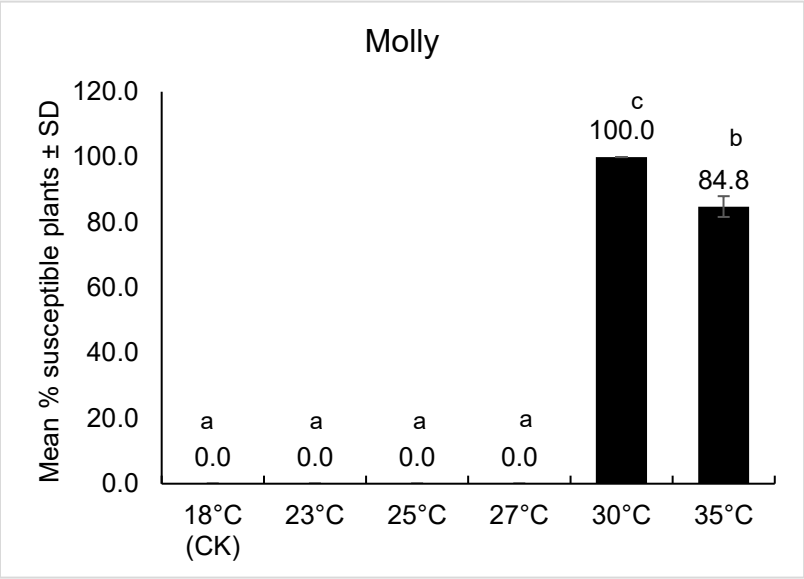
Figure legends

Fig. 1. Impact of 24 h temperature treatment on resistance phenotypes of wheat cultivar Molly and Caldwell, which contains the *R* genes *H13* and *H6,* respectively. All plants were infested with a HF population named 'white eye' that consists chiefly of the avirulent biotype *GP*. X-axis indicates degrees of temperatures. Y-axis indicates mean percentage of susceptible plants of all replicates in each treatment. For each replicate, percentage of susceptible plants was calculated as "No. susceptible plants x 100/No. infested plants). Higher percentage of susceptible plants indicates severer loss of plant resistance. Fig. 1a: Molly; Fig. 1b: Caldwell. Scale bars marked with different letters are significantly different in values at $\alpha = 0.01$.

Fig. 2. Hierarchical cluster of differentially expressed genes based on log10 (FPKM+1) value. Red denotes genes with high expression level, and blue denotes genes with low expression level. CK: the control plants without Hessian fly infestation growing at 18ºC. Infest: HF infested plants growing at 18ºC. Heat: Plants heat treated for 6-h at 35ºC. Heat+Infest: Plants under the combination of HF infestation and heat stress.

Fig. 3. Gene ID and fold change of transcript abundance of genes involved in JA pathway that are regulated by heat stress in the HF infested plants.
LOX: Lipoxygenase; AOS: Allene oxidase synthase; OPR:2-Oxo-PDA-reductase.

Fig. 1


Molly
Mean % susceptible plants ± SD
120.0
100.0
80.0
60.0
40.0
20.0
0.0
a
0.0
a
0.0
a
0.0
a
0.0
c
100.0
b
84.8
18°C (CK)
23°C
25°C
27°C
30°C
35°C

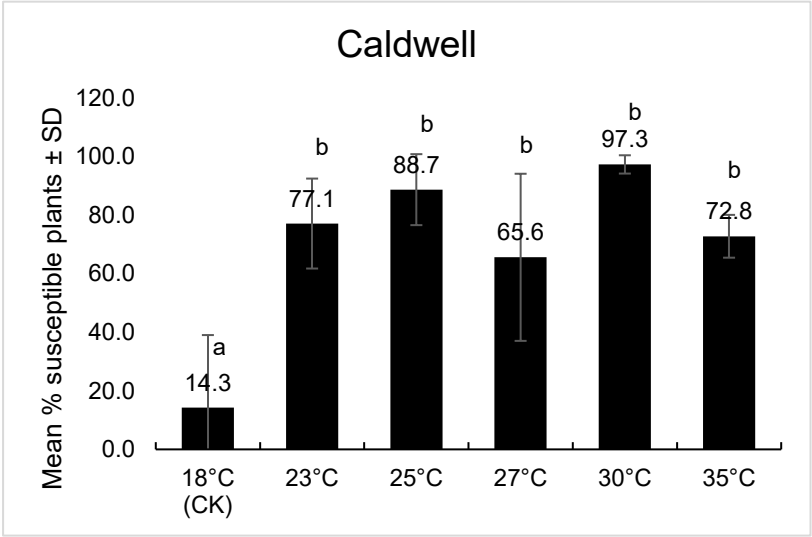

Caldwell
Mean % susceptible plants ± SD
120.0
100.0
80.0
60.0
40.0
20.0
0.0
a
14.3
b
77.1
b
88.7
b
65.6
b
97.3
b
72.8
18°C (CK)
23°C
25°C
27°C
30°C
35°C

Fig. 2

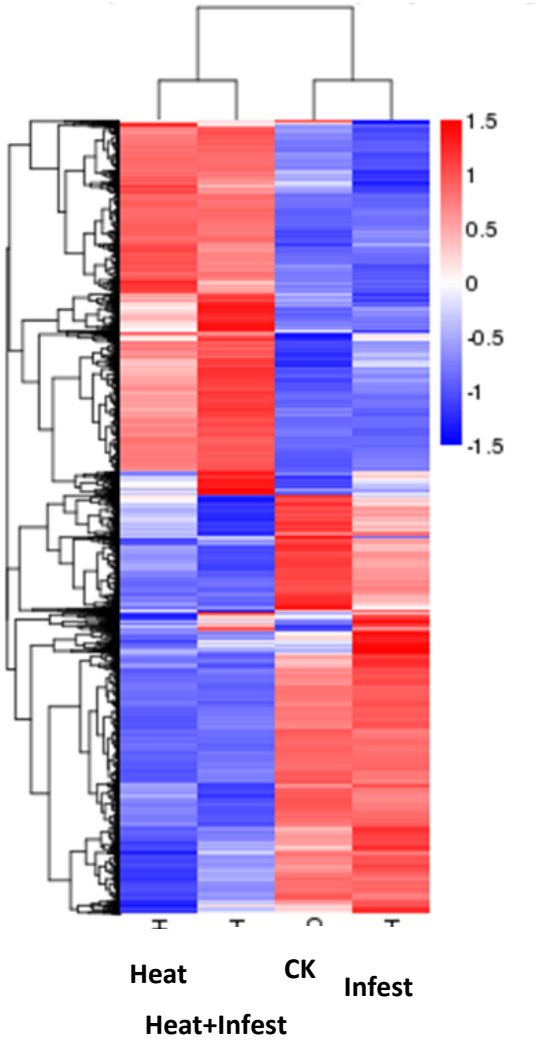
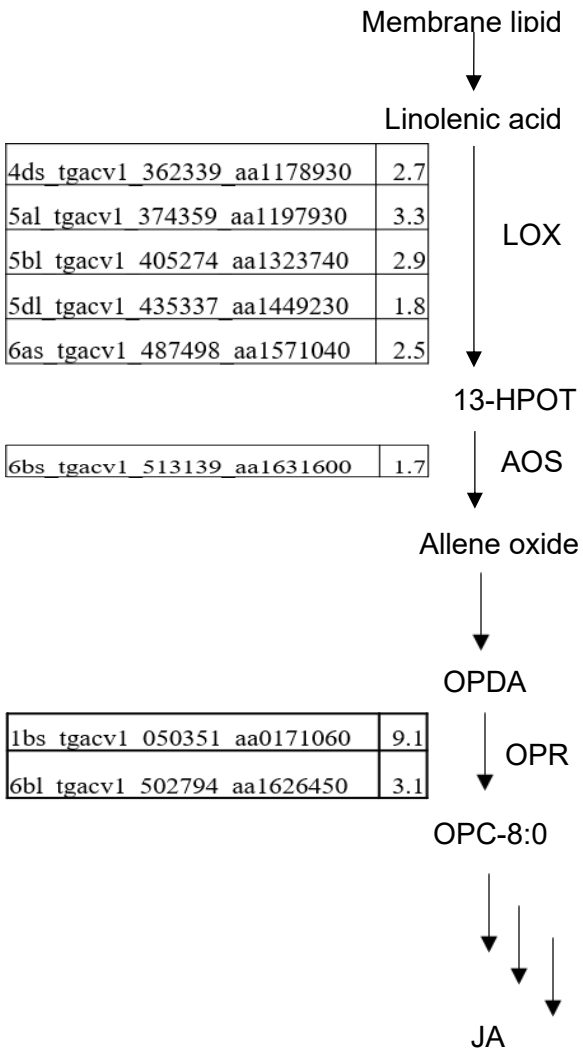


Fig. 3


Membrane lipid
Linolenic acid
4ds_tgacv1_362339_aa1178930 2.7
5al_tgacv1_374359_aa1197930 3.3
5bl_tgacv1_405274_aa1323740 2.9
5dl_tgacv1_435337_aa1449230 1.8
6as_tgacv1_487498_aa1571040 2.5
LOX
13-HPOT
6bs_tgacv1_513139_aa1631600 1.7
AOS
Allene oxide
OPDA
1bs_tgacv1_050351_aa0171060 9.1
6bl_tgacv1_502794_aa1626450 3.1
OPR
OPC-8:0
JA