High temperature effects on *Pi54* conferred resistance to *Magnaporthe oryzae* in two genetic backgrounds of *Oryza sativa*

Geoffrey Onaga^{ac,*}, Kerstin Wydra^b, Birger Koopmann^a, Dmytro Chebotarov^c, Yakouba Séré^d, and Andreas Von Tiedemann^a

^a*Division of Plant Pathology and Crop Protection, Department of Crop Sciences, Georg-August-University Göttingen, Germany*

^b*Erfurt University of Applied Sciences, Horticulture - Plant Production and Climate Change, Leipziger Str. 77, 99085 Erfurt, Germany;*

^c*International Rice Research Institute (IRRI), DAPO Box 7777, Metro Manila, Philippines*

^d*Africa Rice Center, P.O Box 33581, Dar es Salaam, Tanzania.*

**Correspondence: Email: gonaga@gwdg.de*

ABSTRACT

The global temperatures are predicted to rise due to climate change. However, knowledge on the mechanisms underlying the effect of high temperature (HT) on plant pathogen interaction is limited. We investigated the effect of elevated temperature on host phenotypic, biochemical and gene expression patterns in the rice-*Magnaporthe oryzae* (Mo) pathosystem using two genetic backgrounds, Co39 (*Oryza sativa*-indica) and LTH (*O. sativa*-japonica) with (CO and LT) and without (Co39 and LTH) R gene (*Pi54*). After exposure to 28°C and 35°C the two genetic backgrounds showed contrasting responses to Mo. At 28°C, CO, Co39 and LTH displayed a more severe disease phenotype than LT. Surprisingly, CO became resistant to Mo after exposure to 35°C. CO and LT were used for further analysis to determine the defence related biochemical and transcriptome changes associated with HT induced resistance, and the contrasting reactions to Mo observed in CO and LT despite carrying the same R gene. Pre-exposure to 35°C triggered intense callose deposits and cell wall fluorescence of the attacked epidermal cells, as well as, increased hydrogen peroxide (H₂O₂) and salicylic acid (SA) levels. On the other hand, transcriptional changes due to combined stress (35°C+Mo) were largely overridden by pathogen infection in both backgrounds, suggesting that the plants tended to shift their response to the pathogen. However, significant differences in global gene expression patterns occurred between CO and LT backgrounds in response to both single (35°C and Mo) and double stress (35°C+Mo). Collectively, our results suggest that rice lines carrying *Pi54* respond to Mo by rapid induction of callose and H₂O₂, and that these resistance mechanisms are amplified at HT. The relative difference in disease severity between CO and LT at 28°C suggests that the genetic background of japonica rice facilitates the function of *Pi54* more than the background of indica rice. The phenotypic plasticity and gene expression differences between CO and LT backgrounds reveal the presence of intricate molecular signatures that may potentially influence adaptation to plant stresses.

Keywords: Genetic background, R genes, climate change, rice, *Magnaporthe oryzae*

1. Introduction

Global climate models project an increase in average global temperatures by 2 to 5°C until the end of the 21st century (Bokszczanin and Fragkostefanakis, 2013). This increase in temperature is predicted to constrain crop productivity in various ways, including alterations in the biology of plants and their interaction with pathogens. In Africa and in other tropical and sub-tropical countries, the rice crop is already exposed to some episodes of high temperatures (35°C or higher), often in combination with pathogen infections. Thus, insights on how plants respond to elevated temperature and other stresses simultaneously are crucial research tasks in practical management of plant production. Such insights will be relevant to plant breeding and pathology, and would provide tools for further understanding of cross talk between biotic and abiotic stress responses.

Many regulatory pathways and networks are activated in plants when temperature and pathogen stresses occur simultaneously, but may function antagonistically, synergistically or unrelated due to high specificity of host–pathogen interactions. Therefore, the precise mechanism of crosstalk between high temperature (HT) and pathogen stress is controversial, and outlines challenges posed by climatic factors on host-pathogen interaction. In light of increased pathogen infections, it has been demonstrated that some pathosystems already bear the brunt of increasing temperatures. For instance, HT suppressed the resistance of wheat to *Puccinia graminis tritici* (Harder et al., 1979) and the resistance of soybean to *Phytophthora sojae* (Gijzen et al., 1996). In addition, the *N* gene of tobacco that confers resistance to tobacco mosaic virus at 22°C was shown to break down at 30°C (Whitham et al., 1996). Likewise, the amount of *EDS1* and *PAD4* transcripts was reported to be temperature dependent in *Arabidopsis*, with lower accumulation at 28°C than at 22°C (Yang and Hua, 2004). Moreover, the hypersensitive response conferred by *Arabidopsis* *RPW8* gene against powdery mildew was shown to be suppressed at temperatures above 30°C (Xiao et al., 2003).

Conversely, the yellow rust resistance gene *Yr36* was reported to be up-regulated at HT, leading to full or partial resistance against stripe rust (*Puccinia striiformis*) in spring wheat (Fu et al., 2009). High temperature also positively modulated *Xa7* and *Rp1-D21* mediated resistance in rice (Webb et al., 2010) and maize (Negeri et al., 2013), respectively. Thus, plant response to both HT and pathogen stress, when occurring simultaneously, can be varied in multiple ways, and indicates the delicate balance between the components that influence disease development in a pathosystem.

Alternatively, the genetic background appears to affect the effectiveness of R genes, and may modulate differently the global expression of genes following pathogen infection (Palloix et al., 2009). For instance, a study involving transgenic rice plants carrying *Xa26* (also known as *Xa3*) found that the background of *japonica* variety, Mudanjiang 8, was more resistant to *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) strains than the gene donor, *indica* variety Minghui 63 (Sun et al., 2004; Cao et al., 2007). Similar findings were reported in

Arabidopsis, in which the function of an allele of Arabidopsis *R* gene *RPS2* was found to be influenced by the genetic background (Banerjee et al., 2001).

Collectively, these findings indicate a potential complexity associated with HT, genetic background and *R* gene mediated resistance in plants against pathogens. Thus, a comparison of phenotypic and transcriptome expression between genetic backgrounds in response to pathogens at elevated temperature could be interesting.

Magnaporthe oryzae (Mo) is the most destructive pathogen of rice, a staple crop for over half of the world's population (Xue et al., 2012). The primary breeding tool in rice blast management is to introduce resistance (*R*) genes into elite rice cultivars. During the past 25 years, genetic and molecular analysis of resistant donors has led to identification and mapping of more than 100 major blast resistance (*R*) genes; 22 of these genes have been cloned and used for crop protection against Mo (Zhang et al., 2015). Several of these *R* genes have also been used for developing independent sets of near-isogenic lines (NILs) in both *O. japonica* and *O. indica* backgrounds, some of which confer broad-spectrum resistance against Mo strains tested under normal growth conditions (Liu et al., 2002; Jiang et al., 2012b; Onaga and Asea, 2015). However, the potential of rice and the introgressed *R* genes to adapt to both HT and Mo stresses remains less studied. Thus, it is important to gain in-depth knowledge about the complexity of phenotypic, biochemical and transcriptional changes that occur in *O. japonica* and *O. indica* genetic backgrounds, introgressed with *R* genes, in response to Mo at elevated temperature. This will provide insights on how combined abiotic and biotic stresses, as influenced by the genetic background, can alter plant productivity.

In this study, we used two rice genetic backgrounds, Co39 and LTH, with and without *Pi54*, to examine the response of rice to HT and Mo at elevated temperature. *Pi54* possesses a nucleotide binding site-leucine rich repeat (NBS-LRR) in addition to a small zinc finger domain, and was previously reported to be constitutively expressed at basal levels in both resistant transgenic as well as susceptible rice lines up to 48 hpi (Gupta et al., 2012). We show that rice lines carrying *Pi54* respond to Mo by rapid induction of callose and H₂O₂, and that these resistance mechanisms are amplified at HT. We also found that plants with Co39 background had a severe disease phenotype than LTH at 28°C, suggesting that the genetic background plays a significant role in rice response to Mo.

2. Results

2.1. Phenotypic and biochemical responses of *M. oryzae* infected plants before and after high temperature exposure

The interaction between rice and Mo was investigated under normal (28°C) and elevated (35°C) temperature conditions, using CO, LT, Co39 and LTH. At 28°C, CO, Co39 and LTH had a severe disease phenotype and

displayed lesions within 5 days post inoculation (dpi), while LT exhibited reduced lesion sizes (Fig. 1A). At 35°C, *Pi54* associated lesion development was rarely observed on both NILs. To exempt any direct effect of HT on the pathogen, plants were grown at 35°C for 7 d prior to Mo inoculation, where after all procedures were carried out at identical temperature conditions (28°C). Pre-exposure to 35°C induced a delay in disease progress and typical HR lesions in both NILs and their recurrent parental backgrounds (Co39 and LTH), although the disease progress remained comparatively higher in the recurrent parents (Fig. 1B).

To further investigate HT modulation of plant defence, CO and LT were used for further analysis to determine the biochemical and transcriptome changes associated with HT induced resistance. The levels of callose deposition and H₂O₂ in CO and LT plants pre-exposed to 35°C and inoculated with Mo was compared with that in plants grown and inoculated at 28°C. Plants pre-exposure to 35°C followed by Mo inoculation showed intense callose accumulation as well as cell wall fluorescence of the attacked epidermal cells in both NILs (Fig. 2). In accordance with callose deposition, both NILs had significantly higher H₂O₂ levels in plants pre-exposed to 35°C and Mo (Fig. 3A). However, H₂O₂ activity declined significantly in CO plants exposed to 28°C and Mo at 48 hpi. Exposure to HT also increased peroxidase (POX) activity in both NILs (Fig. 3B). However, in spite of showing consistently higher activity at 48 hpi, POX activity was not significantly different between CO plants pre-exposed to 28°C and 35°C prior to Mo inoculation at 24 hpi. We also found significantly lower POX activity in LT plants infected with Mo at 48 hpi despite showing higher induction at 24 hpi.

The phenolic phytohormone, salicylic acid (SA), has beneficial effects on plant growth and development under stress conditions (Awasthi et al., 2015). We compared the levels of SA in plants exposed to 35°C and Mo using CO and LT NILs. HT induced significant increases in free SA in both NILs prior to Mo inoculation (Fig. 4A). Alternatively, for plants exposed to Mo, we found consistently higher levels of free SA in CO than LT, regardless of the temperature difference. However, at 48 hpi, plants pre-exposed to 35°C had a significant reduction in SA in both NILs; although SA remained comparatively higher in CO than LT. In contrast to SA levels, LT had significantly higher PAL activity at 24 hpi than CO in plants inoculated at 28°C (Fig. 4B). Interestingly, pre-exposure to 35°C followed by Mo inoculation induced higher PAL activity in CO, and this was consistently higher up to 48 hpi. Conversely, PAL activity significantly declined in LT plants exposed to 35°C and Mo at 48 hpi.

2.2. *Transcription profiles of CO and LT infected with Mo at 28°C and 35°C*

To determine temperature-induced transcriptome changes, genome-wide gene expression patterns were investigated in CO and LT using mRNA-seq following exposure to 35°C and Mo. Three biological replicates of each treatment were sequenced, yielding an average of sixty nine million reads. For a global view on the sample

relationships, principal component analysis was computed based on one third of the genes with highest variance in expression levels (Fig. S1). Principal component 1 and 2 accounted for 38% of the inherent data variance between the samples, and some of the replicates segregated distinctively for one material origin. On average, 32,000 genes were detected. Fold changes were calculated for the genes obtained, and a false discovery rate (FDR) of 0.05 was set for the calling of differentially expressed genes (DEG). The number of DEG in plants exposed to Mo (CO+28°C+Mo, LT+28°C+Mo, CO+35°C+Mo and LT+35°C+Mo) was significantly higher than in plants exposed to only HT stress (CO+35°C and LT+35°C; Additional file S1). A small subset of genes (600) was shared between plants exposed to HT and Mo (Additional file S2), and hierarchical clustering on the log₂ transformed fold change values clearly showed a considerable transcript profile separation between plants exposed to HT and Mo (Fig. 5). Likewise, substantial differences were observed between gene ontology (GO) terms enriched in plants exposed to HT and Mo in both NILs (Table S1). However, most of the GO terms that were enriched in the up-regulated gene set in response to HT were similar between CO and LT (Fig. 6A, B), suggesting that LT and CO do not substantially differ in the biological processes responding to HT treatment. Oxidation-reduction process was the most enriched and common GO term identified, containing over 400 DEGs from LT and 390 from CO. Defence response to fungus, incompatible interaction (GO:0009817) was significantly enriched in LT+28°C+Mo, LT+35°C+Mo and CO+35°C+Mo but not in CO+28°C+Mo.

2.3. Transcriptome differences between CO+35°C+Mo and CO+28°C+Mo

An important part of this investigation was to identify transcripts that are differentially expressed between CO+35°C+Mo, CO+28°C+Mo and LT to provide an indication of what molecular mechanisms may be associated with resistance at HT. The HT-treated (CO+35°C+Mo) plants were somewhat separated from CO+28°C+Mo: with a total of 1613 DEG specifically expressed in CO+35°C+Mo (Additional file S3). Most of the DEG specific to CO+35°C+Mo were related to cell-cycle regulation (e.g., *MAK16*; LOC_Os07g17040 and *KIP1*; LOC_Os07g49480), trafficking (e.g., *EXO7*; LOC_Os01g55799 and *ABC-1* domain containing protein; LOC_Os02g56200), photosynthesis (e.g., Chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase activase; LOC_Os11g47980, carbonic anhydrase; LOC_Os01g45274, isopropylmalate dehydrogenases; LOC_Os03g45320) and carbohydrate metabolism (e.g., glycoside hydrolase; LOC_Os01g63810 and bidirectional sugar transporter *SWEET1a*; LOC_Os01g65880). A significant up regulation of Peptidyl-prolyl isomerases (*OsFKBP65*) was also detected in CO+35°C+Mo compared to CO+28°C+Mo. Moreover, *OsFKBP65* was induced in concert with heat shock factors/proteins e.g., *HSFA2*, *HSP81-1* (*HSP90*), HSP70s, and several small HSPs. A total of 1391 genes expressed in CO+28°C+Mo were suppressed in CO+35°C+Mo (Additional file S3).

Conceptually, the suppression of these genes suggests sensitivity to heat stress, but could otherwise suggest that some of these genes negatively play a role in rice disease resistance.

2.4. Co-expression network analysis

Genes often act in networks to regulate biological processes. To identify co-expression modules (clusters) within all the genes expressed, we extracted the normalized, log transformed gene expression values in each treatment (CO+28°C+Mo, LT+28°C+Mo, CO+35°C+Mo, LT+35°C+Mo, CO+35°C and LT+35°C) and performed Weighted Gene Co-expression Network Analysis (WGCNA). All possible pairwise correlations were calculated for all the genes expressed in all the treatments and converted into measures of connection strength by taking their absolute values and raising them to a power, β (soft thresholding). The soft thresholding power of 6 seemed a minimal number for which R^2 had a good fit (model fit >0.5) that best approximates a scale free topology of the resultant networks, and is also the default setting in WGCNA (Fig. S2). After fixing the power, genes were split into 4 blocks, and within each block we ran WGCNA clustering using topological overlap measure to determine modules of co-expressed genes (Fig. S3). Sets of genes (modules) with common expression patterns that were associated with particular treatments were identified based on the correlation between ME and treatments (Fig. 7). We identified nine modules that significantly associated with Mo infection, 5 modules associated with genetic background and 3 modules associated with HTxMo interaction ($r \geq 0.5$, $P \leq 0.01$). Although the r value was lower than 0.5, module 1 correlated positively to HT and negatively to Mo infection [ME(HT), $r=0.46$, $P=0.02$; ME(Mo), $r=-0.69$, $p=2e-04$], module 7 correlated positively to Mo infection, module 27 correlated positively to genotype x Mo and negatively to HT [ME (GxMo), $r=-0.45$, $P=0.03$; ME (HT), $r=-0.52$, $P \leq 0.01$] and module 0 correlated positively to genotype x HT x Mo [ME₍₀₎: $r=0.32$, $P=0.03$].

The modules that closely associated with treatments might be very useful for understanding the biological processes associated with these treatments. Module 7 genes are mainly involved in signal transduction (GO:0007165), defence response to bacterium (GO:0042742) and nucleobase-containing compound metabolic process (GO:0006139). Module 1, which significantly associated with HT, had its genes significantly enriched in translation regulator activity (GO:0045182), cellular aromatic compound metabolic process (GO:0006725), nitrogen compound metabolic process (GO:0046482), calcium ion binding (GO:0005509), ATPase activity, coupled to transmembrane movement of substances (GO:0042626) and signal transduction (GO:0007165).

Modules 15 and 25 were apparently the only consensus co-expression modules identified but also with weak positive association with all the traits. Module 15 genes are mainly involved in DNA binding, regulation of gene expression and RNA metabolic process, whereas module 25 genes are mainly involved in transition metal ion binding and cation binding. Module 13 was significantly associated with the genetic background, and had its

genes significantly enriched in cell cycle, O-acyltransferase activity, S-adenosylmethionamine biosynthetic process, adenosylmethionine decarboxylase activity and DNA metabolic process.

To determine genes that are centrally located in the modules associated with treatments, we calculated the intramodular connectivity for each gene by summing the connection strengths with other module genes and dividing this number by the maximum intramodular connectivity. Genes with high intramodular connectivity are referred to as hub genes. The hub genes in the 35 modules were listed in Table S2. These hub genes may provide signature targets for further analysis, and we propose them as potential candidates for stress response in rice.

2.5. Identification and classification of DEG contributing to resistance in LT and CO+35°C+Mo

We categorized the DEG into six classes according to function. Class I included reactive oxygen species (ROS) production and antioxidant encoding genes, Class II are hormone production and signaling genes, class III are resistance (R) genes, receptor kinases, calcium and MAPK signalling genes, class IV includes transcription factors, class V are energy and metabolism genes, and class VI are effector proteins which include defence-related proteins and enzymes involved in secondary metabolism.

In class I, the ROS production genes *OsNox9* (LOC_Os12g35610) and *RbohD* (LOC_Os10g40020) were significantly induced (>5-fold) in CO+35°C+Mo, LT+35°C+Mo and LT+28°C+Mo but not in CO+28°C+Mo (additional file S3). Genes encoding for *POX51* (LOC_Os03g55410), *POX6* (LOC_Os07g02440), ferredoxin (LOC_Os03g48040), *prx117* (LOC_Os08g02110) and *TRX-1* (LOC_Os04g44830) were also more induced in CO+35°C+Mo than CO+28°C+Mo. On the other hand, class II peroxidase encoding genes, e.g., *prx62* (LOC_Os04g59200), genes encoding for ferredoxins (e.g., LOC_Os07g30670 and LOC_Os03g45710), and *APXs* (e.g., LOC_Os08g41090) were more induced in LT than CO after HT treatment. Differential induction of these antioxidant encoding genes indicates a substantial effect of the genetic background on antioxidant activity, and this is consistent with the aforementioned differential POX enzyme activities between CO and LT at 48 hpi.

In class II, genes involved in the synthesis and signaling pathway of SA, jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and other hormonal pathways were considered. We detected a significant up-regulation of genes encoding for SAM dependent carboxyl methyltransferases in response to both HT and Mo, including LOC_Os06g13310 and *OsBISAMT1* (LOC_Os11g15040), the enzyme that catalyzes the conversion of SA to MeSA. LOC_Os06g13310 and *OsBISAMT1* were more induced in plants pre-exposed to 35°C than 28°C in both NILs (additional file S2). We also looked at the differential expression of PAL genes (e.g., *OsPAL5*; LOC_Os02g41670, *OsPAL1*; LOC_Os02g41680, *ZB8*; LOC_Os05g35290, *OsPAL2*; LOC_Os04g43760 and *OsPAL7*; LOC_Os04g43800) and *NPR1* genes (e.g., LOC_Os01g09800) in Mo inoculated plants. We detected reduced expression of these genes in response to Mo and HT in both NILs.

197 The JA pathway genes, e.g., 12-oxophytodienoate reductase 9 (*OPR9*; LOC_Os01g27240), were more induced
198 in CO+35°C+Mo and LT+35°C+Mo than CO+28°C+Mo. Genes encoding for jasmonate O-methyltransferases
199 (e.g., LOC_Os12g13800 and LOC_Os09g17560) and jasmonate induced jacalin-like lectins (e.g.,
200 LOC_Os11g32210) were also more induced in CO+35°C+Mo than CO+28°C+Mo. Interestingly, similar genes
201 (e.g., LOC_Os06g2182, LOC_Os12g14440 and an orthologue of maize O-methyltransferase *ZRP4*;
202 LOC_Os10g02880) were more induced in LT than CO in response to Mo at 28°C.

203 For ET pathway, oxidoreductases from 2OG-Fe(II) oxygenase family (e.g., LOC_Os06g07914), ethylene
204 responsive transcriptional coactivator named multiprotein bridging factor 1c (*MBF1c*; LOC_Os06g39240),
205 dehydration-responsive element-binding protein 1G (*DREB1G*; LOC_Os02g45450), ethylene-responsive
206 transcription factor 5 (*ERF5*; LOC_Os10g41330), 1-aminocyclopropane-1-carboxylate deaminase
207 (LOC_Os02g53330) and basic helix-loop-helix transcription factors (TFs; e.g., *bhlh65*, *bhlh108* and *bhlh56*) were
208 more induced in plants pre-exposed to 35°C than 28°C in both NILs.

209 Among the genes involved in other hormonal pathways, auxin response genes (e.g., *OsIAA13*;
210 LOC_Os03g53150, *OsGH3.2*; LOC_Os01g55940, *OsGH3.5*; LOC_Os05g50890, *OsSOUR1*; LOC_Os01g06230
211 and *ARF19*) were significantly induced in CO+35°C+Mo than CO+28°C+Mo. In ABA pathway, *GEM*
212 (LOC_Os02g42430) was more induced in CO+35°C+Mo and LT+35°C+Mo than CO+28°C+Mo. In contrast,
213 *HVA22* (LOC_Os11g05800), *OsBBD1* (LOC_Os01g50622), *ABA3* (LOC_Os09g38772) were more induced in
214 LT than CO in response to HT+Mo.

215 In class III, we considered significantly regulated genes encoding for nucleotide-binding and leucine-rich
216 repeat (NB-LRR) proteins and receptor like kinases (RLKs). In our dataset, 61 and 75 genes encoding for R like
217 proteins were up-regulated (>4-fold) in LT+28°C+Mo and CO+28°C+Mo, respectively (additional file S2). This
218 number however decreased to 50 and 63 in LT+35°C+Mo and CO+35°C+Mo, respectively, and their expression
219 was significantly lower at 35°C. Transcript levels of *Pi54* (LOC_Os11g42010) were significantly lower at 35°C
220 than 28°C (Fig. 8). Exceptions were observed in LT, in which *Pit* (LOC_Os01g05620) was evidently more
221 induced at 35°C compared to 28°C. Further, NBS-LRR disease resistance protein (LOC_Os01g05600) which
222 shares 91.9% sequence homology to *Pit*, an orthologue of *RPM1* (LOC_Os11g11770), *Yr10* (LOC_Os12g17090)
223 and a GTPase of unknown function (LOC_Os08g10649) were highly induced in LT but not in CO. Alternatively,
224 orthologues of *Avena sativa* NBS-LRR type disease resistance protein O2 (LOC_Os04g43440), *RPM1*
225 (LOC_Os06g48520), *RGA3* (LOC_Os11g10770) and *Xa1* (LOC_Os04g53120) were more induced in
226 CO+35°C+Mo than CO+28°C+Mo. The high induction of these genes in CO+35°C+Mo compared to
227 CO+28°C+Mo may suggest a potential role in rice resistance against Mo.

228 Receptor like kinases (RLKs) are among the largest class of proteins, with about 1100 reported in rice, so far
229 (Kawano and Shimamoto 2013). Our transcriptome analysis revealed a large number of RLK genes expressed in

230 response to Mo than HT (Additional file S2), and their expression was significantly lower at 35°C. Exceptions
231 included transcription of genes encoding for *SHR5* receptors, some of them were relatively less affected by
232 alterations in temperature. For instance, transcript levels of *SHR5* (LOC_Os05g17810) were significantly higher
233 in CO+35°C+Mo than CO+28°C+Mo. The same was true for a leucine-rich repeat receptor-like protein kinase 1
234 (LOC_Os10g02990) and S-domain receptor-like protein kinase (LOC_Os11g10290). In LT, senescence induced
235 RLKs (e.g., LOC_Os09g19380 and LOC_Os05g17604), glutamate receptor (LOC_Os06g08900), *OsWAK36* and
236 *OsWAK43* were more induced in HT treated plants than plants grown and inoculated at 28°C.

237 In class IV, several TFs were more induced in response to Mo than HT (additional file S2). MYB family genes
238 were the most regulated at HT. This was consistent with a high frequency (>90%) of *cis*-elements for water stress
239 (MYBCORE) and dehydration stress (*MYBIAT*) response in both sense and antisense strands of most temperature
240 stress responsive DEG. *MYBAS1* (LOC_Os11g47460) and *RI-MYB/MYB-like* subfamily genes (e.g.,
241 LOC_Os01g60850, LOC_Os03g55840 and LOC_Os01g74600) were highly induced at HT in both NILs. Among
242 *WRKY* genes, *OsWRKY111* (LOC_Os05g50700) was strongly induced in both NILs, whereas *OsWRKY84* and
243 *OsWRKY65* (LOC_Os12g02470) were more induced in CO+35°C+Mo than CO+28°C+Mo. HT treated plants also
244 showed increased transcript levels of splicing factor U2af small subunit A (LOC_Os09g31482) involved in
245 mRNA processing, and a B-Box Zinc Finger Gene (*OsBBX22*) in both NILs.

246 Alternatively, bHLH genes, (*bHLH-68*; LOC_Os04g23550, *bHLH-114*; LOC_Os08g38210 and
247 LOC_Os02g45170) were significantly induced in CO than LT at HT. Genes encoding for *ONAC12*
248 (LOC_Os05g37080), *ONAC43* (LOC_Os02g06950), a zinc binding protein (LOC_Os06g45540) and *ROC6*
249 (LOC_Os09g35760) were more induced in CO+35°C+Mo than CO+28°C+Mo. In contrast, transcript levels of
250 *SWIB/MDM2* complex (LOC_Os03g55310) involved in chromatin remodeling, bZIP genes (e.g., *bZIP62*;
251 LOC_Os07g48660 and LOC_Os05g16640), *OsMADS25* (LOC_Os04g23910), *WRKY4* (LOC_Os03g55164),
252 *bHLH-58*, *bHLH-114* and *bHLH-63* were generally more induced in LT than CO.

253 In class V, LT showed increased expression of most photosynthesis related genes compared to CO. Genes
254 encoding for oxygen evolving enhancer protein 3 (*OEE3*; LOC_Os02g36850) required for photosystem II
255 assembly/stability, chloroplastic group IIA intron splicing facilitator (*CRS1*; LOC_Os07g30670) involved in
256 chloroplast protein translation and 2Fe-2S iron-sulfur cluster binding protein (LOC_Os07g30670) were generally
257 >10-fold induced in response to HT. In addition, plastid transcriptionally active12 (*PTAC12*; LOC_Os01g56350),
258 phosphoenolpyruvate phosphate translocator2 (*PPT2*; LOC_Os08g25624), a metal iron binding protein (*MT3B*;
259 LOC_Os05g11320), cytochrome P450 (LOC_Os03g55230) and proliferating cell nuclear antigen (*PCNA*;
260 LOC_Os02g56130) were more induced in response to HT than Mo. A putative photosystem Q
261 (LOC_Os10g21192) and a photosynthetic reaction center protein (LOC_Os10g39880) were however specifically
262 induced in CO but not in LT. Several cytochrome P450s, including LOC_Os01g36294 and LOC_Os10g30390,

and photosystem II 10 kDa polypeptide encoding genes (e.g., LOC_Os07g05360 and LOC_Os07g05365) were induced >30-fold in both NILs. These genes likely play a significant role in protecting rice PSII from perturbations resulting from heat stress and pathogen infection.

Several genes involved in lipid metabolism were background specific. For instance, the GDSL like gene (LOC_Os01g11730) was stronger induced in LT than CO at 35°C. However, amino acid metabolism genes encoding for asparagine synthetase, alanine-glyoxylate aminotransferase, isovaleryl-CoA dehydrogenase and 2-aminoethanethiol dioxygenase were >40-fold induced in both NILs in response to Mo. Genes encoding for fumarylacetoacetase, 2-isopropylmalate synthase B and D-3-phosphoglycerate dehydrogenase were specifically induced in response to HT.

In contrast, genes involved in the TCA cycle, carbon and amino acid metabolism were largely suppressed by HT treatment. Nonetheless, several genes encoding for S-adenosyl-L-methionine, and one gene encoding for starch mobilization (*Amy3B*; LOC_Os09g28420) were induced >8-fold in both NILs in response to HT. In addition, genes encoding for fumarylacetoacetase (LOC_Os02g10310), 2-isopropylmalate synthase B (LOC_Os12g04440) and D-3-phosphoglycerate dehydrogenase (LOC_Os04g55720) were more induced in response to HT than Mo. Some genes encoding for enzymes involved in the transfer of glucose from UDP-glucose to lipids were background specific. For instance, genes encoding for UDP-glucuronosyl (LOC_Os09g34250) and UDP-glucosyl transferase (LOC_Os02g10880) were

2.6 Expression of PRs and secondary metabolism genes

Pathogenesis related (PR) proteins are induced by pathogenic infection through the coordinated action of SA, JA and ET signaling pathways. We found a significant overlap in transcript accumulation of several PR encoding genes between HT and Mo treated plants (Fig. 9). At HT, we detected a significant induction of several PR6 genes in both backgrounds. Some PR genes were background specific. For instance, PR3 (e.g., *CHIT17*; LOC_Os05g33130) and PR2 (Gns6; LOC_Os01g71350) were significantly induced in CO+35°C but not in LT+35°C, whereas PR-5 (TLP-D34; LOC_Os12g43380) was >10-fold induced in LT+35°C but not in CO+35°C. Transcript accumulation of the gemine like protein (GLP) encoding genes also overlapped between the two backgrounds. For instance, *GLP1* (LOC_Os04g22080) was >100-fold expressed in LT+35°C+Mo, but was not significantly expressed in CO. A subtilisin-like serine proteinase, *OsSub13*, belonging to a class of pathogenesis-related proteins (Jorda and Vera 2000), and a protein-disulfide isomerase (*OsPDIL1-1*), involved in the biosynthesis of a group of plant defence peptides called cyclotides (Jennings et al., 2001), were more induced in LT than in CO in response to Mo.

Secondary metabolism, including the production of phytoalexins, plays a significant role in plant defence against pathogens. Activated secondary metabolism sub-pathways included the phenylpropanoid, flavonoid and terpenoid metabolites (Additional file S2). The expression of genes encoding for the biosynthetic enzymes of diterpenoid phytoalexins was either low or down-regulated in plants exposed to HT. Nonetheless, terpene synthase (LOC_Os04g27430) was induced >30-fold in CO+35°C and LT+35°C. On the other hand, Mo infection increased transcript levels of phytocasin biosynthetic gene (*OsKSL7*; LOC_Os02g36140) in both NILs. Furthermore, we detected the induction of a NADPH-dependent codeinone reductase (LOC_Os10g02490) after HT treatment in both NILs. We speculate that codeinone reductase, together with the aforementioned 12-oxophytodienoate reductase and OMTs may play major roles in the production of secondary metabolites.

2.7 Validation of differential gene expression of known temperature and infection responsive rice genes by qRT-PCR

The expression profiles of known responsive rice genes were validated by analyzing their up-regulation at 35°C and during 35°C+Mo interaction (Fig. S4A-I). The response of all the measured genes was similar but the magnitude of responses was greater in RNA-Seq than in qPCR. This analysis confirmed the high induction of nine genes, including *OsFKBP65*, in plants exposed to 35°C compared to 28°C, prior to Mo inoculation.

3. Discussion

In this study, we tested the hypothesis that elevated temperature would weaken rice defence against Mo, irrespective of the genetic backgrounds in which the R gene (*Pi54*) is introgressed. We used two rice genetic backgrounds with (CO and LT) and without (Co39 and LTH) *Pi54*. After exposure to 28°C and 35°C for 7 d, the two backgrounds showed contrasting responses to Mo infection. At 28°C, CO, Co39 and LTH displayed a more severe disease phenotype than LT. CO and LT were used for biochemical and transcriptome analysis to determine the cause of contrasting reactions to Mo despite carrying the same R gene. At 28°C, LT plants showed more intense callose deposits and cell wall fluorescence than CO, suggesting that the genetic background of LT facilitated the function of *Pi54* more than the background of CO. Interestingly, upon pre-inoculative exposure to 35°C, resistance responses were enhanced in both NILs. Moreover, callose deposits and cell wall fluorescence of the attacked epidermal cells were more intense at 35°C than 28°C.

Intriguingly, these HT induced rice defence responses were contrasted by a decrease in *Pi54* expression at HT. The reduced expression of *Pi54*, in spite of enhanced resistance at HT, suggested that HT probably enhanced plant resistance by either increasing the baseline levels of endogenous *Pi54* downstream products thereby decreasing its expression or acted independent of *Pi54*.

328 In view of these findings, it was critical to further define through biochemical and transcriptome analysis the
329 factors that reinforce plant defences against pathogens at HT. We will jointly discuss the gene expression data and
330 the defence related biochemical changes to ascertain their functional connection in this study.

331 Our data showed a substantially higher number of DEG in Mo infected plants than HT treated plants,
332 suggesting that the rice-Mo interaction underlies a more intense genome transcriptional regulation than HT stress.
333 Exposure to 35°C+Mo was also significantly different from HT (single stress) induced transcriptional changes,
334 and genes expressed in 35°C+Mo clustered closely with 28°C+Mo, indicating that transcriptional changes due to
335 HT were overridden by Mo infection. Thus, plants probably tend to shift their response to pathogens when HT and
336 pathogen stresses occur simultaneously. A strong and simultaneous induction of some heat and pathogen stress
337 response genes in combined stress (35°C+Mo), however, suggested a considerable additive role of temperature in
338 invoking plant defence response. Several of these genes were enriched in oxidation-reduction processes
339 (GO:0055114), suggesting that oxidation-reduction reactions were the likely upstream signals in rice defence
340 response to both HT and Mo. However, oxidation-reduction was similarly enriched in a susceptible reaction (CO
341 plants infected with Mo at 28°C). This suggests that other mechanisms appeared to be necessary for successful
342 restriction of pathogenesis at 35 °C. These include, but probably not limited to, the induction of callose deposition.
343 Luna et al. (2010) previously reported that callose deposition is preceded by PAMP triggered H₂O₂. Our
344 biochemical analysis showed significantly higher H₂O₂ levels in both NILs after HT treatment, suggesting that an
345 earlier activation of a defence response associated with callose accumulation was prompted by HT associated
346 induction of ROS prior to Mo infection. The higher induction of cell membrane NADPH oxidases (*OsNox9* and
347 *RbohD*) in plants pre-exposed to HT followed by Mo inoculation is consistent with increased callose
348 accumulation. However, the role of HT in inducing plant defence against pathogens has been challenged
349 previously. HT was reported to cause downregulation of superoxide, NADPH oxidases and hypersensitive
350 response (HR) in *Nicotiana tabacum* and compromised N gene mediated resistance to *Tobacco mosaic*
351 *virus* (Király et al., 2008). Contrary to these findings, it is possible that HT reprograms signal transduction
352 components, including stress-generated ROS, to induce or compromise disease resistance depending on the host
353 plant and probably the duration of HT stress. The plasticity of CO in this study attests to this, and suggests that
354 this plasticity could be employed by some crops for adaptation to both HT and pathogen infection in a changing
355 climate. This may also include adaptability to ROS accumulation and signal transductions that modulate redox
356 balances by activating either peroxidases (POX), plasma membrane calcium ion channels or both.

357 More than 20 POX genes were differentially expressed in this study. However, POX activity was not
358 significantly different between CO plants exposed to 28°C and 35°C followed by Mo inoculation at 24 hpi.
359 Moreover, POX activity was significantly lower in LT than CO plants infected with Mo at 48 hpi. This
360 inconsistency, despite higher POX activity in CO at 35°C than 28°C, showed ambiguity in POX involvement in

rice defence against Mo, and would require further experimental proof. On the other hand, calcium metabolism is associated with ROS signaling, and ROS are apparently required to prime Ca^{2+} influx after elicitation (Torres et al., 2006). Calcium entering the cytoplasm is also implicated in activating the plasma membrane localized and Ca^{2+} dependent 1,3-B-glucan synthase, thereby effecting callose deposition in a localized manner (Kauss 1990). We detected several up regulated genes involved in calcium signaling in response to HT and Mo infection; and cellular response to calcium ion (GO:0071277) was enriched consistently with hydrogen peroxide biosynthetic process (GO:0050665). It appears therefore that Ca^{2+} is involved in callose synthesis, and the interplay between ROS, Ca^{2+} and callose likely determines the dynamics associated with pre-penetrative defence at HT.

There are also several reports on abiotic and biotic stress induction of ROS and their interaction with phytohormones and associated signaling pathways involved in local and systemic plant defence (reviewed by Ben Rejeb et al., 2014). With respect to systemic defence, conventional responses include production of SA, JA, ET and ABA, and subsequently, their derivatives, e.g., MeSA and MeJA and catalytic enzymes. We therefore also focused on these phytohormones and their corresponding genes. Salicylic acid is the most reported phytohormone in plant defence; however, there are conflicting reports on its role against pathogens, with some reports suggesting that SA appears to be simply an effective therapeutic agent (Rivas-San and Plasencia, 2011). Our data showed decreased levels of SA in LT compared to CO at 28°C, despite increased levels in both NILs at HT (single stress). Considering that CO had a more severe disease phenotype compared to LT, it is possible that the increased level of SA in CO at 28°C did not induce effective defence response against Mo. This finding raises the possibility that SA may not directly confer rice blast resistance.

Earlier reports have shown that JA-induced *AtBSMT1* probably depletes the SA pool in plants by antagonizing the SA synthesis pathway (Chen et al., 2003). It is tempting to suggest that the strong induction of *OsBISAMT1*, which catalyzes the conversion of SA to MeSA, at HT is consistent with this. In addition, it has been suggested that HT may promote JA synthesis and signaling to repress SA signaling leading to a suppression of SA-mediated defence responses (Wang et al., 2009). Studies have also shown that exogenous SA promotes the JA-dependent induction of the defensin gene *PDF1.2*, when applied at low concentrations (Kazan and Manners 2008). Considering that MeSA is a critical mobile signal in plant tissues and occurs at low concentrations compared to SA (Park et al., 2007), our results suggest that MeSA instead of SA modulates JA in activating genes encoding for defensins (e.g., *DEFL9*; LOC_Os06g22919 and *DEFL49*; LOC_Os04g31250) at 35°C. A key question is whether the interaction between MeSA and JA is sufficient to induced effective resistance or other phytohormones are involved? Mechanistic details on such interaction are still lacking. However, it should be noted that ABA is involved in signaling processes inducing JA-dependent defence responses in systemic tissues through its activity on MYCs (Nguyen et al., 2016). MYCs induce plant resistance by regulating many genes encoding JA metabolic enzymes such as LOX, allene oxide synthase (AOS), allene oxide cyclase (AOC) and 12-oxophytodienoate

394 reductase (OPR) (Takagi et al., 2016). A strong induction of *OsBBD1*, involved in ABA derived callose
395 deposition, and 12-oxophytodienoate reductase, which catalyzes the last step in the biosynthesis of JA, in this
396 study, suggests that ABA and JA signalling are key regulatory steps in HT induced resistance pathway. JA and its
397 methyl ester, methyl jasmonate (MeJA), probably complement each other in tailoring downstream defence
398 pathways, considering that Jasmonate-O-methyltransferase (JMT), which converts JA to MeJA and other lipid
399 derivatives, was strongly up-regulated in plants exposed to 35°C+Mo. Endogenous JA/MeJA has also been
400 reported to regulate phytoalexin biosynthesis in rice (Shimizu et al., 2012). Plants pre-exposed to 35°C followed
401 by Mo infection, exhibited increased expression of JA regulated genes, including flavonoid-7-OMT
402 (LOC_Os09g17560) and LOC_Os12g13800, that have been implicated in sakuranetin biosynthesis (Shimizu et
403 al., 2012; Park et al., 2013). This suggests that sakuranetin is one of the crucial components contributing to rice
404 resistance against Mo at HT.

405 Additional signals from HT exposure could be explained by other mechanisms such as ethylene signaling
406 pathways. Ethylene is synthesized from methionine and has been shown to increase in methionine treated rice
407 leaves (Nakazato et al., 2000). The induction of a putative methionine-S-oxide reductase (LOC_Os03g04370, >8-
408 fold), which reverses the oxidation of methionine to methionine sulfoxide (MetSO), and several SAM dependent
409 carboxyl methyl transferases, in this study, suggests that methionine is synthesized in response to HT for multiple
410 purposes. Consistent with this, Module 13 is associated with cell cycle and S-adenosylmethionamine biosynthetic
411 process. It is possible that increased synthesis of methionine and its derivatives contributes to host resistance.
412 Thus, in addition to ABA, H₂O₂, JA, callose deposition, defensins and sakuranetin biosynthesis, we speculate that
413 the integrated signals from methionine and its derivatives are crucial for inducing seedling resistance to Mo.

414 Correlating with this was the increased transcript levels of *MBF1c* in CO plants exposed to 35°C compared to
415 28°C. *MBF1c* is a transcriptional co-activator required for basal thermotolerance and functions upstream to
416 ethylene during heat stress (Suzuki et al., 2011). Constitutive expression of *MBF1c* in transgenic Arabidopsis
417 plants increases resistance against bacterial infections, heat and osmotic stress by partially activating the ethylene-
418 response signal transduction pathway, but without significant increases in SA levels (Suzuki et al., 2008).
419 Considering that ethylene burst is one of the earliest events in HR (Seo et al., 2011), the up-regulation of *MBF1c*
420 in plants exposed to 35°C probably fine-tunes ET signaling pathway. An increased transcript level of *ERF5*, an
421 orthologue of Arabidopsis *ERF96*, is consistent with this, and suggests that ET is involved in HT induced plant
422 resistance. However, ET-mediated responses are also synergistically regulated by gibberellins (GAs),
423 brassinosteroids (BRs) and Auxins (AUXs). Increased expression of genes in the AUX, GA and BR signaling
424 pathways and antagonistic regulators such as *OsIAA13*, *OsGH3.2*, *OsGH3.5*, *ARF19* and *ERF5* was detected in
425 this study, suggesting that internal dynamics are involved in fine-tuning the defence responses associated with
426 these multiple hormones.

Downstream of these hormonal dynamics are various kinds of defence related transcription factors, including U2 small nuclear ribonucleoprotein auxiliary factor (*U2AF*), WRKYs, zinc finger RING type, OsEREBPs, MYBs, HB, bHLH, zinc finger (ZF) family and basic region/Leu zipper motif (bZIP) family. *U2AF* plays a crucial role in the recognition of a 3'-splice site which contributes to the fine-tuned control of pre-mRNA splicing. *U2AF* was expressed in all treatments and could be an important candidate to explore the crosstalk between HT and pathogen response. One of the *OsWRKY IIa* encoding genes, *OsWRKY84*, was also strongly induced in CO+35°C+Mo than CO+28°C+Mo. *OsWRKY84* was previously reported to be over-expressed in response to xylanases in suspension-cultured rice cells. Mo is known to induce endoxylanases for horizontal expansion in infected leaves (Nguyen et al., 2011). Since the functional role of *OsWRKY84* is not well understood, it could be a potential target for further investigation.

Downstream of these TFs are other HT and Mo response genes. Perhaps the most compelling observation was the significant higher expression of Peptidyl-prolyl isomerases (*OsFKBP65*) in CO+35°C+Mo and LT+35°C+Mo. *OsFKBP65* positively modulates heat tolerance by interacting with *AtHSP90.1*, and increases *AtHsfA2*-mediated accumulation of small HSPs (Meiri and Breiman, 2009). Interestingly, HSP90 is a hub gene of module 7. This module was highly correlated with Mo infection. The GO analysis indicated that the genes in module 7 are involved in signal transduction and defence response to bacterium. Thus, HSP90 probably contribute to resistance at HT through its interaction with *OsFKBP65*. Previous studies also implicate *FKBPs* in disease resistance. A peptidyl-prolyl, named microbial factor 3 (*MF3*), was previously shown to induce resistance to *A. longipes* and TMV in tobacco, and *Bipolaris sorokiniana* in wheat (Shumilina et al., 2006). A recent study by Narsai et al., 2013, also showed over representation of peptidyl isomerases in rice against bacterial and parasitic infection. Thus, we speculate that *OsFKBP65* is involved in multiple roles that may need to be examined further.

Other genes identified in response to HT included LOC_Os03g62720, an exosome complex exonuclease involved in the degradation of unstable mRNAs containing AU-rich elements (AREs) within their 3'-untranslated regions. This gene is a hub gene of module 1, and this module positively correlates with HT treatment. Genes in module 1 are significantly enriched in translation regulator activity (GO:0045182), suggesting that LOC_Os03g62720 may play a key role in regulating protein synthesis at HT.

Overall, the present study provided a broad insight into potential defence responses of rice to HT and Mo. We have shown that rice lines carrying *Pi54* respond to Mo by rapid induction of callose and H₂O₂, and these resistance responses are stronger at 35°C than 28°C. Previously we demonstrated that a rice cultivar Nipponbare is susceptible to *M. oryzae* at HT (Onaga et al., 2017). Importantly, in the same study, pathogen putative effector transcript abundance correlated with susceptibility at HT. Contrary to the hypothesis that in planta pathogen effector transcript abundance increases host susceptibility at HT, our results demonstrate that plant plasticity is

one of the main strategies for adaptation to both HT and pathogen infection in a changing climate. For instance, we have shown that despite the impact of HT on *Pi54* transcription, the accumulation of modest *Pi54* transcripts or downstream products reliably detect the pathogen when augmented with pre-exposure to HT. Among the defence networks probably associated with HT are the defence dynamics associated with phytohormone interactions. Crosstalk between MeSA, ABA, MeJA/JA and ET and their signaling pathways appears to be a key defence mechanism of rice against Mo. We hypothesize that ABA accumulates at HT and its signaling pathway is required to increase callose accumulation in infected plants. The defence response associated with MeJA/JA and ET is secondary and ABA possibly fine tunes their synthesis and signaling. Figure 10 shows a hypothetical example of a reaction pathway leading to a defence response in rice against Mo. Due to the strong mutual antagonism between phytohormones, e.g., ET and ABA, our findings do not necessarily rule out the possibility that some defence pathways may be affected negatively, although this may depend on the host plant. Additional focused analyses on these hormones will be required in the future to further evaluate our multi-hormone interaction hypothesis.

4. Material and methods

4.1. Plant and fungal material and rice blast inoculation

Two genotypes, Li-Jiang-Xin-Tuan-He-Gu (LTH, *Oryza japonica*) and Co39 (*Oryza indica*) with and without *Pi54*, were used as hosts in this experiment. LTH is widely identified as a genetic background for the development of NILs, whereas Co39, a short duration *O. indica* cultivar from India with high susceptibility to tropical blast isolates, was recently introduced as a second background for the development of NILs. The experiment included three biological replicates per treatment. Seeds of LTH and Co39 (lacking or having *Pi54*) were soaked in water, incubated and pre-germinated on filter papers in Petri dishes in the dark at 32°C until the seeds sprouted at about 72 hours. Seedlings were transferred to soil filled pots at a rate of 3 seedlings per pot. The plants were grown in the greenhouse at 27±2°C with a photoperiod of 14 h for 21 d and then transferred to growth chambers at 28°C and 35°C for 7 days. After HT exposure, all plants were kept at 28°C for inoculation to allow fungal infection at equal conditions. Inoculation was performed using *M. oryzae* isolate, TAN211.16, from Tanzania. This strain was selected for rice-Mo interaction at high temperature study because it was aggressive on most of the isogenic lines tested in our greenhouse, including *Pi54* in Co39 background. TAN211.16 was grown in petri dishes containing V8 agar under 14 h of fluorescent light at 25°C for 12 days. Three days prior to inoculation, cultures were exposed to low levels of visible and near-UV radiation to maximize sporulation. Inoculation was performed by spraying a conidial suspension containing 0.05% Tween 20 onto rice seedlings. The inoculated plants were incubated for 24 h at 28°C and 90% relative humidity in the dark and then transferred

back to 12 h of light for additional 24 h before leaf sampling. Some plants were maintained at 35°C and 28°C for 10 days after inoculation for disease assessment. Disease assessment was repeated two times to confirm the presence of lesions. Disease reaction ratings and disease progress calculations were conducted as previously described by Onaga *et al.* (2015).

4.2. Histochemistry and microscopy

Callose staining was carried out according to the method of Hao *et al.* (2008). Observations were made with a light microscopy (Leica, Bensheim, Germany) equipped with a Leica DC300F camera. The yellow spots that corresponded to the stained callose were analyzed for number of pixels with the GNU Image Manipulation program (GIMP, <http://www.gimp.org/>). Callose intensity was expressed as the percentage of yellow pixels per total pixels on the digital photographs of the infected areas.

4.3 Measurement of hydrogen peroxide (H_2O_2)

Leaf samples were collected 24 and 48 hpi and immediately ground in liquid nitrogen. The ground powder was extracted in 5% w/v metaphosphoric acid. The samples were centrifuged at $5000 \times g$ for 60 min. The supernatant was adjusted to pH 6.5 using 1 M tricine in 6 M NaOH, and the initial amount of NADH was measured at 340 nm (OD_1) using SPECORD 40 (Analytik, Jena, Germany). Horseradish peroxidase ($350 \mu l l^{-1}$; $2500 U ml^{-1}$) dissolved in 25mM sodium phosphate buffer (pH 7.0) was added and the mixture was incubated at room temperature. The absorbance changes at 340 nm were measured at 25°C, 60 min after the addition of horseradish peroxidase (OD_2). The H_2O_2 contents were determined by a calibration curve using known amounts of H_2O_2 and expressed as micromoles per gram of fresh weight (FW).

4.4 Guaiacol peroxidase activity

Fresh leaf samples weighing 300 mg were homogenized in 3 ml of cold 100mM Na-phosphate buffer (pH 7.0). The homogenates were centrifuged at $5,000 \times g$ for 30 min, and the dialyzed enzyme extract was used for the assay. Assay mixture in a total volume of 3 ml contained 100 mM Na-phosphate buffer (pH 7.0), 50 μl of 20mM guaiacol solution, 30 μl of 12.3mM hydrogen peroxide and 50 μl of enzyme extract. Increase in absorbance was measured at 420 nm (extinction coefficient of $26.6 mM^{-1} cm^{-1}$) at 30 s intervals up to 3 min, using SPECORD 40, Analytikjena AG (Germany). Enzyme specific activity was expressed as mKatal.

526 4.5 *Salicylic acid (SA) determination*

527

528 Accumulation of SA was examined using a Merck-Hitachi high performance liquid chromatography (HPLC;
529 Merck KGaA) as described previously (Kamble and Bhargava, 2007).

530

531 4.6 *Real-time RT-PCR analysis*

532 Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's
533 instructions. Gene-specific primers were designed using the software Primer3Plus
534 (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>). Primer sequences are listed in (Additional
535 file S2). Relative gene expression was determined by quantitative real time PCR (RT-PCR) on a Bio-Rad
536 CFX384 real time system using SYBR Green dye (Bio-Rad, California, USA). Reaction was performed in a 10
537 μ L reaction mixture that consisted of 5 μ L of 2 X SYBR Green PCR Master Mix, 400 nM of both forward and
538 reverse primers and 20ng of cDNA. Thermal cycling was started at 95°C for 15 min, followed by 35 cycles of
539 95°C for 20 s, 62 °C for 30 s and 72°C for 20 s. The final elongation was performed for 5 min at 72°C. The
540 melting curve was obtained by heating the reaction temperature to 95°C for 1 min, cooling to 55°C for the next
541 minute, then slowly increasing the temperature from 65°C to 95°C in a rate of 0.5°C s⁻¹. The primer efficiency
542 was determined using a standard curve. Standard curves with a correlation ≥ 0.98 and efficiency between 90%
543 and 110% were considered as appropriate for calibration, and the efficiency was used for the calculation of
544 relative gene expression. The data were normalized to the geometrical mean of a reference gene
545 (LOC_Os07g02340), previously found to show a stable expression pattern across biotic and abiotic stress (Narsai
546 et al., 2010). The Pfaffl method was used to analyse the qRT-PCR data in Microsoft Excel, and the calculated
547 values were expressed relative to the control.

548

549 4.7 *Illumina HiSeq2000 sequencing*

550

551 The RNAseq gene expression analysis experiment was designed as a three-factor experiment with eight
552 treatments: (1) LT+35°C+Mo; (2) LT+28°C+Mo; (3) LT+35°C; (4) LT+28°C; (5) CO+35°C+Mo; (6)
553 CO+28°C+Mo; (7) CO+35°C; (8) CO+28°C. These treatments represent the genetic background (LTH or Co39),
554 temperature (28°C and 35°C) and *M. oryzae* inoculation (Mo). The experiment included three biological
555 replicates. Each sample represented leaf material from five plants. Total RNA was isolated using Trizol reagent
556 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The integrity of each RNA sample
557 obtained was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a
558 Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

Two micrograms of total RNA were subjected to library preparation using the ‘TruSeq RNA Sample preparation v2 Guide’ following the manufacturer’s instructions. Libraries were clustered for sequencing using Illumina cBOT Cluster generation system and TruSeq v3 chemistry. Fifty bp single-end sequencing was performed on the HiSeq2000 using three barcoded libraries per lane.

563

4.8 Preprocessing, mapping, and analysis of illumina reads and subsequent gene expression data

565

Sequence images were transformed with Illumina software BaseCaller to bcl files, which were demultiplexed to fastq files with CASAVA v1.8.2 allowing for one mismatch in the indices. Sequences were aligned by Bowtie2 v2.0.2 to the Ensembl cDNA release 16 for *Oryza sativa*. Subsequently, conversion and sorting of the resulting sam files to sorted bam files was conducted with samtools 0.1.18 followed by counting the reads to each gene. Genes exceeding 50 counts for at least one sample were filtered, followed by normalization via Trimmed Mean and Median-values. In addition, significant differences in the sequencing depth were normalized by expressing the counts in counts-per-million (CPM) so that the counts for each profile are normalized by the total read count of the profile. Counts were then more naturally compared between profiles. Data was analyzed in the R/Bioconductor environment loading DESeq, lattice, estrogen, gplots and colorRamps, biomaRt packages. Estimation of dispersions and testing for differentially expressed genes based on a χ^2 likelihood ratio test, assuming negative binomial data distribution, were computed via DESeq. Candidate genes were filtered to a minimum of 4x fold change and FDR-corrected p -value<0.05. Gene annotation was conducted using Ensembl Biomart via R.

Functional association enrichment analysis was conducted via the R package goseq. The identification of cis elements was performed using Osiris software (available at <http://www.bioinformatics2.wsu.edu/cgi-bin/Osiris/cgi/home.pl>), which searches known cis elements from PLACE - Plant Cis-acting Regulatory DNA Elements (Higo et al., 1999) database. Gene co-expression modules (clusters) were identified using Weighted Gene Co-expression Network Analysis (WGCNA) (see the result section for details).

584

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592

593 **List of figures and tables**

594 **Fig. 1. (A).** Disease phenotypes of *M. oryzae* (Mo) on LT and CO at 28°C and 35°C at 5 dpi **(B)** Disease progress
595 of *M. oryzae* on Pi54(CO) and Pi54(LT, and their background parents LTH and Co39 at 28 and 35°C. Same letters
596 above standard error bars indicate no significant difference. Plants were pre-exposed to 35°C and 28°C for 7 d
597 prior to Mo inoculation and all plants were incubated at 28°C after Mo inoculation for 24 hours prior to re-
598 exposure to 35°C and 28°C.

599 **Fig. 2.** Callose deposition visualized by aniline blue staining of epidermal cells 48 hpi. CO+35°C+Mo and
600 LT+35°C+Mo represent plants grown at 35°C for 7 d before inoculation; CO+28°C+Mo and LT+28°C+Mo are
601 plants grown at 28°C before inoculation. Callose deposits were more pronounced at 35°C compared to 28°C in
602 both genetic backgrounds. Quantification of callose was performed by determining the percentage of yellow
603 pixels related to total pixels of photographs. Data show means $\pm$ standard deviation. Letters indicate significant
604 differences between treatments in each time point ($p < 0.05$; least-significant difference test). The pictures of
605 callose deposition are presented below the corresponding treatments.

606 **Fig. 3. (A)** Hydrogen peroxide (H_2O_2) quantification in micromoles per gram and **(B)** POX activity measured in
607 mkatal (kat) per gram, of fresh leaf tissue weight (FW) collected from healthy (Mock), *M. oryzae* inoculated
608 (28°C+Mo), high temperature (35°C), and high temperature plus *M. oryzae* inoculated (35°C+Mo). The samples
609 were taken at 24 and 48 hours post inoculation (hpi). Mock28 and 35°C samples were taken at 0 hpi. One katal
610 (kat) corresponds to the quantity of enzyme activity that transforms 1 mol S^{-1} of substrate. Data are presented as
611 mean $\pm$ standard deviation. Different letters denote significant differences at $P \leq 0.05$.

612 **Fig. 4. (A).** Comparison of SA accumulation in CO and LT at 28° and 35°C and **(B)** PAL activity expressed as
613 micromolar change in cinnamic acid per minute and gram of fresh leaf tissue weight (FW) collected from healthy
614 (Mock), *M. oryzae* inoculated (28°C+Mo), high temperature (35°C), and high temperature plus *M. oryzae*
615 inoculated (35°C+Mo). The samples were taken at 24 and 48 h after inoculation (hpi). Mock and 35°C, represent
616 mock samples exposed to 28°C and 35°C without pathogen inoculation. 28°C+Mo and 35°C+Mo represent
617 samples infected with *M. oryzae* at 28 and 35°C. Samples were analyzed at 24 and 48 hours post inoculation
618 (hpi). Different letters denote significant differences at $P \leq 0.05$.

619 **Fig. 5.** Heatmap showing the gene expression clusters and sample clusters of DEG. Samples represent fresh leaf
620 tissue collected from healthy (Mock28°C), 7 day high temperature treatment (CO+35°C, LT+35°C), and Mo
621 inoculated (LT+28°C+Mo, LT+35°C+Mo, CO+28°C+Mo and CO+35°C+Mo), respectively. The color bar
622 represents the log2 of fold change values compared to Mock28°C, ranging from blue to yellow.

623 **Fig. 6.** Significant Gene Ontology (GO) enriched terms from differentially up-regulated genes (fold change 4;
624 FDR, 0.05) in rice plants exposed to 35°C for 7 days. Comparison of the two genetic backgrounds: (A), CO+35°C
625 and (B), LT+35°C, respectively.

626 **Fig. 7.** Correlation matrix of module eigengene values obtained for DEG and treatments **based on** patterns of
627 their co-expression. Infect represents Mo infection, hitemp; high temperature and genotype; genetic background.
628 Within each cell, upper values are correlation coefficients between module eigengene and the treatments; lower
629 values are the correspondent P-value. Each of the cells was labelled with color that signifies the level of
630 correlation. Red represents strong positive correlation whereas blue represents strong negative correlation.

631 **Fig. 8.** The effect of temperature on transcript levels of *Pi54* in LT and CO. The samples were taken at 24 and 48
632 hours post inoculation (hpi). Mock and high temperature samples were taken at 0 hpi. Data are presented as mean
633 fold increases over control $\pm$ standard deviation. Different letters denote significant differences at $P \leq 0.05$.

634 **Fig. 9.** Heatmap view of the previously reported PR genes differentially expressed in the six treatments I;
635 CO+35°C, II; LT+35°C, III; CO+28°C +Mo, IV; CO+35°C +Mo, V; LT+35°C +Mo, VI; LT+28°C +Mo. Cluster
636 analysis of expression profiles of the 78 genes. Yellow indicates higher expression while blue represents lower
637 expression.

638 **Fig. 10.** Hypothetical defence responses activated in rice–*M. oryzae* interaction. Prior to infection, ROS are
639 induced by high temperature. In an attempt to manipulate the plant, the fungus secretes effectors to avoid being
640 detected. The R genes and associated RGAs perceive the signals, amplifying ROS. The resulting ROS serve two
641 functions; ABA associated induction of callose at the site of pathogen entry and neighboring cells; and JA
642 synthesis. JA together with methionine probably regulates biosynthesis and activity of JOMTs, SAMTs and
643 ethylene. SAMTs modulate SA conversion to MeSA while JOMTs modulate JA conversion to a more potent
644 MeJA, which probably complements the triggering of phytoalexins, defensins and PRs. Both MeSA and ethylene
645 mobile signals may prime the neighboring cells defences. Increased expression of negative defence regulators,
646 e.g., Auxins, is counteracted by the activity of *OsIAA13*, *OsGH3s*. The red lines indicate the first defence
647 responses that restrict the pathogen. The purple lines indicate the subsequent defences (in the order of 1, 2, 3 and
648 4) that further restrict and probably kill the pathogen. The blue lines indicate interactions that may be necessary to
649 modify cellular response under stressful conditions for multi-protection. The black lines indicate additional
650 probable changes that may be required to induce a defence response.

651

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35°C+Mo

28°C+Mo

CO

35°C+Mo

28°C+Mo

LT