Title

Rice early molecular response against M. oryzae

Journal

Molecular Plant Pathology, 17(3)

ISSN

1464-6722

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Publication Date

2016-04-01

DOI

10.1111/mpp.12286

Peer reviewed

Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) 17(3), 354–368 DOI: 10.1111/mpp.12286

The durably resistant rice cultivar Digu activates defence gene expression before the full maturation of *Magnaporthe oryzae* appressorium

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SUMMARY

Rice blast caused by the fungal pathogen *Magnaporthe oryzae* is one of the most destructive diseases worldwide. Although the rice–*M. oryzae* interaction has been studied extensively, the early molecular events that occur in rice before full maturation of the appressorium during *M. oryzae* invasion are unknown. Here, we report a comparative transcriptomics analysis of the durably resistant rice variety Digu and the susceptible rice variety Lijiangxintuanheigu (LTH) in response to infection by *M. oryzae* (5, 10 and 20 h post-inoculation, prior to full development of the appressorium). We found that the transcriptional responses differed significantly between these two rice varieties. Gene ontology and pathway analyses revealed that many biological processes, including extracellular recognition and biosynthesis of antioxidants, terpenes and hormones, were specifically activated in Digu shortly after infection. Forty-eight genes encoding receptor kinases (RKs) were significantly differentially regulated by *M. oryzae* infection in Digu. One of these genes, *LOC_Os08g10300*, encoding a leucine-rich repeat RK from the LRR VIII-2 subfamily, conferred enhanced resistance to *M. oryzae* when overexpressed in rice. Our study reveals that a multitude of molecular events occur in the durably resistant rice Digu before the full maturation of the appressorium after *M. oryzae* infection and that membrane-associated RKs play important roles in the early response.

Keywords: blast disease, durable resistance, *Magnaporthe oryzae*, receptor kinase, rice, transcriptional profiling.

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INTRODUCTION

Rice blast caused by the fungal pathogen *Magnaporthe oryzae* is one of the most destructive diseases of rice, reducing global yields annually by 10%–15% (Dai *et al*., 2007; Skamnioti and Gurr, 2009; Talbot, 2003). Advancing knowledge of the molecular events governing the rice–*M. oryzae* interaction will contribute to methods for improving resistance to this serious disease (Chen and Ronald, 2011).

Magnaporthe oryzae is able to infect all rice tissues, including roots, leaves, stems and panicles (Ribot *et al*., 2008; Sesma and Osbourn, 2004). Infection is initiated by conidia that attach firmly to rice leaves and germinate within a few hours. After the germ tube ceases polar growth, the tip begins to swell, 2–4 h post-inoculation (hpi), and then forms a mature appressorium, which penetrates the underlying tissue within 24 hpi (Ebbole, 2007; Howard and Valent, 1996; Talbot, 2003). The primary hyphae differentiate into bulbous invasive hyphae in the cells at 32–36 hpi. The fungus then spreads into neighbouring cells to form necrotic lesions throughout the next several days (Kankanala *et al*., 2007).

To withstand *M. oryzae* attack, rice has evolved two main immune systems, namely pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm *et al*., 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006). PTI is mediated by pattern recognition receptors (PRRs) through the recognition of PAMPs. Because PAMPs are widely conserved in pathogens and are often essential for pathogen virulence or survival, PTI is predicted to confer durable and broad-spectrum resistance. ETI is typically mediated by nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins that recognize effectors secreted into the plant cell by the pathogen. Unlike PAMPs, effectors are highly variable and thus the disease resistance mediated by ETI is generally race specific (Dodds and

Table 1 Summary of previous transcriptomic data from the analysis of the rice–*Magnaporthe oryzae* interaction.

Reference	Rice cultivars (resistant/susceptible)	*Magnaporthe oryzae* race	Time point after *Magnaporthe oryzae* inoculation
Li *et al*. (2006)	Near-isogenic lines H7R(*Pi-k*)/H7S	ZB1	24 h
Vergne *et al*. (2007)	Near-isogenic lines R-IR64(*Pi33*)/S-IR64	PH14	24 h and 48 h
Bagnaresi *et al*. (2012)	Gigante Vercelli/Vialone Nano	It2, It3 and It10 (mixed)	24 h
Vijayan *et al*. (2013)	None/HR-12	Mo-si-63	6 h
Wei *et al*. (2013)	LTH/IRBL18 (*Pi1*) and IRBL22 (*Pi9*)	CH63	24 h
Wang *et al*. (2014a)	Jinheung/Jinheung	KJ401 (incompatible) and KJ301 (compatible)	12 h and 48 h

Rathjen, 2010; Jones and Dangl, 2006; Tao *et al*., 2003; Vergne *et al*., 2010).

In rice, a well-studied PTI system is mediated by the LysM domain-containing receptor CEBiP (chitin elicitor-binding protein) and its co-receptor OsCERK1 (Miya *et al*., 2007; Shimizu *et al*., 2010). On recognition of the synthetic or pathogen-derived chitin, CEBiP and CERK1 form a receptor complex on the rice cell membrane and induce a series of defence responses, including the activation of mitogen-activated protein kinases (MAPKs) (Kishi-Kaboshi *et al*., 2010), reactive oxygen species (ROS) production, defence gene expression, phytoalexin production and the accumulation of phosphatidic acid (PA), a signal molecule important for the plant response to both biotic and abiotic stresses (Kaku *et al*., 2006; Testerink and Munnik, 2005; Yamaguchi *et al*., 2004, 2005). A chimeric receptor, CRXA, consisting of the extracellular portion of CEBiP and the intracellular portion of the rice XA21 PRR, is also able to perceive the chitin signal and gains the ability to confer resistance to *M. oryzae* (Kishimoto *et al*., 2011).

In ETI, rice NBS-LRR proteins interact directly or indirectly with *M. oryzae* effectors (Liu *et al*., 2013). The interactions trigger downstream defence reactions, such as increased production of antifungal secondary metabolites (Peters, 2006), cell wall thickening (Huckelhoven, 2007), pathogenesis-related (PR) protein expression (Jwa *et al*., 2006) and programmed cell death at sites of invasion (Greenberg and Yao, 2004). Although PTI and ETI use different receptors at the early stages of infection, they share common molecular processes, such as the activation of MAPK cascades, utilization of a shared set of transcription factors (TFs) and induced expression of *PR* genes (Eulgem and Somssich, 2007; Mishra *et al*., 2006).

Previous transcriptional profiling studies have been performed on the rice–*M. oryzae* interaction using many approaches, including expressed sequence tag (EST) sequencing (Jantasuriyarat *et al*., 2005), robust-long serial analysis of gene expression (Gowda *et al*., 2007), proteomics (Kang *et al*., 2009; Kim *et al*., 2004), RNA-seq (Bagnaresi *et al*., 2012) and microarray (Vergne *et al*., 2007; Wei *et al*., 2013). These studies identified diverse biological processes and defence-related genes responsive to *M. oryzae* (Bagnaresi *et al*., 2012; Wei *et al*., 2013). However, almost all of these studies focused on the molecular events occurring in the host at 24 hpi or later, at which time the appressorium is already well developed. The transcriptome results obtained from previous studies are shown in Table 1. Very recently, a transcriptomic study on rice with the earlier time point (12 hpi) included after infection with *M. oryzae* has been performed using microarray. Although this study found some TFs and receptor-like kinases up-regulated at 48 hpi, and probably associated with the defence response in incompatible rice, it did not identify significant gene expression changes at an earlier time point (12 hpi) (Wang *et al*., 2014a). To date, the molecular events triggered at the early stages (before 24 hpi) of the rice–*M. oryzae* interaction, before the full formation of the appressorium, are still unknown.

Digu, a Chinese *indica* rice variety, confers robust and durable resistance to all of the tested blast isolates, including approximately 1000 *M. oryzae* strains collected from China, Japan, the Philippines, the UK and USA. It has been long and widely used as an important genetic resource for blast resistance breeding in China and south Asia (Chen *et al*., 2004). Previous studies have identified three blast resistance genes, *Pid1*, *Pid2* and *Pid3*, from Digu using three different *M. oryzae* strains as inocula: ZB13, ZB15 and Zhong-10-8-14, respectively (Chen *et al*., 2006; Shang *et al*., 2009). Of these, *Pid2* and *Pid3* have been isolated. *Pid2* encodes a B-lectin receptor kinase (RK) and *Pid3* encodes a typical NBS-LRR protein (Chen *et al*., 2006; Shang *et al*., 2009). Because each of these three identified resistance genes confers race-specific disease resistance (Chen *et al*., 2004, 2006; Shang *et al*., 2009), whereas Digu confers strong, broad-spectrum and durable resistance to *M. oryzae*, we hypothesized the presence of additional resistance mechanism(s), distinct from those mediated by these three genes, in Digu.

In this study, we examined the infection-associated development of *M. oryzae* in rice and found that the development of both the penetration peg and the primary invasive hyphae were inhibited at 24 hpi in Digu compared with the susceptible rice Lijiangxintuanheigu (LTH). We then performed comparative transcriptional profiling analyses between Digu and LTH at early stages of infection (5, 10 and 20 hpi). Our study reveals significant transcriptomic changes as early as 5 hpi that differ between these two rice varieties. Gene ontology (GO) and pathway analyses of the microarray data revealed that several biological processes,

including extracellular recognition and biosynthesis of antioxidants, terpenes and hormones, were specifically activated in Digu in response to *M. oryzae*, suggesting that these processes might be involved in blocking fungal penetration peg formation and inhibiting invasive hyphal development. We also identified 48 genes encoding predicted RKs whose expression was specifically up- or down-regulated by *M. oryzae* infection in Digu. One of these RK genes, *LOC_Os08g10300*, conferred enhanced resistance to *M. oryzae* when overexpressed in the susceptible rice variety, TP309. Our study reveals that the molecular interaction between pathogen and rice plant occurs hours before full development of the appressorium, and that RKs play important roles in the durably resistant rice cultivar Digu in the early defence response against infection by *M. oryzae*.

RESULTS

Differences in *M. oryzae* development between compatible and incompatible interactions with rice are apparent within 24 hpi

The invasion processes of *M. oryzae* in compatible rice have been well studied (Ebbole, 2007; Howard and Valent, 1996; Talbot, 2003). To explore whether the development of *M. oryzae* is different when grown on compatible and incompatible rice, we examined the development of *M. oryzae* during the first 52 h after infection on the durably resistant rice Digu and the susceptible rice LTH. To facilitate the observation, we used an *M. oryzae* strain carrying green fluorescent protein (GFP) as a marker. We used rice sheath tissue for microscopic observations of *M. oryzae* penetration of the rice cuticle because this tissue has reduced autofluorescence when compared with green leaves. By 5 hpi, the formation of the appressorium was evident as a dome-shaped structure at the tip of the germ tube of *M. oryzae* on both Digu and LTH (Fig. S1, see Supporting Information). By 10 hpi, the appressorium had initiated a cell wall melanization process, which is essential for the accumulation of the enormous turgor pressure needed to rupture the rice leaf cuticle. The autophagic fungal conidial cell death necessary for appressorium maturation was also detected on both Digu and LTH, as indicated by the disappearance of GFP fluorescence within the conidial cells at 10 hpi. At 16 hpi, the appressorium was fully melanized on both rice varieties (Figs 1 and S1). These results demonstrate that, before penetration of the host cuticle, the early invasion processes of *M. oryzae* are similar between the incompatible and compatible interactions, including conidial germination, germ tube extension, appressorium formation and maturation, and autophagic conidial cell death.

We found significant differences in the development of *M. oryzae* 16 h after inoculation on Digu and LTH. At 20 and 24 hpi, the penetration peg and primary invasive hyphae emerged frequently from the appressorium on LTH, but rarely on Digu (Fig. 1). At 36 hpi, the secondary invasive hyphae of *M. oryzae* on the LTH sheath expanded into rice cells neighbouring the cell ruptured by the appressorium. In striking contrast, *M. oryzae* on Digu only showed the initial emergence of a short penetration peg (Fig. S1). These results indicate that differences in *M. oryzae* infection on LTH and Digu could be observed at the penetration and post-penetration stages. Based on these results, we hypothesize that the molecular events that differentiate the compatible and

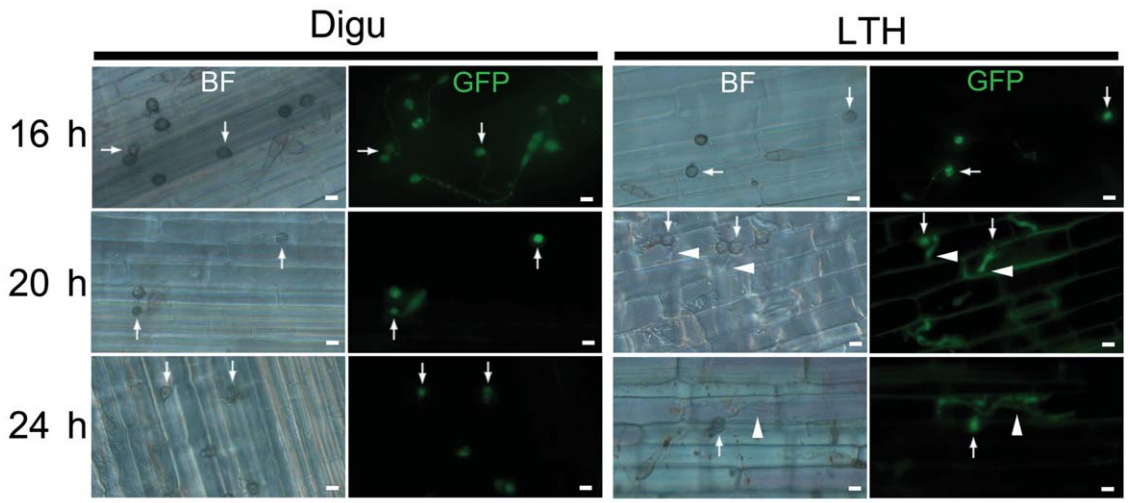

Fig. 1 Development of *Magnaporthe oryzae* on rice leaf sheaths post-inoculation. The leaf sheaths of durably resistant rice Digu and susceptible rice Lijiangxintuanheigu (LTH) were inoculated with spore suspensions of *M. oryzae* strain Zhong-10-8-14-GFP that expresses the *green fluorescent protein* (*GFP*) gene. The inoculated leaf sheaths were examined under a fluorescence microscope at the time points 16, 20 and 24 h post-inoculation (hpi), as indicated. Arrows and arrowheads indicate infection structure appressorium and invasive hyphae, respectively. Scale bar, 10 μm. BF, bright field.

incompatible interactions of rice with *M. oryzae* would occur before 20 hpi when the fungal penetration peg forms and invasive hyphae begin to develop.

Several *PR* genes in rice have been reported to be up-regulated in response to biotic stress as a result of an enhanced immune response (van Loon *et al*., 2006). To investigate whether the rice immune response is activated before 20 hpi, we compared the transcriptional expression levels of *PR* genes between Digu and LTH challenged with *M. oryzae*. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed on rice leaf samples collected at 5, 10 and 20 hpi for expression of the following *PR* genes: *PR10* (*LOC_Os12g36830*) and *BETV1* (*LOC_Os12g36850*). The expression levels of *PR10* and *BETV1* increased over time and reached a peak at 20 hpi in both Digu and LTH. Importantly, the expression of both *PR* genes reached higher levels in Digu compared with LTH during fungal infection (Fig. S2, see Supporting Information). These results reveal that the durably resistant rice Digu and the susceptible rice LTH respond to *M. oryzae* differently in the activation of *PR* genes.

Based on these findings, we hypothesize that the rice immune response to *M. oryzae* infection is activated within 20 h and is differentially regulated in the durably resistant rice Digu compared with the susceptible rice LTH. These differences result in reduced penetration peg formation and invasive hyphal growth on Digu relative to LTH, as observed at approximately 1 day post-inoculation (dpi).

Identification of rice genes differentially or specifically expressed in early compatible or incompatible interaction with *M. oryzae*

To understand the molecular events underlying the early response of Digu to *M. oryzae*, we performed a transcriptomic study to compare the responses between the durably resistant rice Digu and the susceptible rice LTH on *M. oryzae* infection. RNA samples were prepared from rice leaf tissues harvested from Digu and LTH at 5, 10 and 20 hpi following *M. oryzae* or mock treatment (Fig. S3A, see Supporting Information). After quality validation, the RNA samples were reverse transcribed and subjected to microarray hybridization for transcriptional profiling analysis. Three independent biological replications were performed. Pearson's correlation coefficients among these replicates ranged from 0.80 to 0.99 with an average of 0.93, showing high consistency among these biological replicates (Table S1, see Supporting Information). Genes whose expression levels increased or decreased by 0.5-fold or more after *M. oryzae* inoculation compared with mock inoculation at each time point were identified as differentially expressed genes (DEGs) (Fig. S3B). Only those DEGs present in all three independent biological replicates were selected for further analyses.

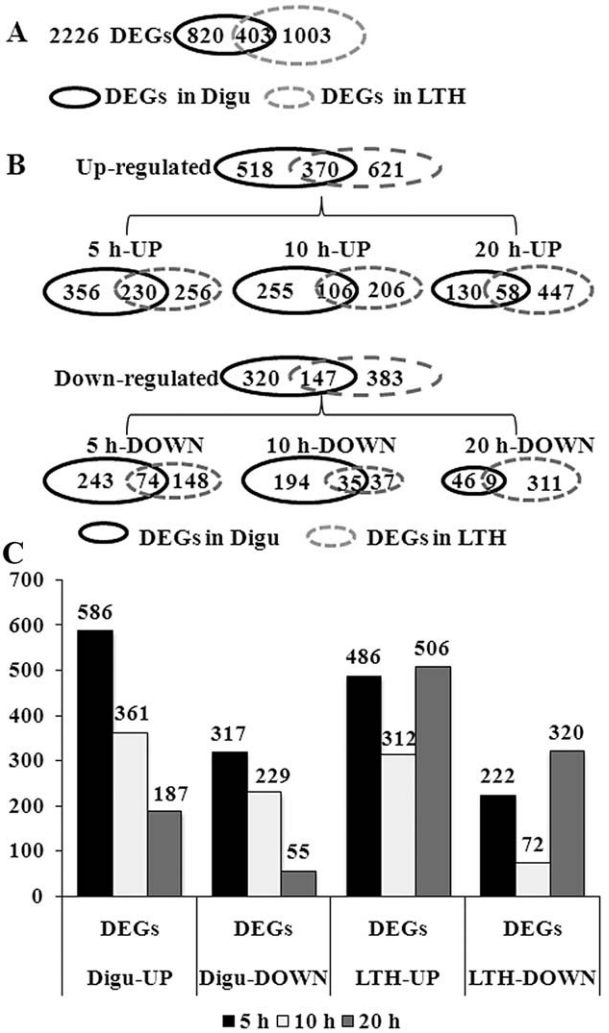

Fig. 2 Identification of differentially expressed genes (DEGs) from rice Digu and Lijiangxintuanheigu (LTH) after *Magnaporthe oryzae* inoculation. Those genes with expression levels increased or decreased by more than 0.5-fold in either Digu or LTH compared with mock inoculation were identified as DEGs. Only those DEGs present in all three independent biological duplicates were considered as real DEGs in our study. Top panel: DEGs with up-regulated expression; bottom panel: DEGs with down-regulated expression. (A) Venn diagram of DEGs in Digu and LTH within 20 h post-inoculation (hpi). (B) Venn diagram of DEGs in both Digu and LTH at 5, 10 and 20 hpi. (C) The column diagram shows the numbers of DEGs with up- and down-regulated expression in DG and LTH at 5, 10 and 20 hpi.

A total of 2226 DEGs were identified from rice Digu and LTH in response to *M. oryzae* infection (Fig. 2A and Table S2, see Supporting Information). Thirteen of 14 randomly selected DEGs were verified by qRT-PCR analysis (Fig. S4 and Table S3, see Supporting Information), suggesting that most of the DEGs (more than 92%) could be validated and the microarray data were highly reliable. Among all DEGs, 403 were commonly shared between Digu and LTH, whereas 820 were Digu specific and 1003 were LTH specific

(Fig. 2A). In detail, among the up-regulated DEGs, 370 (230 at 5 hpi, 106 at 10 hpi and 58 at 20 hpi) were commonly shared between Digu and LTH, whereas 518 (356 at 5 hpi, 255 at 10 hpi and 130 at 20 hpi) were Digu specific and 621 (256 at 5 hpi, 206 at 10 hpi and 447 at 20 hpi) were LTH specific (Fig. 2B). Similarly, among the down-regulated DEGs, 147 (74 at 5 hpi, 35 at 10 hpi and 9 at 20 hpi) were shared, whereas 320 (243 at 5 hpi, 194 at 10 hpi and 46 at 20 hpi) were Digu specific and 338 (148 at 5 hpi, 37 at 10 hpi and 331 at 20 hpi) were LTH specific (Fig. 2B). These results suggest that most DEGs are either Digu or LTH specific. Furthermore, we found that more DEGs in Digu were up-regulated at the earliest time point (586 genes at 5 hpi) than at later time points (361 and 187 genes at 10 and 20 hpi, respectively). Similarly, among the down-regulated DEGs, more were present at the earliest than the later time points (317, 229 and 55 genes at 5, 10 and 20 hpi, respectively) in Digu (Fig. 2C). These results indicate that changes in gene expression can be observed in response to *M. oryzae* infection as early as 5 hpi in Digu and fewer changes are present at later time points (e.g. 20 hpi). In contrast, in LTH, more genes were up-regulated (506 vs. 486) or down-regulated (320 vs. 222) at the latest time point (20 hpi) than at the earlier time points (5 and 10 hpi) (Fig. 2C). These results indicate that the majority of gene expression changes in LTH in response to *M. oryzae* infection are delayed relative to the rapid response of Digu.

Specific molecular pathways in durably resistant rice are involved in the early response against *M. oryzae*

To identify the specific molecular pathways involved in the early response against *M. oryzae*, we performed GO analysis on all DEGs. We identified 169 (at 5 hpi), 122 (10 hpi) and 84 (20 hpi) enriched GO terms for the up-regulated DEGs, and one (5 hpi), 18 (10 hpi) and 30 (20 hpi) enriched GO terms (Fig. 3A) for the down-regulated DEGs in Digu. In LTH, we identified 110 (5 hpi), 113 (10 hpi) and 122 (20 hpi) enriched GO terms for the up-regulated DEGs, and five (5 hpi), 13 (10 hpi) and zero (20 hpi) GO terms for the down-regulated DEGs (Fig. 3A). Interestingly, of these Digu-specific GO terms enriched from the up-regulated DEGs, 71 GO terms were present at 5 hpi, 32 at 10 hpi and 15 at 20 hpi, showing that more GO terms were used in the resistant rice in response to *M. oryzae* at the earlier time points than at the later points. In contrast, among the LTH-specific GO terms, only 12 GO terms were present at 5 hpi, whereas 23 were present at 10 hpi and 53 were present at 20 hpi (Fig. 3B), showing that less GO terms were used at the earlier time points than at the later points in LTH. These results suggest that the GO terms associated with compatible interaction are different from those associated with incompatible interaction, and that the earliest time point (5 hpi) is important for the Digu-specific response against *M. oryzae* invasion. However, fewer GO terms were associated with the down-regulated DEGs in both Digu and LTH. At 5 hpi, Digu and LTH shared one from the common down-regulated DEG. Four LTH-specific GO terms and no Digu-specific GO terms were observed for down-regulated DEGs. At 10 hpi, there were 16 in common, 12 specific for Digu and seven specific for LTH. At 20 hpi, there were none in common, 30 specific for Digu and none specific for LTH (Fig. 3B). These results indicate that the down-regulated DEGs are less important than the up-regulated DEGs, in both resistant and susceptible rice, in response to *M. oryzae*. Collectively, these results suggest that many molecular pathways are activated instead of inhibited in Digu in defence against *M. oryzae*. These results also reveal that the molecular events activated at the early time point (at 5 hpi) are more important than those at later time points for resistance in Digu.

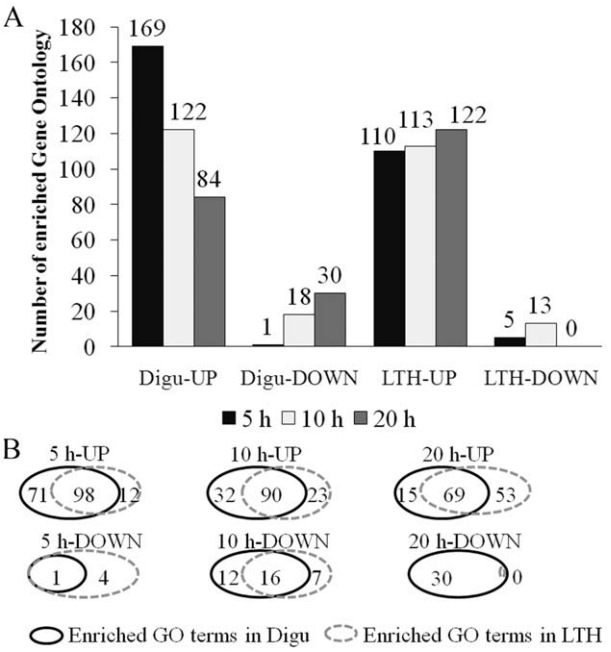

Fig. 3 Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in Digu and Lijiangxintuanheigu (LTH). GO full assignments to the DEGs were retrieved from the BiNGO database (Maere *et al*., 2005). (A) GO term enrichment analysis of up- and down-regulated DEGs in Digu and LTH at 5, 10 and 20 h post-inoculation (hpi). The number of GO terms enriched from the up-regulated DEGs was greater than those enriched from the down-regulated DEGs in both the durably resistant rice Digu and the susceptible rice LTH at each time point post-inoculation. (B) Venn diagram of GO terms in both DG and LTH at 5, 10 and 20 hpi.

Among the GO terms associated specifically with the up-regulated DEGs in Digu at 5 hpi, we identified the GO term 'response to stimulus' in biological process, the GO terms 'molecular transducer activity' and 'transporter activity' in molecular function, and the GO term 'extracellular region' in cellular component. Because these processes have been reported to be involved in the perception and transduction of extracellular signals (Chasis *et al*., 1988; Nürnberger *et al*., 1994; Karnchanaphanurch *et al*., 2009),

Table 2 Enrichment analysis of functional gene ontology (GO) categories of the Digu-specific and Lijiangxintuanheigu (LTH)-specific differentially expressed genes (DEGs) during rice early responses to *Magnaporthe oryzae*.

GO-ID	Enriched GO terms	Digu-5 h	Digu-10 h	Digu-20 h	LTH-5 h	LTH-10 h	LTH-20 h
23052	Signalling	—	UP	—	—	—	—
60089	Molecular transducer activity	UP	—	—	—	—	—
30234	Enzyme regulator activity	UP	UP	—	—	—	—
43190	ATP-binding cassette (ABC) transporter complex	UP	—	—	—	—	—
5576	Extracellular region	UP	UP	—	—	—	—
9987	Cellular process	—	—	DOWN	—	—	—
8152	Metabolic process	—	—	DOWN	—	—	—
3824	Catalytic activity	—	DOWN	DOWN	—	—	—
43231	Intracellular membrane-bound organelle	—	DOWN	—	—	—	—
43227	Membrane-bound organelle	—	DOWN	—	—	—	—
43229	Intracellular organelle	—	DOWN	—	—	—	—
43226	Organelle	—	DOWN	—	—	—	—
44424	Intracellular part	—	DOWN	—	—	—	—
5622	Intracellular	—	DOWN	—	—	—	—
44444	Cytoplasmic part	—	DOWN	—	—	—	—
5737	Cytoplasm	—	DOWN	—	—	—	—
5364	**Nucleus**	—	—	—	—	—	**UP**

Note: GO full assignments to these Digu- (normal) and LTH-specific (bold) DEGs were retrieved from the BiNGO database. 'UP' indicates that the enriched GO terms are up-regulated at the time points post-inoculation, whereas 'DOWN' indicates that the enriched GO terms are down-regulated. '—' shows that the enriched GO terms are not significantly regulated.

our finding indicates that the early response in the durably resistant rice Digu is probably activated through rice receptor(s) perceiving extracellular signal molecules derived from *M. oryzae*.

To better understand the molecular pathways associated with the GO terms, we analysed the GO categories with enrichment analysis. We found 19 up-regulated and 12 down-regulated GO categories in Digu, as well as 15 up-regulated and two down-regulated GO categories in LTH (Table S4, see Supporting Information). Among these, 16 GO categories were Digu specific. Interestingly, of these 16 GO categories, five were up-regulated and 11 were down-regulated (Table 2). These results suggest that Digu employs unique molecular pathways in response to *M. oryzae* infection as compared with LTH. Interestingly, we also identified several new enriched Digu-specific pathways that were activated after *M. oryzae* inoculation, including 'antioxidant activity' as up-regulated pathways and 'cellular process', 'metabolic process' and 'catalytic activity' as down-regulated pathways. Among these pathways, only 'transporter activity' was found to be associated with the race-specific response of resistant rice IRBL18 and IRBL22 to *M. oryzae* infection (Table S4). These results suggest that the new pathways identified might contribute to the durable resistance observed in Digu. These results also indicate that the defence response of Digu identified by transcriptomics differs from the race-specific response of IRBL18 and IRBL22.

Specific metabolic processes in Digu are involved in the early response against *M. oryzae*

The above GO-based analyses suggest that many 'metabolic processes' are part of the response of Digu to *M. oryzae* infection. To identify specific metabolic processes in Digu, we used the RiceCyc (http://pathway.gramene.org/) database to analyse the metabolic profiles of the up- and down-regulated genes in Digu and LTH after *M. oryzae* inoculation. We identified a total of 38 Digu-specific and up-regulated metabolic pathways (29 at 5 hpi, nine at 10 hpi and 11 at 20 hpi) within 20 hpi (Table S5, see Supporting Information). Interestingly, most of these pathways are associated with metabolic processes, including carbon source (10 pathways), antioxidant (five pathways), nitrogen source (three pathways), terpene biosynthesis (three pathways) and hormone biosynthesis (two pathways) (Tables 3 and S5), suggesting that these metabolic processes are involved in the Digu response to *M. oryzae* invasion. Because most metabolic processes (29 of 38) were activated within 5 hpi, we hypothesize that these metabolic process are critical for the early defence response of Digu against *M. oryzae*.

We also identified eight pathways that were specifically activated in Digu, but inhibited in LTH, during the rice–*M. oryzae* interaction. These pathways are mainly associated with energy metabolic processes, including energy carriers (two pathways), nitrogen source (one pathway), and detoxification and nitrogen utilization (one pathway) (Tables 3 and S5). This result indicates that these energy metabolic processes are more active in Digu in response to *M. oryzae* invasion, as compared with LTH, and might contribute to durable resistance to *M. oryzae*.

In addition, we found that five pathways [lysine biosynthesis VI, lysine biosynthesis II, lysine biosynthesis I, UDP-galactose biosynthesis (salvage pathway from galactose using UDP-glucose) and haem biosynthesis] were activated in LTH, but repressed in Digu, during the rice–*M. oryzae* interaction (Table S5). This result

Table 3 The Digu-specific pathways associated with differentially expressed genes (DEGs) during the Digu early responses to *Magnaporthe oryzae*.

	Related metabolic process	Pathway
Digu-specific pathways with up-regulated DEGs	Carbon source	Galactosylcyclitol biosynthesis
		2-Keto-glutarate dehydrogenase complex
		Branched-chain α-keto acid dehydrogenase complex
		Sucrose degradation I, III
		Glycerol degradation IV
		Reductive tricarboxylic acid cycle (TCA) cycle I
		Pentose phosphate pathway
	Carbon source, nitrogen source	Arginine degradation X
		Aldoxime degradation
	Nitrogen source	Urea transport (into cytosol)
	Terpene	Leucopelargonidin and leucocyanidin biosynthesis
		Leucodelphinidin biosynthesis
		Linamarin degradation
	Hormone	Indole-3-acetic acid (IAA) biosynthesis VI
		Phaseic acid biosynthesis
	Antioxidant	Coumarin biosynthesis
		Flavin biosynthesis
		Sulfate reduction II
		Sulfate activation for sulfonation
		Sulfite oxidation III
Pathways with up-regulated DEGs in Digu but down-regulated DEGs in LTH	Energy carriers	Salvage pathways of purine nucleosides II (plant)
		Salvage pathways of purine nucleosides
	Nitrogen source in the xylem sap	Ureide biosynthesis
	Detoxification and nitrogen utilization	Cyanate degradation
Pathways with down-regulated DEGs in Digu but up-regulated DEGs in LTH	Potential extracellular signalling molecule	UDP-galactose biosynthesis
	Oxidase activity	Haem biosynthesis II

Note: The rice metabolic pathway analysis was performed on the Digu-specific DEGs using RiceCyc ver 3.2.

suggests that the biosyntheses of lysine, UDP-galactose and haem in rice might facilitate *M. oryzae* infection in the susceptible rice LTH and are highly repressed in Digu, leading to the inhibition of *M. oryzae* invasion.

TFs are up-regulated at early stages in response to *M. oryzae* infection

TFs can directly regulate the expression of defence-related genes. In our study, we identified 37 TFs whose expression was responsive to *M. oryzae* specifically in the durably resistant rice Digu. These TFs belong to WRKY, AP2, MYB, bHLH, GRAS, NAC, ARF, C2H2, E2F, GATA, MADS, ARR-B, CAMTA and CO-like subfamilies. Among these TF genes, most were up-regulated at early time points (Fig. 4A). Four of these TF families were specific to the defence response of Digu: ARF (*LOC_Os01g13520* up-regulated and *LOC_Os12g41950* down-regulated), ZF-HD (*LOC_Os09g24810* down-regulated), GATA (*LOC_Os02g56250* up-regulated) and E2F (*LOC_Os12g06200* up-regulated) (Fig. 4B). GATA is associated with the hypersensitive response (Dangli *et al*., 1996), programmed cell death and callus differentiation (Wang *et al*., 2005). ARF regulates the expression of auxin-responsive genes (Tiwari *et al*., 2003; Ulmasov *et al*., 1997). ZF-HD is expressed in the mesophyll specifically (Windhovel *et al*., 2001). E2F is important for cell proliferation (van den Heuvel and Dyson, 2008). Taken together, our results suggest that the hypersensitive response, programmed cell death, callus differentiation, inhibition of rice growth and development might be associated or required for the Digu defence response against *M. oryzae*, prior to fungal penetration peg formation and invasive hyphal development.

RKs play key roles in defence against *M. oryzae* infection leading to durable resistance

RKs play important roles as PRRs and regulators of PTI (Chen and Ronald, 2011; Ronald and Beutler, 2010; Zipfel *et al*., 2006). For instance, the LRR XII RKs, such as FLAGELLIN-SENSITIVE 2 (FLS2) (Gomez-Gomez and Boller, 2000), elongation factor-Tu receptor (EFR) (Zipfel *et al*., 2006) and XA21 (Song *et al*., 1995), function with the LRR II subfamily regulatory RKs, BAK1 (SERK3), BKK1 (SERK4) and OsSERK2, to perceive the signal molecules derived from bacteria and activate the defence response (Chen *et al*., 2014; Chinchilla *et al*., 2007; Roux *et al*., 2011; Wang *et al*., 2014b).

To determine whether RKs are involved in the early response against *M. oryzae* in Digu and, if so, which RKs, we searched for RKs in the DEGs. We found 48 RK genes belonging to the subfamilies of LRR, WAK, SD, DUF26, L-LEC, LRK10L and RKF3, which were specifically up- or down-regulated in Digu post-inoculation (Fig. 5). We verified the *M. oryzae* responsiveness of these DEGs by qRT-PCR analysis on eight genes randomly selected from these

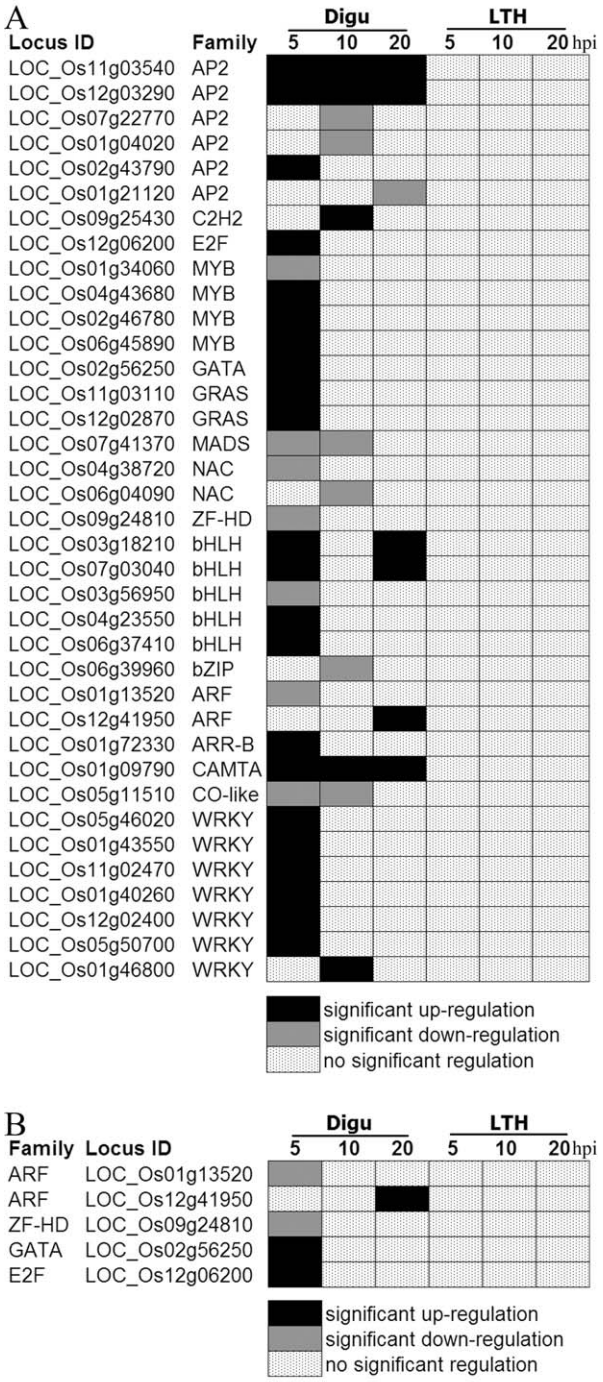

Fig. 4 Transcription factors encoded by Digu-specific differentially expressed genes (DEGs). The gene IDs of the transcription factors were obtained from the database of Plant TFDB (Jin *et al*., 2014). The family names of the DEGs are shown next to their Locus IDs. (A) Transcription factors encoded by Digu-specific DEGs. The subfamilies WAKY, AP2, MYB, bHLH, GRAS, NAC, ARF, C2H2, E2F, GATA, MADS, ARR-B, CAMTA and CO-like are indicated. (B) Four transcription factor families activated specifically in rice Digu on *Magnaporthe oryzae* inoculation.

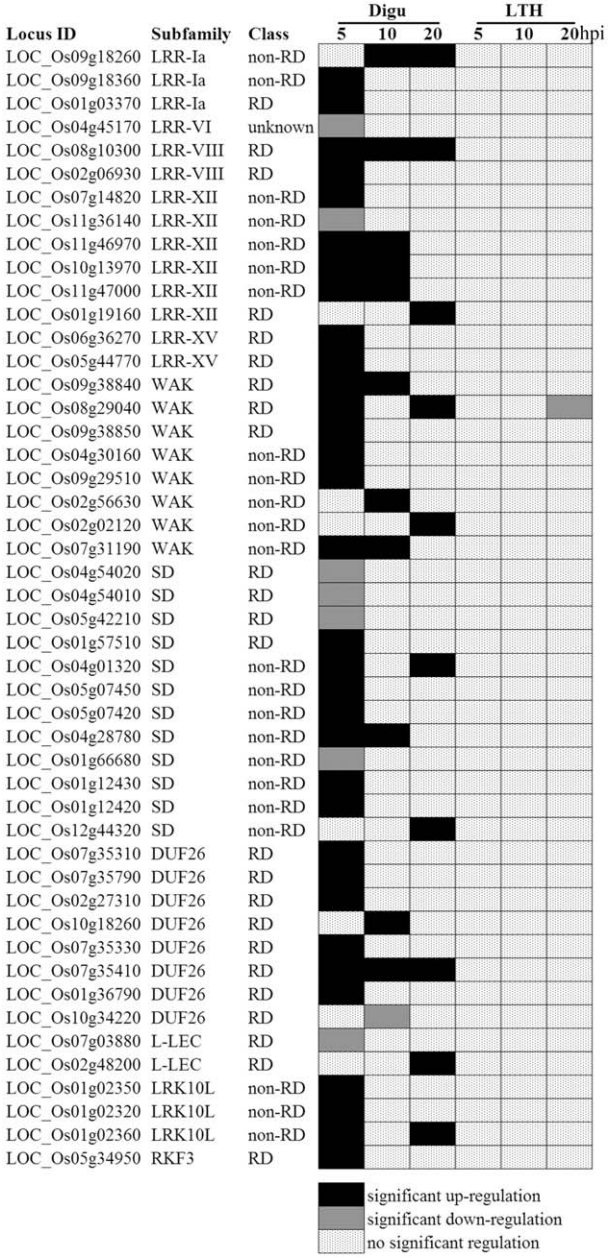

Fig. 5 Receptor kinase (RK) genes encoded by Digu-specific differentially expressed genes (DEGs). The IDs of the RK genes were retrieved from the rice kinase database (Dardick *et al*., 2007). The information on the subfamily and class of the genes was also obtained from the rice kinase database. The RKs encoded by these genes belong to LRR (leucine-rich receptor), WAK (cell wall-associated kinase), SD (S-domain receptor-like protein kinase), DUF26 (domain unknown function 26), L-LEC (lectin-like receptor kinase), LRK10L and RKF3 subfamilies. Up- or down-regulated expression of these genes at each time point post-inoculation is indicated. Among these genes, most are up-regulated, specifically in Digu overall, especially at the point of 5 hpi.

RK genes. The expression patterns of the eight DEGs were consistent when compared between the qRT-PCR and microarray results (Fig. S5 and Table S3, see Supporting Information). Forty of these RK genes were up-regulated, including 13 from the LRR subfamily, eight from the WAK subfamily, eight from the SD subfamily, seven from the DUF26 subfamily, one from the L-LEC subfamily, three from the LRK10L subfamily and one from the RKF3 subfamily (Fig. 5). These results suggest that most of these RK genes play positive regulatory roles in Digu in response to *M. oryzae*. Most of these RK genes (35 of 40) were rapidly up-regulated as early as 5 hpi, suggesting that these RKs are important for the early signal perception or signal transduction. Only eight RKs were specifically down-regulated in Digu, indicating that a small number of RK genes might negatively regulate the early defence response of Digu.

We identified two RK genes that were specifically up-regulated in Digu at all three time points: *LOC_Os07g35410*, encoding an RK belonging to the DUF26 subfamily, and *LOC_Os08g10300*, encoding an RK with eight extracellular LRRs belonging to the VIII-2 subfamily. One well-characterized representative of the VIII-2 subfamily is the sugarcane *SHR5* gene. *SHR5* expression is down-regulated in the interaction between sugarcane and endophytic, N_2-fixing bacteria and is up-regulated in response to infection with pathogenic bacteria and fungi (Vinagre *et al*., 2006).

To assess the biological function of *LOC_Os08g10300*, we overexpressed this gene in the susceptible rice variety TP309 (Fig. S6, see Supporting Information) and assayed the resulting transgenic plants for resistance to *M. oryzae*. We inoculated seven independently transformed rice lines and the TP309 control. We found that all seven independently transformed rice lines (*Os08g10300ox*) conferred enhanced disease resistance to five different *M. oryzae* strains (WJ9, WJ10, YC7, YC26 and Zhong-10-8-14). We further analysed their T1 and T2 progeny plants. The expression levels of *PR* genes were up-regulated in the transgenic plants in comparison with that in TP309 (Fig. S7, see Supporting Information). We found that the progeny carrying the transgene exhibited much smaller disease lesion spots on leaves, whereas null segregants showed clear disease lesion spots, similar to those of the susceptible parental control TP309 to these strains (Figs 6 and S8, see Supporting Information). These results indicate that the observed resistance co-segregated with the transgene *Os08g10300ox* and that this gene confers enhanced resistance to *M. oryzae*.

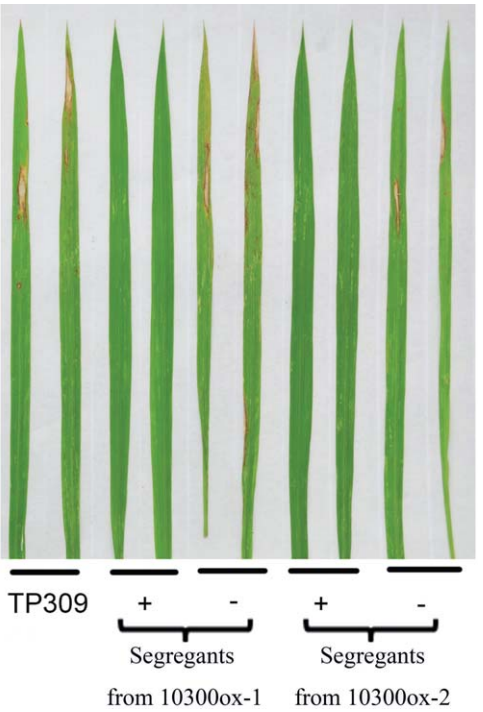

Fig. 6 Determination of the blast disease resistance of the transgenic plants carrying *Os08g10300ox*. The seedlings from 2-week-old plants were inoculated by spraying with *Magnaporthe oryzae* strain Zhong-10-8-14. Disease resistance was determined on two representative transgenic lines overexpressing *Os08g10300* (abbreviated as 10300ox-1 and 10300ox-2, respectively) in the TP309 genetic background at day 8 post-inoculation. The progeny carrying the transgene and the null segregants are indicated by '+' and '–', respectively. TP309 was used as susceptible control for the blast disease resistance determination.

DISCUSSION

Molecular interaction between rice and *M. oryzae* occurs within 5 h after pathogen inoculation

Previous studies have suggested that 24 hpi is a critical point for *M. oryzae* invasion (Talbot, 2003), and thus many transcriptional profiling studies have focused on this time point for studies of the rice response to *M. oryzae* (Kim *et al*., 2013; Wei *et al*., 2013). However, recent studies have also demonstrated that *M. oryzae* forms a mature appressorium in order to develop a penetration peg and invasive hypha prior to 24 hpi (Kankanala *et al*., 2007; Wilson and Talbot, 2009). No studies have analysed the molecular events that occur prior to this early time point. In our study, we used microarray-based expression profiling to assess the molecular events underlying the rice–*M. oryzae* interaction before 24 hpi. We identified more than 2000 DEGs before 24 hpi in Digu and LTH. The DEGs in Digu are largely different from those in LTH. Of the 586 DEGs at 5 hpi, 356 are Digu specific and 256 are LTH specific. DEGs specific to the durably resistant rice Digu encode RKs, signal transducers, TFs and PR proteins, involved in cell wall organization, signal transduction and the downstream defence response. Some of the DEGs also encode enzymes involved in sulfation pathways, such as sulfate reduction II (assimilatory) and sulfite oxidation III, which have also been shown previously to be associated with defence responses. Some of the Digu-specific DEGs identified at 5 hpi

encode TFs of GATA and E2F types with known function in the defence response against biotic stress. In summary, our results indicate that the key molecular events that govern the Digu defence response occur very early (as early as 5 hpi) on *M. oryzae* infection. We hypothesize that these early responses inhibit key aspects of fungal development, including full maturation of the appressorium, formation of the penetration peg and the spread of invasive hyphae (Fig. 1), leading to durable resistance in Digu to *M. oryzae*.

Durably resistant rice Digu employs a set of defence responses that are distinct from those mediated by LysM and NBS-LRR proteins

In rice, the characterized immune systems against *M. oryzae* are mediated either by LysM motif-containing proteins (CEBiP, OsLYP4 and OsLYP6), a lectin motif-containing RK (Pid2) or NBS-LRR proteins. The receptors CEBiP, OsLYP4 and OsLYP6 perceive chitin molecules derived from *M. oryzae* (Liu *et al*., 2012; Liu and Zhao, 2012) to initiate immune signalling. The signal is then transmitted downstream through a MAPK cascade, leading to the activation of the immune response. In this immune system, the *OsLYP4* and *OsLYP6* receptor genes and MAPK cascade genes (*OsMAPK5* and *OsMAPK12*) are rapidly induced on challenge by chitin or *M. oryzae* (Liu *et al*., 2012; Liu and Zhao, 2012). Despite their importance in the CEBiP-mediated defence response, none of these genes are present among our identified DEGs from Digu at 5, 10 or 20 hpi (Table S6, see Supporting Information). Further, qRT-PCR analysis also revealed that the expression of the genes CEBiP, OsLYP4 and OsLYP6 in rice Digu was not induced or repressed at the early time points post-inoculation with *M. oryzae* (Fig. S9, see Supporting Information). These results suggest that the molecular events in Digu underlying the early interaction between rice and *M. oryzae* before 20 hpi are unlikely to be dependent on LysM motif proteins, and that Digu may employ defence mechanisms distinct from those mediated by LysM motif proteins.

Previous analyses of transcriptional profiles mediated by NBS-LRR proteins (Vergne *et al*., 2007; Wei *et al*., 2013) have demonstrated that the responses of resistant and susceptible plants are quite similar overall, but with more genes prominently regulated in incompatible interactions. In contrast, our study reveals that the transcriptional profiles are largely different between the durably resistant rice Digu and the susceptible rice LTH at the early stages (5, 10 or 20 hpi) (Fig. 2 and Table S2). We also noted several activated molecular processes in Digu that were not detected in resistance mediated by NBS-LRR proteins. For example, we found that the GO terms 'extracellular region' and 'peroxidase activity' were associated with Digu in response to *M. oryzae*, but not with the transcriptional responses observed in NBS-LRR-mediated, race-specific resistance.

Previous studies have also demonstrated that some NBS-LRR genes are induced after pathogen infection. These include *Pib* (Wang *et al*., 1999), *Pi5-1* (Lee *et al*., 2009) and *Pi-k^h* (Sharma *et al*., 2005). We thus analysed whether NBS-LRR genes were up-regulated in DEGs. However, we did not identify any NBS-LRR genes that were up-regulated specifically in Digu on *M. oryzae* inoculation. Although we found five NBS-LRR genes whose transcriptional expression was induced in the susceptible rice LTH (Fig. S10, see Supporting Information), these NBS-LRR genes are unlikely to contribute to resistance to *M. oryzae*, because LTH is fully susceptible to the tested *M. oryzae* strains. Collectively, these findings indicate that rice Digu employs resistance mechanisms that are distinct from those employed by characterized NBS-LRR proteins (Shang *et al*., 2009).

Plasma membrane kinases are expressed early in Digu after *M. oryzae* infection

In our study, we identified 48 RK genes that were significantly differentially regulated in Digu at the early stages of infection; most were activated as early as 5 hpi. These RK genes encode proteins belonging to LRR, WAK, SD, DUF26, L-LEC, LRK10L and RKF3 subfamilies (Fig. 4). The up-regulation of these RKs was not observed in NBS-LRR-mediated resistance (Wei *et al*., 2013) (Fig. S11, see Supporting Information) or other race-specific resistance (Wang *et al*., 2014a) to *M. oryzae*. Thus, these RK genes appear to be induced specifically in the durably resistant rice Digu. Alternatively, these RKs may be expressed in other resistant rice varieties, but are only activated early in the rice response to infection (e.g. 5 hpi). In this case, such changes would not have been identified in studies that examined only later time points. We found that 23 of these RKs fall into the non-RD kinase subclass, a molecular signature tightly associated with the immune response in both plants and animals (Dardick and Ronald, 2006). Transgenic rice plants overexpressing *LOC_Os08g10300*, encoding an RK, conferred enhanced resistance to five strains of *M. oryzae* (Fig. 6), including strain Zhong-10-8-14. Because previous studies have shown that rice plants carrying Pid2 confer resistance to *M. oryzae* strain ZB15, but not to strain Zhong-10-8-14 (Chen *et al*., 2006), our results demonstrate that *LOC_Os08g10300* employs a distinct mechanism of resistance or perception of *M. oryzae* when compared with Pid2. However, we could not rule out the possibility that this RK may function as an important partner instead of resistant protein in defence against the invasion of *M. oryzae*. Further loss-of-function studies would help to uncover the mechanism mediated by this RK gene. The protein structure of LOC_Os08g10300 is similar to that of sugarcane SHR5, which belongs to the VIII-2 subclass of LRR RKs. Sugarcane *SHR5* is induced by infection with pathogenic bacteria and fungi, and is thus predicted to be involved in the defence response (Vinagre *et al*., 2006). Thus, we

hypothesize that VIII-2 subclass LRR RKs may serve as important regulators of the immune response.

In addition to LRR RKs, RKs from subfamilies WAK, SD and DUF26 were differentially regulated in Digu in response to *M. oryzae* infection. Of the eight WAK RK genes specifically regulated in Digu, six (*LOC_Os09g38840*, *LOC_Os08g29040*, *LOC_Os09g38850*, *LOC_Os04g30160*, *LOC_Os09g29510* and *LOC_Os07g31190*) responded to the invasion of pathogens within 5 hpi (Fig. 5). Among the 12 SD RK genes, eight were up-regulated and four were down-regulated after *M. oryzae* infection (Fig. 5), indicating that the SD subfamily plays dual roles early in defence against the invasion of *M. oryzae* through both positive and negative regulation. Of the eight DUF RKs, seven were up-regulated on *M. oryzae* infection, suggesting that these DUF RKs positively regulate the immune response. Our results are consistent with previous studies showing that members of the Arabidopsis WAK subfamily (WAK1, WAK2, WAK3 and WAK5) (He *et al*., 1998, 1999), the SD subfamily [ARK1, ARK3, Brassica S family receptor 2 (SFR2) and SFR3] (Pastuglia *et al*., 1997, 2002) and the DUF26 subfamily (Czernic *et al*., 1999; Du and Chen, 2000; Ohtake *et al*., 2000) are implicated in pathogen defence responses.

In summary, our study reveals the activation of molecular events in Digu before the full formation of the appressorium of *M. oryzae* in response to pathogen infection, and demonstrates that many of these events are distinct from those observed in the susceptible control or those mediated by CEBiP, Pid2 or NBS-LRR proteins. Our study also identifies a large set of RKs that are up-regulated in *M. oryzae* before the appressorium is well developed, suggesting that these RKs play important roles in the durably resistant rice Digu in defence against the invasion of *M. oryzae* before the formation of the appressorium.

EXPERIMENTAL PROCEDURES

Fungal growth on rice sheath

The durably resistant *indica* rice (*Oryza sativa*) Digu and the susceptible rice LTH were grown in a growth chamber at 28 °C in a 12-h light/12-h dark photoperiod with 75% humidity. For microscopic monitoring of fungal development, a *GFP*-expressing *M. oryzae* strain, Zhong-10-8-14-GFP (kindly provided by Professor Zhu, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China) was used to inoculate the detached rice sheaths from 4-week-old rice plants. The fungus stably expressing *GFP* was grown on complete agar medium for 2 weeks before producing spores. Spores were collected via flooding of the fungal agar cultures with sterile water, and the spore concentration in the suspension was adjusted to approximately 5×10^5 conidia/mL. The detached rice sheath assay was performed as described previously (Mosquera *et al*., 2009). All images of conidial germination, appressorium development, penetration peg formation and invasive hyphae growth were recorded using a fluorescent microscope of ZEISS industry, and microscopic examinations of the infected sheath were carried out in three independent experiments.

Blast infection procedures

The Digu and LTH varieties were grown in two tubs in a growth chamber at 28 °C in a 12-h light/12-h dark photoperiod with 75% humidity. Two-week-old rice plants in tub I were used for inoculation with mixed isolates of *M. oryzae* strains (WJ9, WJ10, YC7 and YC26). These isolates carry high virulence and are prevalent in Sichuan, China. The spore concentration was adjusted to 5×10^5/mL with 0.2% Tween-20. Two-week-old rice plants in tub II were used for mock inoculation with Tween-20 (0.2%) lacking *M. oryzae* spores. The fungal- and mock-inoculated rice seedlings were kept in a dark chamber at 95% humidity and 28 °C. After 24 hpi, the plants were maintained in the growth chamber at 28 °C in a 12-h light/12-h dark photoperiod with 95% humidity. Leaves were harvested at 5, 10 and 20 hpi for experimentation. To ensure that the inoculation was successfully performed, the remaining seedlings were kept for another 8 dpi for disease evaluation (Fig. S3A). The harvested leaves were ground in liquid nitrogen and immediately stored at −80 °C until RNA extraction. The experiment was repeated three times with 1-month intervals, representing three independent biological replicates.

Microarray analysis

We used Affymetrix Genechip Rice Genome Arrays (Affymetrix, Santa Clara, CA, USA) in this transcriptomic study. RNA quality assessment, RNA labelling and microarray hybridization were performed at Capitalbio Ltd. (Beijing, China) following the manufacturer's instructions. After normalization, a non-specific filtering step was carried out (Wei *et al*., 2013). We identified those genes with an expression level increased or decreased by 0.5-fold in either Digu or LTH compared with mock inoculation as DEGs, according to the method described previously (Liu and Zhao, 2012). Only those DEGs present in all three independent biological replicates were considered as real DEGs in our study (Fig. S3B). The probe sets without gene annotations of MSU Rice Genome Annotation Project database release 7.0 (http://rice.plantbiology.msu.edu/) (Kawahara *et al*., 2013) were removed. Hierarchical clustering was performed using cluster version 3.0 (http://www.falw.vu/huik/cluster.htm) with the average linkage clustering method, and was illustrated by TreeView software (Saldanha, 2004).

Computational gene function analysis

GO analysis was performed on DEGs based on gene annotations. GO full assignments to these genes were retrieved from the BiNGO database (Maere *et al*., 2005). Rice metabolic pathway analysis was performed using RiceCyc ver 3.2 (Jaiswal *et al*., 2006). The plant TFDB (Jin *et al*., 2014) database was used for analyses of rice TFs; the rice kinase database (Dardick *et al*., 2007) was used for the analysis of rice kinase; the MSU database was used for the determination of rice gene families.

qRT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Shanghai, China) according to the manufacturer's protocols. cDNA

was synthesized using an RNA reverse transcription kit (Invitrogen Life Technologies, Shanghai, China). The PCR program was run as follows: 5 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, 30 s of annealing at 60 °C, polymerization at 72 °C for 50 s and a final extension step at 72 °C for 3 min. The expression of the reference *UBQ5* gene was used for the normalization of all qRT-PCR data (Jain *et al*., 2006). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels with three experimental repeats (Livak and Schmittgen, 2001). Primer sequences for qRT-PCR are listed in Table S7 (see Supporting Information).

Generation of transgenic plants

The full-length cDNA of RK *LOC_Os08g10300* (abbreviated as *Os08g10300*) was cloned into pENTR/D to yield pENTR-*Os08g10300*, which was then recombined with the Ubi-C1300GTW vector to generate the over-expression construct, Ubi-C1300-*Os08g10300* (abbreviated as *Os08g10300ox*). The construct, *Os08g10300ox*, was introduced into the blast susceptible rice TP309 variety through *Agrobacterium*-mediated transformation according to the method described previously (Chern *et al*. 2005). The regenerated transgenic plants carrying *Os08g10300ox* were selected with hygromycin. PCR-based genotyping was performed to determine whether the transgenic plants contained the transgene, as described previously (Chen *et al*., 2010). Overexpression of *Os08g10300* in the transgenic lines carrying *Os08g10300ox* was confirmed by qRT-PCR (Fig. S6).

ACKNOWLEDGEMENTS

We thank Professor Lihuang Zhu (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China) for helpful discussions. This study was conceived by X. W. Chen. The experiments were designed by Y. Liu, W. T. Li, S. G. Li, M. S. Chern, P. C. Ronald and X. W. Chen. The experiments were performed by Y. Liu, W. T. Li, J. Wang, M. He, X. G. Zhou, C. Yao, C. Yuan, J. C. Wang, J. J. Yin, W. L. Chen, B. T. Ma, Y. P. Wang and P. Qin. Data were analysed by W. T. Li, S. G. Li and X. W. Chen. The paper was written by W. T. Li, M. S. Chern, P. C. Ronald and X. W. Chen.

This work was supported by grants from the Transgenic Projects from the Chinese Ministry of Agriculture 2014ZX0800903B, National Nature Science Foundation of China 31171622 and 31371705, 'Hundred Talents Plan Foundation, Youth Foundation 13QNJJ0076 and Youth Innovation Team Foundation 14CXTD0039 from Sichuan, China to X. W. Chen. This work was also supported by the Specialized Research Fund for Doctoral Program of Higher Education 20135103120004 to J. Wang and 20125103120011 to W. T. Li, Key Project of Sichuan Education Department 15ZA0020 to W. T. Li, NSFC 31301626, International Cooperation and Exchange Program 2014HH0066 to M. He, and NIH GM59962 to P. C. Ronald.

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SUPPORTING INFORMATION

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

Fig. S1 Development of *Magnaporthe oryzae* on rice leaf sheath post-inoculation. The leaf sheaths of durably resistant rice Digu and susceptible rice Lijiangxintuanheigu (LTH) were inoculated with a spore suspension of *M. oryzae* strain Zhong-10-8-14-GFP that expresses the *green fluorescent protein* (*GFP*) gene. The inoculated leaf sheaths were examined under a fluorescence microscope at the time points 0, 5, 8, 10, 16, 20, 24, 30, 36 and 52 h post-inoculation (hpi), as indicated. Arrows and arrowheads indicate infection structure appressorium and invasive hyphae, respectively. Scale bar, 10 μm.

Fig. S2 Determination of the relative expression levels of *pathogenesis-related* (*PR*) genes in rice Digu and Lijiangxintuanheigu (LTH) post-infection with *Magnaporthe oryzae*. Inoculation, sampling and expression analyses were carried out as described in Fig. S1A. The relative expression of genes after *M. oryzae* inoculation was normalized to the expression with mock inoculation. All quantitative reverse transcription-polymerase chain reaction (qRT-PCR) data were normalized to the expression of the reference *UBQ5* gene before the determination of relative expression. The expression of *PR* genes *PR10* and *BETV1* was included for this assay. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels with three repeats (Livak and Schmittgen, 2001). SD represents the standard deviation of three technical replicates. This experiment was biologically repeated three times with similar results.

Fig. S3 The experimental design for screening differentially expressed genes (DEGs) from rice Digu and Lijiangxintuanheigu (LTH). (A) Preparation of seedling samples of rice Digu and LTH. The samples in tub I were inoculated by spraying with *Magnaporthe oryzae* spores. The concentration of spores was 5×10^5/mL with 0.2% Tween-20. The samples in tub II were sprayed with 0.2% Tween-20 without spores for mock inoculation. Sampling was performed at the time points 5, 10 and 20 h post-inoculation (hpi), as indicated. The seedling samples marked with 'left' were kept for disease evaluation to ensure that the inoculation was successfully performed. The disease evaluation was performed at day 8 post-inoculation. (B) The formula used for the determination of DEGs. Genes whose expression on *M. oryzae* inoculation was increased or decreased by 0.5-fold or more at each time point were selected as DEGs. Only those DEGs present in all three independent biological replicates were selected as DEGs in our study.

Fig. S4 Validation of randomly selected differentially expressed genes (DEGs) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR was performed on 14 randomly selected genes using the leaf samples collected from rice Digu and Lijiangxintuanheigu (LTH) at the time points 5, 10 and 20 h post-inoculation (hpi). The experimental designs of inoculation and sampling were the same as described in Fig. S3A. The expression comparison was performed between *Magnaporthe oryzae* inoculation and mock inoculation at each time point of 5, 10 and 20 hpi for rice Digu or LTH after the qRT-PCR data had been normalized to the expression of the reference *UBQ5* gene. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels with three repeats (Livak and Schmittgen, 2001). SD represents the standard deviation of three technical duplicates. This experiment was biologically repeated three times with similar results.

Fig. S5 Validation of differentially expressed, Digu-specific receptor kinase (RK) genes using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The leaf tissues of Digu and Lijiangxintuanheigu (LTH) were harvested at the time points 5, 10 and 20 h post-inoculation (hpi) from *Magnaporthe oryzae*-inoculated and mock-inoculated plants. qRT-PCR experiments and

data analyses were performed in the same way as described in Fig. S3. SD represents the standard deviation of three technical duplicates. This experiment was biologically repeated three times with similar results.

Fig. S6 Determination of expression of *Os08g10300* in the transgenic plants carrying *Os08g10300ox*. The leaf samples were collected at the seedling stage for RNA extraction, and real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCRs) were performed using the extracted RNA samples. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels with three repeats (Livak and Schmittgen, 2001). SD represents the standard deviation of three technical duplicates. Two representative transgenic lines overexpressed *Os08g10300* (abbreviated as 10300ox-1 and 10300ox-2, respectively) in the TP309 genetic background.

Fig. S7 Determination of the relative expression levels of *pathogenesis-related* (*PR*) genes in transgenic plants carrying *Os08g10300ox*. Inoculation and expression analyses were carried out as described in Fig. S1A. The leaf samples were collected at the seedling stage for RNA extraction, and real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCRs) were performed using the extracted RNA samples. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels with three repeats (Livak and Schmittgen, 2001). SD represents the standard deviation of three technical duplicates. Two representative transgenic lines overexpressed *Os08g10300* (abbreviated as 10300ox-1 and 10300ox-2, respectively) in the TP309 genetic background.

Fig. S8 Determination of the blast disease resistance of the transgenic plants carrying *Os08g10300ox*. The seedlings from 2-week-old plants were inoculated by spraying with *Magnaporthe oryzae* strains (WJ9, WJ10, YC7 and YC26). Disease resistance was determined on two representative transgenic lines overexpressing *Os08g10300* (abbreviated as 10300ox-1 and 10300ox-2, respectively) in the TP309 genetic background at day 8 post-inoculation. The progeny carrying the transgene and the null segregants are indicated by '+' and '–', respectively. TP309 was used as the susceptible control for blast disease resistance determination.

Fig. S9 Determination of the relative expression levels of *CEBiP*, *OsLYP4* and *OsLYP6* in Digu. Inoculation, sampling and expression analyses were carried out as described in Fig. S1A. The relative expression of genes after *Magnaporthe oryzae* inoculation was normalized to the expression with mock inoculation. All quantitative reverse transcription-polymerase chain reaction (qRT-PCR) data were normalized to the expression of the reference *UBQ5* gene before the determination of relative expression. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels with three repeats (Livak and Schmittgen, 2001). SD represents the standard deviation of three technical replicates.

Fig. S10 Nucleotide-binding site and leucine-rich repeat (NBS-LRR)-containing genes encoded by differentially expressed genes (DEGs). Four NBS-LRR genes in Lijiangxintuanheigu (LTH), but none in Digu, are responsive to infection with *Magnaporthe oryzae*.

Fig. S11 Comparative analyses of the receptor kinases (RKs) encoded by the Digu-specific differentially expressed genes (DEGs) [compared with Lijiangxintuanheigu (LTH)] and those expressed differentially in other resistant rice lines in response to *Magnaporthe oryzae*. The RK genes of the resistant rice Gigante Vercellior were retrieved from the study reported previously (Bagnaresi *et al*., 2012), whereas the RKs of the resistant rice *Pi1* and *Pi9* were from the study reported previously (Wei *et al*., 2013). GV, Gigante Vercelli; VN, Vialone Nano; IRBL18, LTH carrying *Pi1*; IRBL22, LTH carrying *Pi9*.

Table S1 Spearman correlations among Digu and Lijiangxintuanheigu (LTH) samples.

Table S2 The differentially expressed genes (DEGs) identified from Digu and Lijiangxintuanheigu (LTH) during rice early responses to *Magnaporthe oryzae*.

Table S3 Comparison of the expression patterns of differentially expressed genes (DEGs) using microarray and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses.

Table S4 The enrichment analysis of functional gene ontology (GO) categories associated with differentially expressed genes (DEGs) in response to *Magnaporthe oryzae*.

Table S5 Pathways specifically present in the durably resistant rice Digu in response to *Magnaporthe oryzae*.

Table S6 Expression evaluation of the genes associated with CEBiP (chitin elicitor-binding protein)-mediated pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) for their responsiveness to *Magnaporthe oryzae* in Digu and Lijiangxintuanheigu (LTH) at 5, 10 and 20 h post-inoculation (hpi).

Table S7 Primers of *pathogenesis-related* (*PR*) genes, randomly selected differentially expressed genes (DEGs) and other genes for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses.