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OsWRKY22, a monocot *WRKY* gene, plays a role in the resistance response to blast

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SUMMARY

With the aim of identifying novel regulators of host and nonhost resistance to fungi in rice, we carried out a systematic mutant screen of mutagenized lines. Two mutant *wrky22* knockout lines revealed clear-cut enhanced susceptibility to both virulent and avirulent *Magnaporthe oryzae* strains and altered cellular responses to nonhost *Magnaporthe grisea* and *Blumeria graminis* fungi. In addition, the analysis of the pathogen responses of 24 overexpressor *OsWRKY22* lines revealed enhanced resistance phenotypes on infection with virulent *M. oryzae* strain, confirming that *OsWRKY22* is involved in rice resistance to blast. Bioinformatic analyses determined that the *OsWRKY22* gene belongs to a well-defined cluster of monocot-specific *WRKY*s. The co-regulatory analysis revealed no significant co-regulation of *OsWRKY22* with a representative panel of *OsWRKY*s, supporting its unique role in a series of transcriptional responses. In contrast, inquiring a subset of biotic stress-related Affymetrix data, a large number of resistance and defence-related genes were found to be putatively co-expressed with *OsWRKY22*. Taken together, all gathered experimental evidence places the monocot-specific *OsWRKY22* gene at the convergence point of signal transduction circuits in response to both host and nonhost fungi encountering rice plants.

INTRODUCTION

WRKY proteins are characterized by the highly conserved WRKYGQK domain and a zinc-finger motif (Eulgem *et al*., 2000). Arabidopsis and rice *WRKY* genes have been classified into three groups depending on the number and type of WRKY domains and zinc-finger motif (Wu *et al*., 2005; Zhang and Wang, 2005).

In plants, the *WRKY*s encode large gene families of transcription factors which are implicated in diverse developmental and physiological functions. They have been shown to be involved in response to wounding (Hara *et al*., 2000), cold (Marè *et al*., 2004) and drought (Pnueli *et al*., 2002). In Arabidopsis, they also play a crucial role in response to infection with *Blumeria graminis* (Lippok *et al*., 2007), *Erwinia carotovora* (Li *et al*., 2004), *Erysiphe cichoracearum* (Li *et al*., 2006), *Hyaloperonospora parasitica* (Knoth *et al*., 2007), *Botrytis cinerea* (Abuqamar *et al*., 2006; Mao *et al*., 2011), *Alternaria brassicicola* (Zheng *et al*., 2006), *Ralstonia solanacearum* (Hu *et al*., 2008) and *Pseudomonas syringae* (Journot-Catalino *et al*., 2006; Murray *et al*., 2007; Xu *et al*., 2006; Zheng *et al*., 2007).

A systematic expression analysis of rice *WRKY*s revealed that 15 of the 45 genes tested were induced on infection with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Magnaporthe oryzae*, the causal agents of bacterial blight and fungal blast, respectively (Ryu *et al*., 2006). Ramamoorthy *et al*. (2008) analysed the rice *WRKY* gene family and showed that several *OsWRKY*s are co-regulated under abiotic stress and on hormone treatments. Custom *OsWRKY* array analysis has recently shown that 24 members of the rice *WRKY* family are differentially regulated in response to biotic or abiotic stress conditions, and some are significantly co-expressed (Berri *et al*., 2009).

Several studies have also suggested the importance of specific *OsWRKY*s (mainly belonging to group 2) in the transcriptional regulation of defence-related genes in rice response to pathogens. More precisely, *OsWRKY71* (group 2A) may function as a transcriptional regulator upstream of *OsNH1* and *OsPR1b* in the defence signalling pathway (Liu *et al*., 2007), while *OsWRKY13* (group 2E) acts as an activator of the salicylic acid

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(SA)-dependent pathway and a suppressor of the jasmonic acid (JA)-dependent pathway (Qiu *et al*., 2007). *OsWRKY6* (group 2D) has been shown to play a role in the pathogen- or SA-inducible expression of *OsPR1a*; indeed, *OsWRKY6* is induced by *Xoo* and SA, and activates *OsPR1* promoter in rice (Hwang *et al*., 2011). Shimono *et al*. (2007) identified a group 3 gene, *OsWRKY45*, which is transcriptionally up-regulated by SA and benzothiadiazole, a functional analogue of SA. Zhang *et al*. (2008) isolated another gene belonging to group 3, *OsWRKY55* (*OsWRKY31* according to the nomenclature of Wu *et al*., 2005), which is induced by the rice blast fungus and auxin. *OsWRKY89* (group 1) was strongly induced by treatments with methyl jasmonate and UV-B radiation (Wang *et al*., 2007). All the *WRKY* genes mentioned above have been defined as positive regulators of resistance as their over-expression has been associated with enhanced resistance to *Xoo* and/or *M. oryzae*. In contrast, Peng *et al*. (2008) identified *OsWRKY62* (group 2A) as a negative regulator of rice defence. Indeed, transgenic plants over-expressing this gene showed compromised *Xoo* resistance. Later, Peng *et al*. (2010) reported that, more generally, *OsWRKY* group 2A members (including *OsWRKY62*) play both positive and negative regulatory roles in tuning rice innate immunity. In most cases, when mutant lines that knocked down the expression of the above-mentioned genes were screened for a phenotype to infection with blast or *Xoo*, clear-cut phenotypes were lacking. For example, *OsWRKY55* over-expressing lines were more resistant to *M. oryzae*, but the RNA-interference knock-down plants did not show the expected specific hypersusceptible phenotypes (Zhang *et al*., 2008). This probably occurs because of the functional redundancy of phylogenetically related and unrelated *WRKY* genes (Berri *et al*., 2009). To decipher the function of a specific gene, it is therefore relevant to ascertain the relation with its closest paralogues and to verify whether it might be part of a co-regulatory network.

In the present study, we screened 35 insertion lines in *OsWRKY*s belonging to different phylogenetic groups for their infection phenotype against two *M. oryzae* isolates. Only one line, corresponding to *OsWRKY22*, exhibited a greater number of enlarged lesions in response to both strains and was analysed further. We showed that *OsWRKY22* is a relevant regulator of rice resistance to *M. oryzae* and is also involved in the rice response to the nonhost barley powdery mildew. To better describe *OsWRKY22*, we analysed the co-regulation and expression profiles after blast infection of this gene together with its closest paralogues belonging to the same subgroup, which is poorly described so far. We highlighted that this *OsWRKY* subgroup within group 3 has closest homologues only in monocot plants. By depicting the genes co-expressed with *OsWRKY22*, we also reported that this gene might play different roles during the rice response to host and nonhost fungi.

RESULTS

wrky22 mutants show increased susceptibility to *M. oryzae*

Seedlings from T2 generations carrying mutations in 23 *WRKY* genes (corresponding to 35 mutant lines) were tested for altered susceptibility or resistance to a *M. oryzae* strain FR13 which is virulent on the corresponding wild-type rice cultivar. A clear-cut phenotype was observed only for two allelic lines in the gene *OsWRKY22* (Os01g60490). The *wrky22* mutants were also challenged with the avirulent *M. oryzae* strain CL3.6.7. When homozygous *wrky22-1* and *wrky22-2* mutants were inoculated with FR13 or CL3.6.7 at 4 and 2 weeks after sowing, respectively, plants reproducibly displayed enhanced susceptibility (Fig. 1a,b) with a significantly increased number of enlarged lesions (Fig. 1c). Reverse transcription-polymerase chain reaction (RT-PCR) analysis using primers located in the second and third exons of *WRKY22* confirmed the highly reduced transcription levels of this gene in both *wrky22-1* and *wrky22-2* homozygous lines (Fig. 1d). The *wrky22* mutants grew at the same rate and flowered at the same time as wild-type plants, and showed normal plant architecture (data not shown). Homozygous *wrky22-1* and *wrky22-2* mutants were also inoculated with barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*) to assess an involvement in nonhost interactions. However, no macroscopic phenotype was observed.

Both allelic mutant lines were further investigated at the cellular level by analysing the formation of H_2O_2 or callose deposition at 24 and 48 h post-inoculation (hpi) (Fig. 2) with *M. oryzae* FR13 (virulent), CL3.6.7 (avirulent) and BR32 (nonadapted). In addition, the two nonhost species *M. grisea* BR29 and barley powdery mildew (*Bgh*) were included in these cytological analyses to learn more about the putative involvement of *OsWRKY22* during nonhost interactions. Figure 2 shows the results obtained with *wrky22-1*, but the same cellular events occurred in both *OsWRKY22* allelic lines.

The frequency of multicellular H_2O_2 production was significantly higher at both 24 and 48 hpi in the mutant plants after inoculation with CL3.6.7 (Fig. 2a). However, 3,3′-diaminobenzidine (DAB) staining did not differ significantly between wild-type and mutants in response to FR13, BR32 and BR29, respectively. In contrast, at 24 hpi, the occurrence of fluorescence corresponding to callose deposition was higher in wild-type plants than in *wrky22* mutants during interactions with *Magnaporthe* CL3.6.7/BR32/BR29 (Fig. 2b). When plants were inoculated with *Bgh*, the occurrence of fluorescence was higher at 24 hpi in *wrky22* mutants.

In conclusion, *wrky22* mutants showed different cellular responses relative to the wild-type with regard to both H_2O_2 formation and callose deposition. This is likely to correlate with the enhanced disease susceptibility phenotype following infection

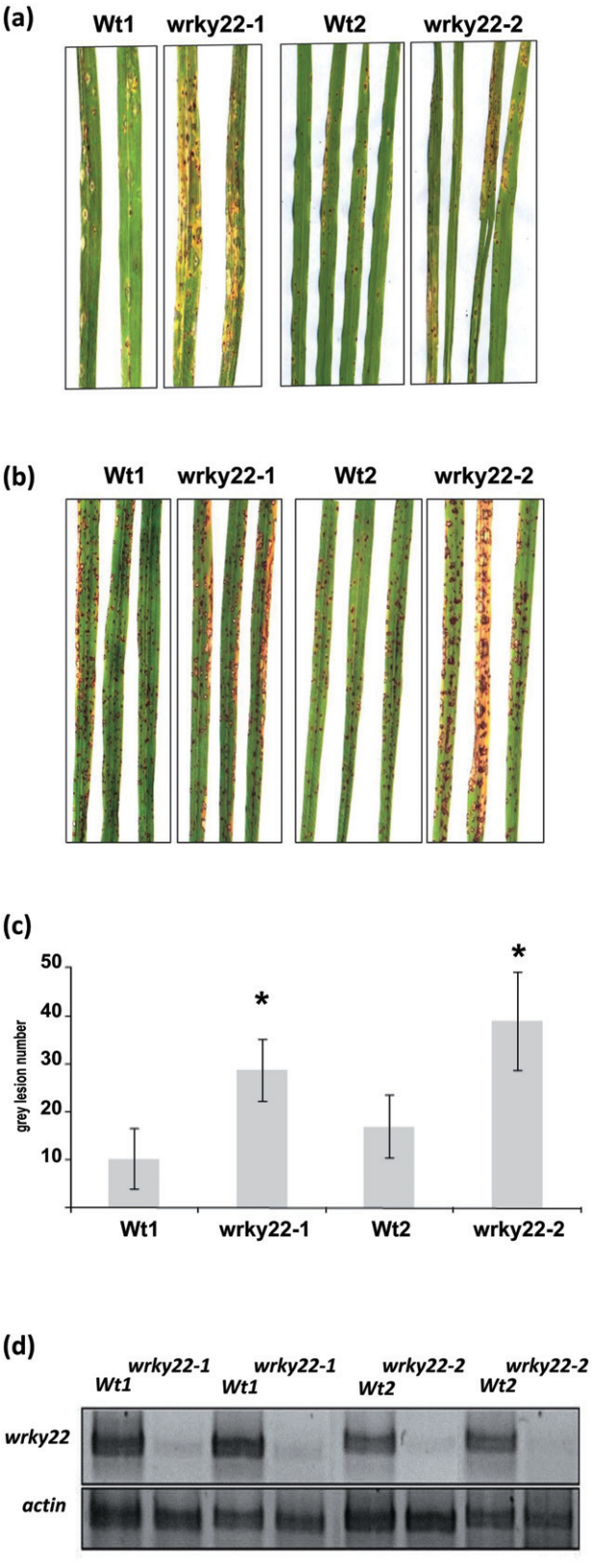

Fig. 1 Phenotypes of the two *wrky22* T-DNA insertion lines and corresponding nullizygous (wild-type, Wt) plants after *Magnaporthe* infection. (a) Symptoms of 4-week-old *wrky22* mutant lines upon *M. oryzae* virulent FR13 infection at 7 days post-inoculation. (b) Symptoms of 2-week-old *wrky22* mutant lines upon *M. oryzae* avirulent CL3.6.7 infection. (c) Number of grey lesions surrounded by brown zones in *wrky22* mutants and corresponding nullizygous (Wt) plants at 7 days post-inoculation with the avirulent *M. oryzae* strain CL3.6.7. (d) Nested reverse transcription-polymerase chain reaction (RT-PCR) of *OsWRKY22* and house-keeping actin expression in *wrky22* mutants and Wt plants before inoculation.

with CL3.6.7 strain as observed at 7 days post-inoculation (dpi) (Fig. 1a–c).

Over-expression of *WRKY22* results in enhanced disease resistance to *M. oryzae*

Transgenic Nipponbare rice lines over-expressing the *OsWRKY22* cDNA were regenerated. None of the over-expressing transformed plants exhibited morphological defects at the vegetative and flowering stages compared with control plants transformed with empty vector (data not shown). The transcription levels of *OsWRKY22* were measured by quantitative RT-PCR (Fig. 3a). In over-expressing (OE) plants, the *WRKY22* transcript level was found to be 600–3000 fold higher than in controls. We tested 24 T0 transformation events for resistance to the compatible strain of blast fungus FR13 in comparison with control plants containing empty vector. Twenty-one (91%) transgenic lines over-expressing *WRKY22* showed significantly enhanced disease resistance (Fig. 3b). Three independent transgenic lines from T0 seeds (bearing only one T-DNA insertion and denoted by #1, #2 and #3 in Fig. 3b) were then multiplied to obtain T1 progeny plants segregating for the introduced T-DNA. Thirteen, 26 and 27 plants for each of these T1 lines were challenged with FR13 and the phenotype of the OE lines was confirmed as resistant (Fig. 4a). Indeed, 75% of the OE analysed plants were totally or partially resistant (Fig. 4b). In addition, this enhanced resistance to FR13 correlated strongly with higher basal WRKY transcript levels measured before inoculation, as shown by the quantitative RT-PCR data in Fig. 4c.

OsWRKY22 is repressed by virulent and induced by avirulent *M. oryzae* isolates

As *wrky22* mutant plants and over-expressing lines showed increased susceptibility and resistance to *M. oryzae*, respectively, the differential regulation of *WRKY22* in rice in response to blast infection was investigated. Gene expression was measured in a time course experiment at early infection stages (between 0 and 24 hpi) using 2-week-old Nipponbare leaves challenged with virulent and avirulent isolates. The inoculation with the virulent isolate FR13 strongly repressed *OsWRKY22* transcription at 8 hpi, whereas transcript levels between infected and mock plants were

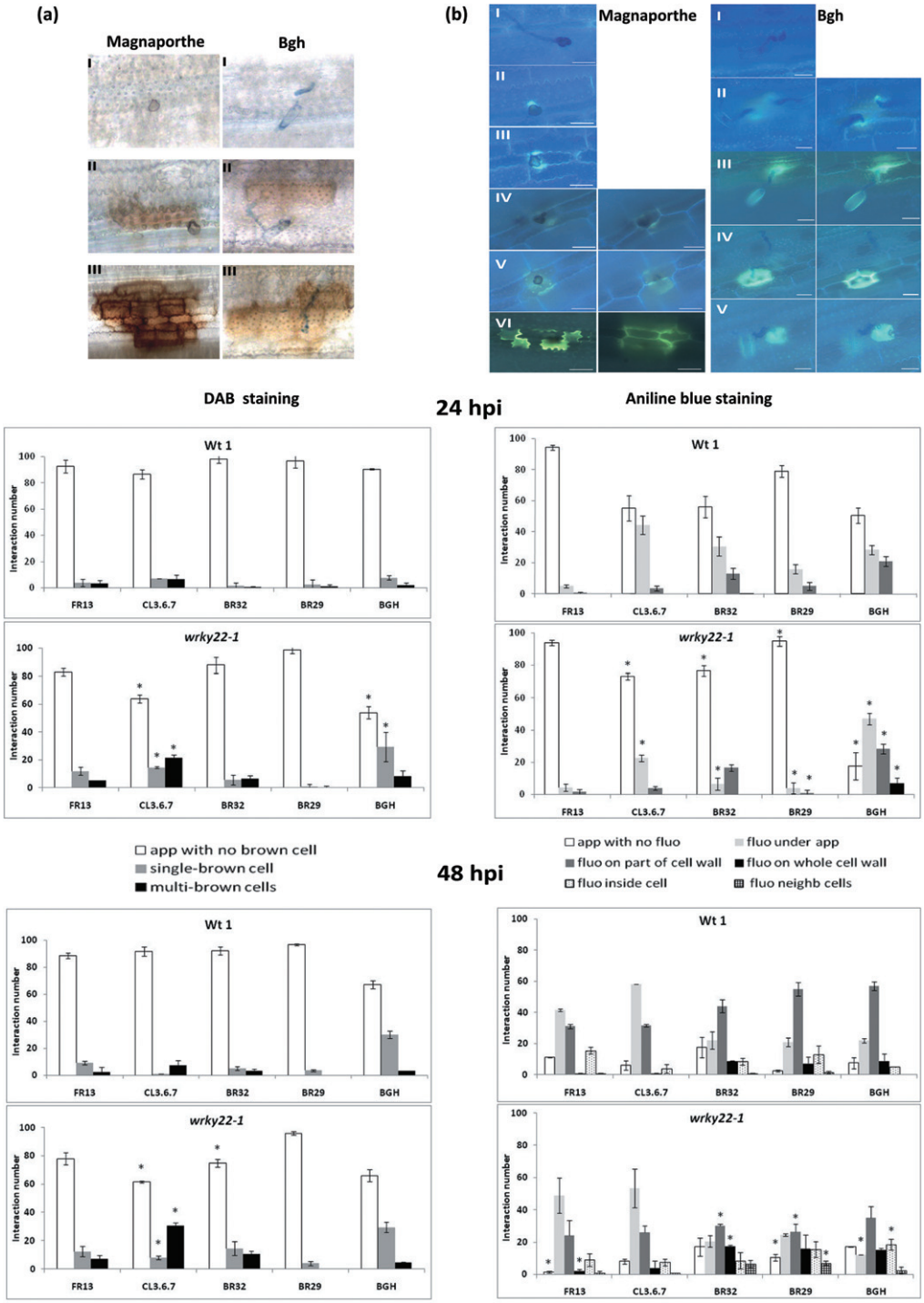

Fig. 2 *Magnaporthe oryzae*, *M. grisea* and *Blumeria graminis* f. sp. *hordei* (*Bgh*) leaf infection process at the cellular level in *wrky22* mutants and corresponding nullizygous wild-type (Wt) plants at 24 and 48 h post-inoculation (hpi). (a) Classification of cytological interaction types revealed after 3,3′-diaminobenzidine (DAB) staining (H_2O_2 production): I, appressorium with no brown cell; II, single brown cell; III, multiple brown cells. (b) Classification of cytological interaction types revealed after aniline blue staining (callose deposition): I, appressorium with no fluorescence; II, fluorescence under appressorium; III, fluorescence on part of the cell wall; IV, fluorescence on the whole cell wall; V, fluorescence inside the cell; VI, fluorescence occurring in the neighbouring cells. Left panels show *Magnaporthe* and right panels *Bgh* infection process. The histograms represent the frequency of DAB-associated cellular phenotypes or callose deposition at 24 and 48 hpi with *Magnaporthe* FR13 (virulent), BR32 and CL3.6.7 (avirulent) isolates, the nonhost *M. grisea* BR29 and *Bgh* strains. The mean and standard deviation of three replicates (constituting 100 interactions) are shown (*$P < 0.05$). Bars, 20 μm.

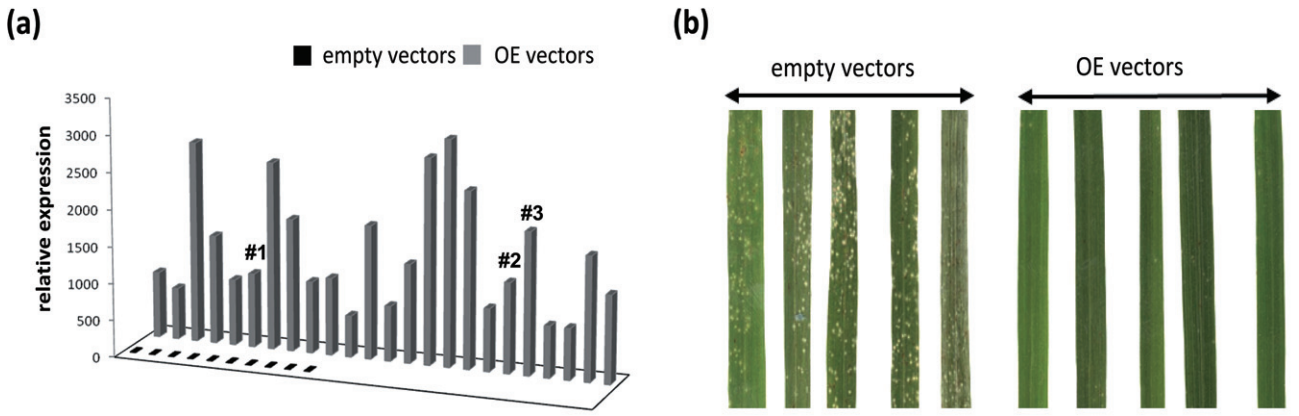

Fig. 3 Over-expressing (OE) *OsWRKY22* rice cv. Nipponbare T0 plants. (a) *OsWRKY22* expression levels in OE transgenic lines and in empty vector control plants before inoculation. #1, #2 and #3 indicate the three selected T0 lines to obtain T1 progeny plants segregating for the introduced T-DNA. (b) Enhanced disease resistance phenotype of the OE lines and phenotype of the empty vector plants upon *M. oryzae* FR13 virulent infection. A representative sample of leaves from OE and empty vector control plants at 7 days post-inoculation is shown.

not statistically significantly different at the later time points. With regard to inoculation with the avirulent CL3.6.7 strain, *OsWRKY22* was found to be statistically up-regulated (compared with the mock plants) only at 12 hpi (Fig. 5).

OsWRKY22 belongs to a monocot subgroup within group 3

To better describe *OsWRKY22* in relation to its closest *OsWRKY* paralogues, the following *in silico* analyses were carried out: (i) hierarchical clustering analyses of rice WRKYs using full protein sequences; and (ii) neighbour-joining (NJ) analysis of all-against-all BLASTp best results of the rice group 3 WRKY.

Using a novel modularity-based clustering method, based on the full protein sequences and tuning to the median of the *E*-value similarities in the entire dataset, the relationships between WRKY family members were obtained (Fig. 6a). The branches in the tree describe the hierarchical structure of the OsWRKY family divided into the three main groups (1, 2, 3) and several subgroups, supported by high modularity values at each node. This hierarchical clustering tree highlights, for the first time, the division of group 3 into three consistent subgroups (I, II and III).

To assess which rice WRKYs had the closest homologues in monocot/dicot plants, an all-against-all BLASTp analysis of the whole OsWRKY family was carried out against the nonredundant protein databases. All OsWRKYs have dicotyledonous closest homologues, except for a small set within group 3. Subsequently, an NJ tree was built using the best hits (*E*-values $> 10^{-30}$) of OsWRKYs belonging to group 3 (Fig. 6b). Two divergent groups were identified: the first contained rice, sorghum, corn, wheat and barley proteins, and the second group included cereal WRKYs as well as proteins from various dicotyledonous plants (Arabidopsis, *Vitis vinifera*, *Lotus japonica*, *Glycine max*, *Ricinus communis*, *Populus trichocarpa*, *Medicago truncatula*). Nineteen OsWRKY proteins, including OsWRKY22, were closest homologues only to other cereal WRKYs and were split into two subgroups, in agreement with our hierarchical clustering analysis. We concluded that these 19 genes within group 3 can be referred to as monocot/cereal-specific (MCS) *OsWRKY*s.

OsWRKY22 is poorly co-regulated with MCS and Os*WRKY* members

To test whether *OsWRKY22* is strongly co-regulated with the other MCS *OsWRKY* members, we gathered a large set of Affymetrix expression data in a matrix [Rice Expression Matrix (REM)] and performed co-regulation analysis using RI-REG, a software tool specifically developed to calculate the Pearson coefficient. A stringent cut-off (0.8) was applied during linear and logarithmic co-regulation analysis to include only the genes best co-regulated with the MCS *WRKY*s. Therefore, 37 genes (from 94 in the Affymetrix dataset) are present in the networks (Fig. 7), including the 14 MCS *WRKY* genes occurring in the Affymetrix chip.

Eighteen and four *WRKY* genes formed one major and one small network, respectively, with very similar structures in both P-lin and P-log analyses, but the number of connections decreased considerably in the P-log network. The remaining 15 genes were outside these two main networks. In the P-lin analysis, four MCS *WRKY*s (*OsWRKY116*, *OsWRKY20*, *OsWRKY82*, *OsWRKY52*) fell only into the major network (Fig. 7a). Most of the genes belonging to the major network were linked by multiple connections, including the four MCS *WRKY*s. Surprisingly, the MCS *WRKY* genes were not directly connected, except for *OsWRKY116* and *OsWRKY20*. In the P-log network (Fig. 7b), *OsWRKY22* belonged to the major and *OsWRKY18* to the small network, suggesting that these genes are co-regulated with other rice *WRKY*s in specific conditions. *OsWRKY22* was connected only with two MCS (*OsWRKY116*, *OsWRKY20*) and two other members of the rice *WRKY* family

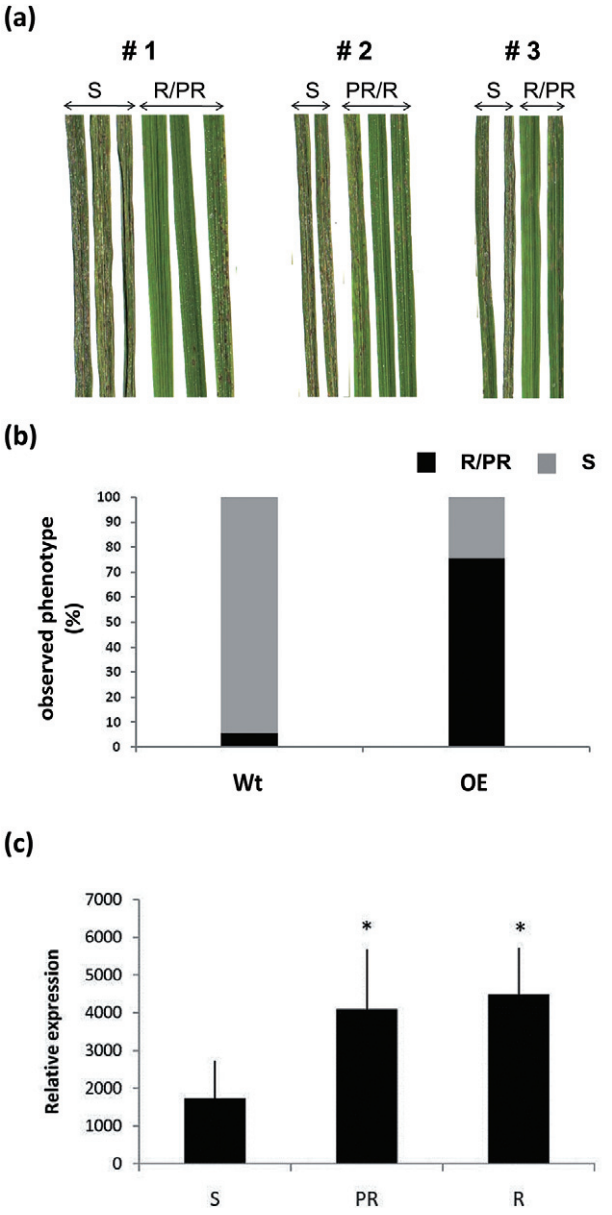

Fig. 4 Over-expressing (OE) *OsWRKY22* rice cv. Nipponbare T1 plants. Three independent transgenic lines (#1, #2 and #3, all carrying only one T-DNA) with 13, 26 and 27 plants for each line were analysed for phenotyping and molecular analysis. (a) Symptoms of three OE transgenic lines on *Magnaporthe oryzae* FR13 virulent infection: S, susceptible; R, resistant; PR, partially resistant. A representative sample of leaves from OE plants is shown. (b) Frequency of observed and expected S/R/PR phenotypes of OE transgenic lines and nullizygous (wild-type, Wt) plants upon infection with FR13. (c) Basal *OsWRKY22* expression levels in OE transgenic lines and nullizygous Wt plants before inoculation. The mean and standard deviation of three replicates are shown (*$P < 0.05$).

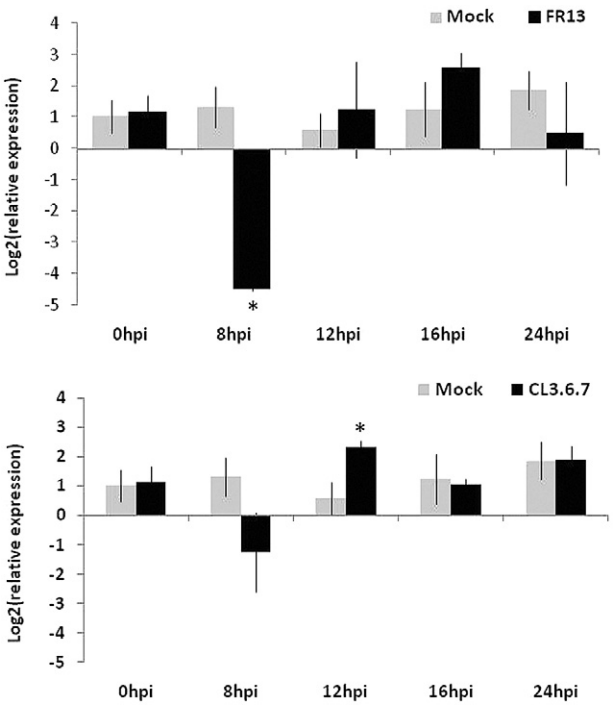

Fig. 5 *OsWRKY22* gene expression following *Magnaporthe* or mock inoculation by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The strains used were *Magnaporthe* FR13 (virulent) and CL3.6.7 (avirulent) isolates. Values represent the means of $\log_2$(relative expression) and standard deviation for four biological replicates. Statistical differences between mock and infected samples were assessed by *t*-test analysis (*$P < 0.05$). All calculations for relative quantification were performed as described in Pfaffl (2001) and the reference gene used was actin (Os03g50885).

(*OsWRKY58* and *OsWRKY43*). Increasing the cut-off to 0.9, only *OsWRKY116* and *OsWRKY20* were connected in P-lin, whereas no connections among MCS *OsWRKY* genes were found in the P-log network (data not shown).

In conclusion, MCS *WRKY*s are poorly co-regulated and especially only in specific conditions. *OsWRKY116* and *OsWRKY20* are the only two MCS *WRKY* genes strongly co-regulated.

WRKY22 and MCS *OsWRKY* involvement in the blast response

To learn more about the potential involvement of *OsWRKY22* and MCS *WRKY*s in response to different *Magnaporthe* isolates, the gene expression of 10 members (*OsWRKY22*, *OsWRKY20*, *OsWRKY108*, *OsWRKY55*, *OsWRKY84*, *OsWRKY112*, *OsWRKY52*, *OsWRKY46*, *OsWRKY50*, *OsWRKY95*) was measured by quantitative RT-PCR. Nipponbare leaves challenged with *M. oryzae* virulent FR13, avirulent CL3.6.7, nonadapted BR32 and *M. grisea* nonhost isolate BR29 were collected at 12 hpi (before fungal penetration of leaf epidermal cells), 24 hpi (beginning of fungus penetration) and 48 hpi (multicellular propagation for host strains) after infection.

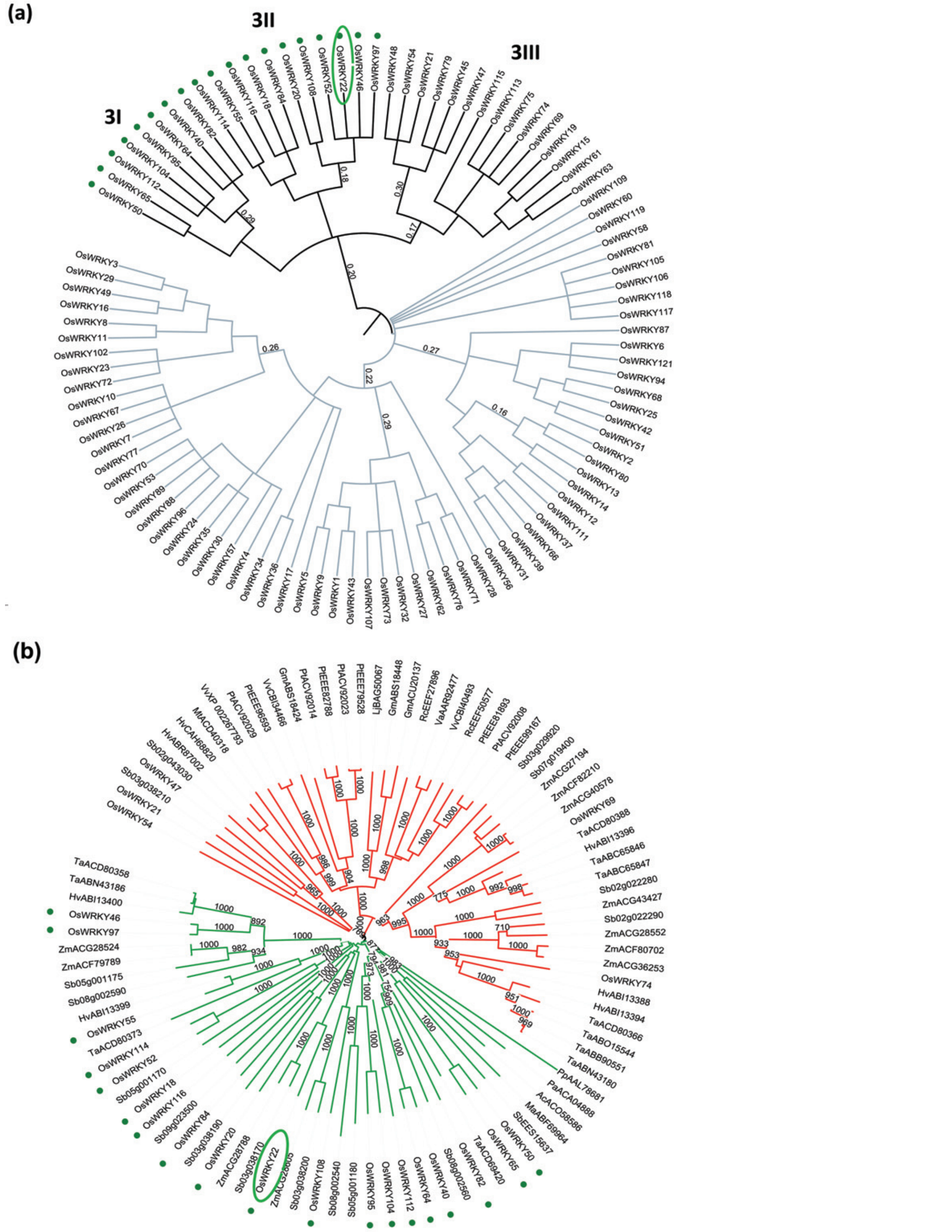

Fig. 6 *In silico* analysis of the monocot-specific OsWRKY proteins. (a) Hierarchical clustering tree of the OsWRKYs obtained using the full protein sequences. Subgroups 3I, 3II and 3III are the three subclusters within group 3 identified in this study. (b) Neighbour-joining tree based on all-against-all BLASTp best hits (*E*-values > 1e^{-30}) of all group 3 OsWRKYs. The 19 WRKYs belonging to subgroups 3I and 3II (green dots) are identifiable within the monocot-specific group (in green) bearing only sequences from cereals. Gm, *Glycine max*; Hv, *Hordeum vulgare*; Lj, *Lotus japonica*; Mt, *Medicago truncatula*; Pt, *Populus trichocarpa*; Rc, *Ricinus communis*; Sb, *Sorghum bicolor*; Ta, *Triticum aestivum*; Vv, *Vitis vinifera*; Zm, *Zea mays*. Outgroups: Ac, *Areca catechu*; Ma, *Musa acuminate*; Pa, *Picea abies*; Pp, *Physcomitrella patens*.

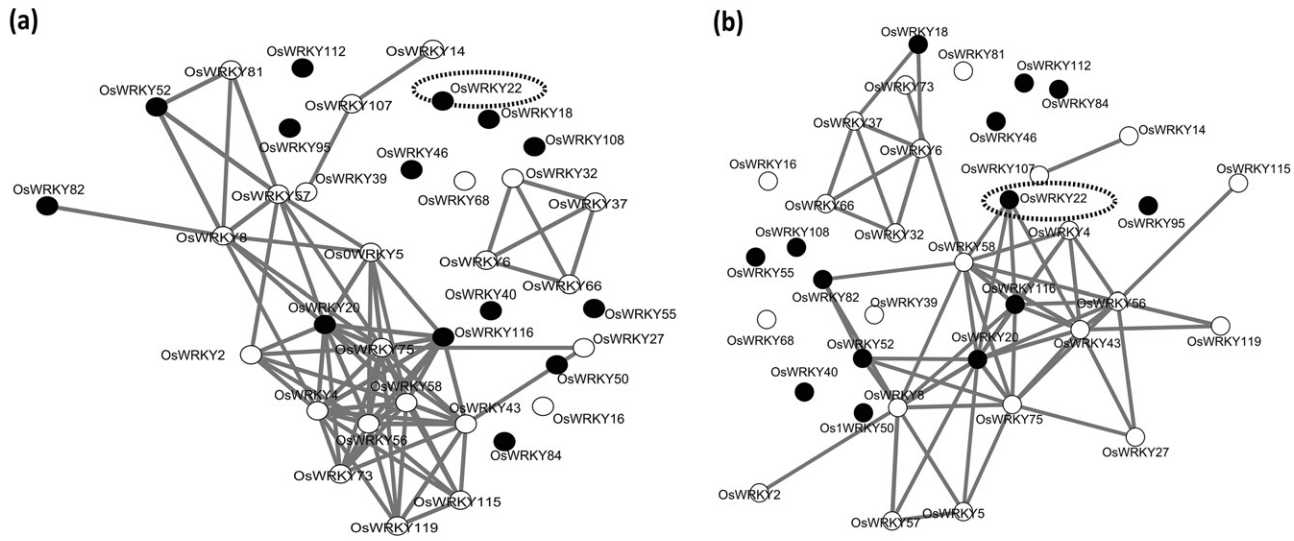

Fig. 7 Co-regulatory networks of the monocot/cereal-specific (MCS) *OsWRKY* genes and other members of the *OsWRKY* family. (a) Linear Pearson analysis. (b) Logarithmic Pearson analysis. The 14 MCS *OsWRKY* genes (black nodes) and their best co-regulated *OsWRKY*s (white nodes) are included in the graphical representation (lines connecting nodes represent Pearson coefficient threshold > 0.8).

The *OsWRKY22* transcript level was not differentially regulated on BR32 and BR29 infection at the considered time points. With regard to the other MCS *WRKY*s, eight of 10 genes were significantly induced or repressed following plant inoculation with virulent, avirulent and nonhost *Magnaporthe* strains (Fig. S1, see Supporting Information). In particular, some of them were involved in the rice response to several isolates at specific time points. *OsWRKY112* and *OsWRKY20* expression profiles did not show differences between infected and mock plants at the tested time points (data not shown).

Genes co-expressed with *OsWRKY22* putatively involved in the rice defence response

OsWRKY22 function was investigated, identifying genes co-expressed with *WRKY22* during the rice defence response. For this purpose, a subset of the Rice Expression Matrix, including only the experiments in biotic stress conditions (286 of 707), was inquired. The resulting 147 genes co-regulated with *OsWRKY22* (41% of those annotated) were classified into eight major classes in relation to their potential defence function in rice (Fig. 8 and Table S1, see Supporting Information). Disease resistance genes were the most abundant (20%), including mainly leucine-rich repeat (LRR) genes. *Mildew resistance locus* a (*Mla1* and *Mla6*) were also present. The pathogenesis-related (PR)/PR-like genes represented 9%, including mainly lipid transfer protein-like (LTP), defensins and subtilisins. The defence-related genes co-expressed with *OsWRKY22* (11%) included genes involved in the synthesis of antimicrobial molecules or pathogen recognition (flavonoids, extensin, terpenes, ankyrin, harpins, lectins). The cell wall-related class represented only 2%. Genes related to primary/secondary metabolism constituted a high proportion of the co-expressed genes (18%) and most were related to protein folding and degradation processes (bric-a-brac tramtrack broad complex, ubiquitin complex, proteases, speckle genes). Genes involved in signalling (13%) during pathogen detection, including kinase receptors and lipase/lipase-like genes, were also co-expressed with *OsWRKY22*. The hormone transduction class included only two genes related to ethylene and jasmonate synthesis. Among regulators (7%), the most abundant were MYB and helix–loop–helix (HLH) transcription factors. Genes indirectly involved in the pathogen response, such as the F-box family, SNARE domain, flavin monooxygenase and reticuline oxidase-like protein, were also present in the list of genes co-expressed with *OsWRKY22* ('others' in Fig. 8).

No *OsWRKY* genes were present among regulators. Interestingly, the two MCS *OsWRKY116* and *OsWRKY20*, shown to be co-regulated with *OsWRKY22* in the P-log network of this study, were not among the genes co-expressed with *OsWRKY22*, when only the subset of biotic stress-related experiments was analysed. This finding confirms that *OsWRKY22* acts independently from other rice *WRKY*s as the master regulator of specific signal transduction pathways leading to defence-related rice-specific responses to fungal pathogens.

DISCUSSION

We have screened several knock-down insertion lines corresponding to 23 *WRKY* genes from different phylogenetic groups to identify novel *OsWRKY* genes involved in plant innate immunity. Altered susceptibility to both virulent and avirulent *M. oryzae*

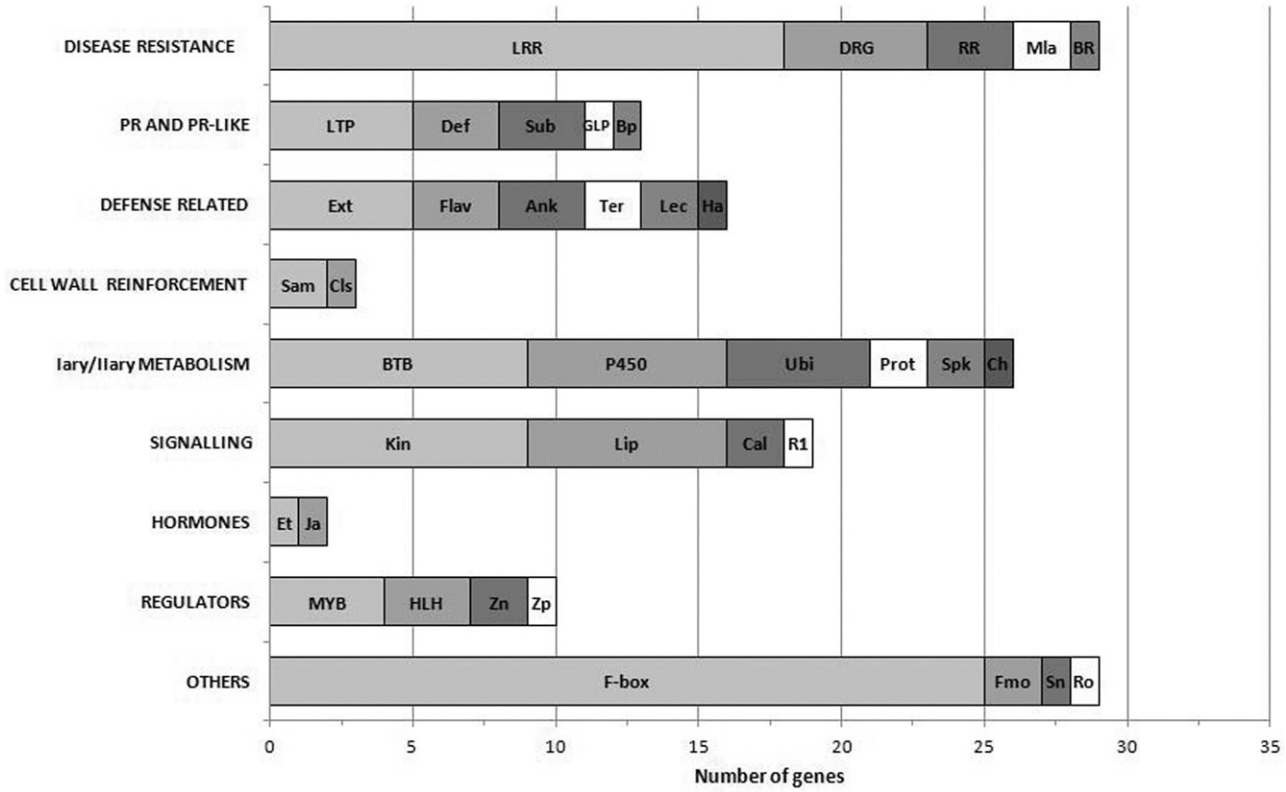

Fig. 8 Bar charts of the genes co-expressed with *OsWRKY22* putatively involved in the rice defence response (147 genes) according to their known or predicted function. The number of genes is shown on the *x*-axis and the main categories on the *y*-axis. LRR: different categories of leucine-rich repeat (LRR) resistance genes; DRG: defence-related genes; RR: rust resistance; Mla: MLA protein; BR: blight resistance. LTP: LTP family; Def: defensins; Sub: subtilisins; GLP: germin-like proteins; Bp: BURP domain. Ext: extensin family; Flav: flavonoids-related genes; Ank: ankyrin repeat family; Ter: terpene synthases putative; Lec: lectin domain; Ha: harpin-induced protein. Sam: SAM-dependent carboxyl methyltransferase; Cls: cellulose synthase-like family. BTB: Bric-a-Brac Tramtrack Broad Complex; P450: cytochrome P450; Ubi: several proteins belonging to the ubiquitin complex; Prot: proteases; Spk: speckle-type POZ proteins; Ch: chorismate mutase. Kin: different categories of receptor kinase; Lip: GDSL-like lipases; Cal: calmodulin-binding proteins; R1: RGA-1. Et: 1-aminocyclopropane-1-carboxylate oxidase; Ja: jasmonate *O*-methyltransferase. MYB: MYB transcription factor; HLH: helix–loop–helix DNA-binding domain; Zn: zinc finger C3HC4-type domain; Zp: bZIP transcription factor domain. F-box: F-box domain; Fmo: flavin mono-oxygenase; Sn: SNARE domain; Ro: reticuline oxidase-like protein.

strains was associated with typical blast lesions for the gene *OsWRKY22*.

At the cellular level, the *wrky22* mutant plants were impaired in callose deposition in response to the avirulent *M. oryzae* and the nonhost *M. grisea* strains at 24 hpi, whereas higher callose deposition occurred in *wrky22* mutants in response to *Bgh*. In plants, the first layer of defence encountered by pathogens is the cell wall (Thordal-Christensen, 2003). When attacked, plants physically reinforce cell walls by lignification (Göhre and Robatzek, 2008) and the deposition of callose (Kudlicka and Brown, 1997). The OsWRKY22 protein does not seem to play the same role at early time points in response to penetration attempts of host and nonhost fungi, which may rely on a different elicitor repertoire of *M. oryzae* or *Bgh*. These differences most probably reflect the different lifestyles of the two pathogens, i.e. facultative versus obligate biotrophy. Ambivalent responses against these pathogens have been reported previously for barley (Jarosch *et al*., 1999; Zellerhoff *et al*., 2008). In addition, a significantly higher H_2O_2 production following inoculation with the avirulent *M. oryzae* strain, as well as with the nonhost *Bgh* isolate, was observed. This higher H_2O_2 accumulation was maintained only in the avirulent interaction at 48 hpi. Reactive oxygen species (ROS), including H_2O_2, are involved in rapid programmed cell death to block pathogen progression (Thordal-Christensen *et al*., 1997), and are usually associated with plant resistance mechanisms (Shetty *et al*., 2008). However, ROS accumulation alone is often insufficient to ensure disease resistance (Heath, 2000; Hückelhoven, 2007). Despite the high level of H_2O_2 production, the *wrky22* mutants also exhibited an increased number of blast lesions compared with wild-type plants. This observation supports the hypothesis that, at late stages of infection, *M. oryzae* benefits from ROS-induced cell death, because the pathogen switches to necrotrophy. A similar scenario has been described for the *mlo* mutant plants inoculated with *M. oryzae*

strains (Jarosch *et al*., 1999). However, this enhanced H_2O_2 production boosts the notion that the avirulent blast pathogen and the nonhost *Bgh* fungus are able to penetrate more easily into *wrky22* mutant epidermal cells, which then respond, activating a second layer of defence responses, i.e. hypersensitive response reflected by whole-cell or multi-cell DAB staining, as also reported for the Arabidopsis *pen* mutants (Collins *et al*., 2003; Lipka *et al*., 2005; Stein *et al*., 2006).

WRKY genes can function as either repressors or activators of different biological processes (Hu *et al*., 2008; Li *et al*., 2004, 2006; Qiu *et al*., 2007; Wang *et al*., 2006) and respond to different pathogens (Tao *et al*., 2009). Our results revealed that *OsWRKY22* is likely to play a central role not only in the regulation of upstream defence responses to virulent and avirulent rice *Magnaporthe* strains, but also in the rice response to the nonhost powdery mildew isolate.

We also showed that rice transgenic plants over-expressing *OsWRKY22* displayed an enhanced resistance to the virulent host *M. oryzae* strain. Several *OsWRKY*s were found to be involved in the rice response to *Xanthomonas oryzae* (Liu *et al*., 2007; Peng *et al*., 2008), *Pseudomonas syringae* DC3000 (Qiu and Yu, 2009) and *M. oryzae* (Chujo *et al*., 2007; Zhang *et al*., 2008). However, most of the enhanced resistance phenotypes mentioned above were observed in ectopic over-expressing lines, as claimed by Pandey and Somssich (2009). In contrast, in the data presented here, rice plants showed opposite phenotypes to *Magnaporthe* virulent infection, depending on whether *OsWRKY22* was knocked-down or over-expressed. The only other two *WRKY* genes previously presenting a different phenotype in mutant and over-expressing lines were *OsWRKY45* (Shimono *et al*., 2007) and *OsWRKY89* (Wang *et al*., 2007).

In our previous phylogenetic analysis using Arabidopsis and rice WRKY domains, OsWRKY22 clustered in group 3 within a subgroup called 3C (Berri *et al*., 2009). Further analyses were performed in the present study to describe *OsWRKY22* in relation to its subgroup. A novel clustering method was used to better define the hierarchical structure of group 3, as there is no consistency with regard to the number of its subclusters and genes so far (Berri *et al*., 2009; Wu *et al*., 2005; Xie *et al*., 2005; Zhang and Wang, 2005). The hierarchical OsWRKY tree was in good agreement with previous phylogenetic classifications (Berri *et al*., 2009; Ross *et al*., 2007; Wu *et al*., 2005). Nevertheless, it consistently established the partition of group 3 into three subgroups (3I, 3II and 3III), taking into account also single conserved residues/motifs. This approach is relevant when analysing poorly conserved proteins, such as WRKYs. The NJ tree of the all-against-all BLASTp showed that the 19 *OsWRKY*s belonging to subgroups 3I–3II had closest homologues only in monocot/cereal plants. Several authors have suggested a monocot/rice-specific group of WRKY proteins (Berri *et al*., 2009; Mangelsen *et al*., 2008; Wu *et al*., 2005; Yang *et al*., 2009; Zhang and Wang, 2005); however, no detailed analyses demonstrating the split of this clade from the rest of the gene family had been carried out before that presented here.

We observed that 74% of MCS *WRKYs* are positioned in segmentally duplicated blocks (data not shown), whereas *OsWRKY22*, *OsWRKY116*, *OsWRKY20* and *OsWRKY108* are reported to be tandemly duplicated genes (Ramamoorthy *et al*., 2008; Ross *et al*., 2007; Xie *et al*., 2005). Although extensively duplicated, only a small portion of MCS *OsWRKY* genes were co-expressed and only at specific conditions in our co-regulation analysis. Nevertheless, the same genes were also connected to *OsWRKY* members of other phylogenetic groups, in agreement with the analysis for the Arabidopsis *WRKY* family (Berri *et al*., 2009). Although *WRKY22* is involved in the blast response, as well as other monocot-specific *WRKYs* (as suggested by the quantitative RT-PCR data), this gene appears to play a unique and independent role in the rice defence response, as supported by the co-regulatory network analysis and the list of genes co-expressed with *OsWRKY22* putatively involved in the response to biotic stress.

To understand the role of a gene, it is crucial to identify the genes associated with a specific regulatory network. Given the phenotype of *wrky22* mutants following blast infection, we explored a subset of the Rice Expression Matrix involved in the pathogen response and identified a list of genes co-expressed with *OsWRKY22*, which are likely to be putative targets, regulators or partners in its network. Indeed, similar correlation analyses have been shown previously to represent a powerful tool to investigate the gene function and to identify new candidate genes involved in specific cellular processes (Gigolashvili *et al*., 2009; Hirai *et al*., 2007; Murgia *et al*., 2011; Vandepoele *et al*., 2009). On the basis of the thorough analysis of the co-expressed genes, we propose a putative defence signalling model involving *OsWRKY22* (Fig. S2, see Supporting Information). Three interaction types are shown: virulent *M. oryzae*, avirulent *M. oryzae* and nonhost *Bgh*. Cell wall components and pathogen-associated molecular patterns (PAMPs) are recognized by specific receptors that activate or repress *OsWRKY22* transcription. These events stimulate or repress the induction of specific sets of defence-related responses involved in the oxidative burst, cell wall reinforcement, secretion of antimicrobial molecules, protein degradation and activation of different transcription factors.

Further validation and investigations of the candidate genes described in the defence signalling model will be an intriguing research perspective. To this aim, high-throughput technologies for expression (RNA-seq) and interactions (ChIP-Seq), modelling networks from sequences, and integrated expression and genetics approaches will facilitate the discovery of the biological functions of *OsWRKY22* and other MCS *WRKY*s implicated in rice–blast (Pandey and Somssich, 2009; Roccaro and Somssich, 2011) as well as rice–powdery mildew interactions.

EXPERIMENTAL PROCEDURES

Identification of *OsWRKY* insertion mutants

Thirty-five *WRKY* insertion mutants from the collections of the Génoplante Oryza Tag Line (http://oryzatagline.cirad.fr/) (Larmande *et al*., 2008; Sallaud *et al*., 2004) and National Institute of Agrobiological Sciences (http://tos.nias.affrc.go.jp/) (Miyao *et al*., 2007), corresponding to 23 different genes belonging to different phylogenetic groups (Table S2, see Supporting Information), were screened in our laboratory for rice–*M. oryzae* interaction. All mutant homozygous lines were derived by self-pollination. The genotype of the progeny was determined by PCR analysis using the primers listed in Table S3 (see Supporting Information). Gene-specific primers for screening wild-type plants and T-DNA/Tos17 border primer combined with the gene-specific primer for screening homozygous lines were used. The number of T-DNA/Tos17 inserts in the mutant lines was determined by Southern blot analysis (see Appendix S1).

Over-expression of *OsWRKY22*

The open reading frame (ORF) of *OsWRKY22* (Wa32) was amplified by hemi-nested PCR using the following primer combinations: Wa32cloF1/R1 and Wa32cloF2/R1 (Table S3). Appropriately sized DNA fragments were gel purified (Machery Nagel, Düren, Germany) and then subcloned between the two *Bam*HI sites of the pCR® 4 Blunt TOPO® Vector (Life Technologies Ltd., Paisley, UK). The binary vector of the *OsWRKY22* ORF was obtained by ligation into the corresponding restriction sites of the multiple-cloning site of pC2300 Ubi Tnos, a pCAMBIA2300 (CAMBIA, Canberra, Australia) derivative [J. C. Breitler, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Montpellier, France, unpublished data]. Positive clones were selected on Luria–Bertani (LB)–kanamycin plates after incubation at 37 °C overnight, and screened for inserts by colony PCR. The *OsWRKY22* over-expressing construct was transferred into the rice variety Nipponbare by *Agrobacterium*-mediated transformation following the protocol of Toki *et al*. (2006). The copy number of each construct in transgenic plants was determined by Southern blot analysis.

DAB and aniline blue staining

The production of H_2O_2 and ROS was detected using the 'DAB-uptake method' described by Thordal-Christensen *et al*. (1997). The inoculated leaf samples of the *wrky22* mutant and wild-type were examined by light microscopy. Callose was stained by the method reported in Zellerhoff *et al*. (2008). Leaves were analysed by epifluorescence microscopy using a Leica (Leica Microsystems GmbH, Wetzlar, Germany) DMRBE microscope and a long-pass filter (excitation filter, 340–380 nm; dichromatic mirror, 400 nm; suppression filter LP, 425 nm). A total of 100 infection sites was inspected per leaf and scored independently for both stains. Three leaves were analysed for each data point and the mean with standard deviation was calculated. Statistically significantly different comparisons between wild-type and mutants were assessed by *t*-test analysis.

Plant material and infection assays

All rice plants used in this study had the Nipponbare genetic background. Rice plants were grown according to Faivre-Rampant *et al*. (2008). The isolates BR29, BR32, CL3.6.7 and FR13 were chosen from the *Magnaporthe* strain collection (CIRAD). Inoculation assays were carried out as described previously (Faivre-Rampant *et al*., 2008). Macroscopic phenotypes (necrosis, lesions) on rice leaves were observed at 7 dpi.

Bgh was maintained on the compatible barley (*Hordeum vulgare*) line Golden Promise by weekly transfer. Inoculation assay on secondary leaves of 2-week-old rice plants was carried out in analogy with barley plants, as described previously by Zellerhoff *et al*. (2010). Rice leaves were examined for macroscopic lesions at 7 dpi.

Leaf samples were harvested at 24 and 48 hpi with *Magnaporthe* and *Blumeria* strains for microscopy, and at 24 hpi for transcriptome analysis.

Computational analysis of the group 3 WRKY protein sequences

A novel modularity-based clustering was developed and applied to the 101 rice WRKY full protein sequences. The procedure consisted of several steps, including the use of BLAST *E* values as a proxy for similarity, the re-scaling of *E* values into similarities using a sigmoid function centred around the median of the data and the repeated re-clustering of clusters with a different median *E* value. Full details are presented in Appendix S1.

An all-against-all BLASTp search was conducted using the complete set of OsWRKY sequences in the query of the nonredundant protein databases of GenBank. Alignment of the 19 monocot-specific OsWRKY proteins and their closest homologues (corresponding to *E* values $< 10^{-30}$) was performed using CLUSTALX2. Bootstrap values were obtained using a random number generator seed of 111 and number of bootstrap trials of 1000. A tree image showing relationships among clusters was obtained using iTOL (http://itol.embl.de/index.shtml).

Transcriptome analysis

Three biological replicates for each interaction type and the corresponding mock were extracted and analysed independently by GeneChip® Rice Genome Array. Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions and purified using Qiagen RNeasy columns. RNA was quantified using a NanoDrop ND-1000 spectrophotometer and quality was tested using a 2100 Bioanalyser (Agilent Technologies, Inc., Santa Clara, CA, USA). For Affymetrix GeneChip® analysis, 8 μg of total RNA were used to make biotin-labelled cRNA targets. All the processes for cDNA and cRNA synthesis, cRNA fragmentation, hybridization, washing, staining and scanning were conducted according to the GeneChip® standard protocol (Eukaryotic Target Preparation, Affymetrix, Affymetrix Inc., Santa Clara, CA, USA). CEL files were included in the Rice Expression Matrix (see below) and analysed together with other raw expression data. Information on the GeneChip® Rice Genome Array can be found in the Affymetrix website http://www.affymetrix.com/estore/index.jsp.

Rice Expression Matrix and clustering analysis

As of October 2010, the Rice Expression Matrix consisted of 728 hybridizations and was constructed by gathering the publicly available Affymetrix

experiments from platform GPL2025 in Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and the 21 hybridizations carried out in this study. The raw microarray data were normalized using the RMA (Robust Multichip Average) Express program, as reported by Bolstad *et al*. (2003). Further details are given in Appendix S1.

Correlation analysis software (RI-REG) and co-regulatory networks

The Visual C++ program (RI-REG), developed by Berri *et al*. (2009), was applied to analyse gene co-regulation. The Rice Expression Matrix was used without further modification, scaling, array normalization or processing of replicates. The Pearson correlation coefficient for each gene pair was calculated using the linear values (linear analysis) or following log transformation (Menges *et al*., 2008). Graphical representations of *WRKY* gene networks were produced using igraph (http://igraph.sourceforge.net; Csárdi and Nepusz, 2006). More details are given in Appendix S1.

Real-time quantitative RT-PCR analysis

RNA (15 μg) was treated with DNase I (Fermentas, Burlington, Ontario, Canada), and 5 μg of RNA were denatured for 5 min at 65 °C in water with oligo(dT)18 (3.5 μM) and deoxynucleoside triphosphate (dNTP) (1.5 μM). Reverse transcription was carried out for 60 min at 37 °C with 200 U of reverse transcriptase M-MLV (Promega, Madison, WI, USA). Two microlitres of cDNA (dilution 1:10) were then used for quantitative RT-PCR. Forward and reverse primers are given in Table S3. All calculations for relative quantification were performed as described in Pfaffl (2001) using a mathematical model to determine the relative quantification of the target gene compared with the reference gene (actin) from inoculated leaves versus controls (mock). The mean and standard deviation of four biological replicates are reported. A *t*-test was performed to establish whether infected plants were significantly different from mock plants ($*P < 0.05$).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Monocot-specific *OsWRKY* gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of 2-week-old Nipponbare leaves following *Magnaporthe* or gelatin inoculation at 12, 24 and 48 h post-inoculation (hpi). The strains used were *Magnaporthe* FR13 (virulent), CL3.6.7 (avirulent), BR32 (nonadapted) and nonhost *M. grisea* BR29. Values represent the means of $\log_2$(relative expression) and standard deviation for four biological replicates. Statistical differences between mock and infected samples were assessed by *t*-test analysis ($*P < 0.05$). All calculations for relative quantification were performed as described in Pfaffl (2001) and the reference gene used was actin (Os03g50885).
Fig. S2 Putative schematic overview of OsWRKY22 involvement in rice responses to blast and barley powdery mildew.
Table S1 List of genes co-expressed with *OsWRKY22* putatively involved in the rice defence response.
Table S2 List of insertion mutant lines analysed in this study.
Table S3 List of primers used in this study.
Appendix S1 Supporting methods.

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