The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection

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SUMMARY

Arbuscular mycorrhizal (AM) symbioses are mutualistic associations between soil fungi and most vascular plants. Their association benefits the host plant by improving nutrition, mainly phosphorus nutrition, and by providing increased capability to cope with adverse conditions. In this study, we investigated the transcriptional changes triggered in rice leaves as a result of AM symbiosis, focusing on the relevance of the plant defence response. We showed that root colonization by the AM fungus *Glomus intraradices* is accompanied by the systemic induction of genes that play a regulatory role in the host defence response, such as *OsNPR1*, *OsAP2*, *OsEREBP* and *OsJAmyb*. Genes involved in signal transduction processes (*OsDUF26* and *OsMPK6*) and genes that function in calcium-mediated signalling processes (*OsCBP*, *OsCaM* and *OsCML4*) are also up-regulated in leaves of mycorrhizal rice plants in the absence of pathogen infection. In addition, the mycorrhizal rice plants exhibit a stronger induction of defence marker genes [i.e. pathogenesis-related (PR) genes] in their leaves in response to infection by the blast fungus *Magnaporthe oryzae*. Evidence indicates that mycorrhizal rice plants show enhanced resistance to the rice blast fungus. Overall, these results suggest that the protective effect of the AM symbiosis in rice plants relies on both the systemic activation of defence regulatory genes in the absence of pathogen challenge and the priming for stronger expression of defence effector genes during pathogen infection. The possible mechanisms involved in the mycorrhiza-induced resistance to *M. oryzae* infection are discussed.

INTRODUCTION

Most agricultural crop species all over the world suffer from a vast array of fungal diseases that cause severe yield losses. Rice blast, caused by the fungus *Magnaporthe oryzae*, is one of the most devastating diseases of cultivated rice (*Oryza sativa* L.), because of

its widespread distribution and destructiveness (Talbot, 2003). The conventional control of rice blast depends on the use of chemically synthesized fungicides. However, the use of such chemicals has several drawbacks, such as their side effects on nontarget organisms, the incidence of development of resistance on prolonged application and the adverse impact on human health and the environment (Yamaguchi and Fujimura, 2005). Breeding for durable resistance to this fungus is a difficult problem, as most of the resistance genes break down in a few years because of their race specificity and the rapid change in pathogenicity of the blast fungus.

Plants have the ability to respond to pathogen infection through a variety of defence reactions (Jones and Dangl, 2006). Pathogens activate basal defence responses in the plant cell through the receptor-mediated recognition of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) and downstream signalling to activate the innate immune response. This innate immune response includes the rapid generation of reactive oxygen species (ROS) and cell wall reinforcement, changes in ion fluxes across the plasma membrane and transient increases in Ca²⁺ levels, and the activation of protein phosphorylation/dephosphorylation cascades. The production of antimicrobial compounds (i.e. phytoalexins) and the accumulation of pathogenesis-related (PR) proteins represent a ubiquitous response to pathogen infection in plants (van Loon *et al.*, 2006). The phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) act as defence signalling molecules (Glazebrook, 2005; Pieterse *et al.*, 2009). The hormone-mediated signalling pathways do not function independently, but rather influence each other through a complex network of regulatory interactions (Glazebrook, 2005; Pieterse *et al.*, 2009). Depending on the type of plant–pathogen interaction, one or more of these hormone-regulated signalling pathways tends to predominate. Although the primary mode of interaction between the SA and JA signalling pathways appears to be mutual antagonism, there is also evidence for synergistic interactions between the two hormone-mediated signalling pathways (Glazebrook, 2005; Pieterse *et al.*, 2009).

Plant resistance responses act not only at the site of pathogen infection, but also at locations remote from this site, referred to as

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systemic acquired resistance (SAR) (Durrant and Dong, 2004). The establishment of SAR is associated with increased levels of SA, and is characterized by the coordinated expression of a set of *PR* genes (van Loon *et al.*, 2006; Ward *et al.*, 1991). The regulatory protein NPR1 (nonexpresser of *PR* genes) has emerged as a master regulator of the SAR response and a transcriptional co-activator of *PR* gene expression (Cao *et al.*, 1994; Delaney *et al.*, 1995). NPR1 has been proposed to play a role in the fine tuning of the antagonistic regulation of SA and JA/ET signalling to control gene expression (Leon-Reyes *et al.*, 2009).

Systemic resistance responses can also be activated by the colonization of roots with certain nonpathogenic microorganisms, such as selected strains of plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF), a phenomenon known as induced systemic resistance (van Loon *et al.*, 1998; Van der Ent *et al.*, 2009). Although the majority of studies on beneficial microbe-induced resistance point to a role for JA and ET in the induced systemic resistance response, several examples of PGPR and PGPF that trigger SA-dependent SAR responses have been documented (Van der Ent *et al.*, 2009).

The arbuscular mycorrhizal (AM) symbiosis is the most widespread symbiotic association formed between plants and fungi (Smith and Read, 2008). Root colonization by AM fungi improves the uptake of water and mineral nutrients, mainly phosphorus and nitrogen, in the host plant in exchange for photosynthetically fixed carbon (Bonfante and Genre, 2010; Parniske, 2008). The AM symbiosis improves plant growth and fitness. Yet another benefit conferred by the AM symbiosis is an improved level of resistance to root-infecting pathogens (Cordier *et al.*, 1998; Pozo and Azcón-Aguilar, 2007; Whipps, 2004). Systemic protection to pathogen infection at the root system level has been demonstrated in tomato plants (Cordier *et al.*, 1998; Pozo *et al.*, 2002; Zhu and Yao, 2004). Enhanced resistance to infection by root pathogens may be a consequence not only of improved plant nutrition, but also of competition for root colonization sites or photosynthates, changes in the rhizosphere microbial population or the activation of plant defence mechanisms.

The AM symbiosis may also have an impact on plant interactions with above-ground pathogens, but the effect of root colonization by AM fungi on foliar pathogens varies depending on the AM fungus and the type of pathogen encountered by the plant. Thus, enhanced resistance or susceptibility to foliar pathogens has been reported in mycorrhizal plants (Fritz *et al.*, 2006; Liu *et al.*, 2007; Shaul *et al.*, 1999; Whipps, 2004). It has been proposed that pathogens with a biotrophic lifestyle, such as powdery mildew and rust fungi, perform better in mycorrhizal plants (Pozo and Azcón-Aguilar, 2007). With regard to hemibiotrophs, the impact of the symbiosis varies from no effect to a reduction in disease (Lee *et al.*, 2005). Protection against the necrotrophic fungus *Alternaria solani* has been described in tomato plants (Fritz *et al.*, 2006; de la Noval *et al.*, 2007).

With regard to the effects of AM colonization on host defence gene expression, different responses can be observed in the mycorrhizal roots of different plant species, as the induction and suppression of host defence mechanisms have been described (Dumas-Gaudot *et al.*, 2000; Gianinazzi-Pearson *et al.*, 1996; Liu *et al.*, 2007; Pozo *et al.*, 2002). As an example, a systemic repression, or a delay in the activation of *PR* gene expression, has been reported in leaves of mycorrhizal tobacco plants challenged with *Botrytis cinerea*, with these plants exhibiting an increase in disease symptom severity (Shaul *et al.*, 1999). Temporal variations in the response of mycorrhizal plants to the challenging pathogen may also occur, the plant defence response being induced during the initial stages of AM symbiosis but subsequently down-regulated (García-Garrido and Ocampo, 2002). The determination of how the expression of defence genes is modulated during root colonization by AM fungi, and how this modulation affects the plant–pathogen relationship in above-ground tissues, is a requisite for a better exploitation of AM fungi in crop protection.

Cereal crops, and rice in particular, are hosts for AM fungi (Campos-Soriano *et al.*, 2010; Sawers *et al.*, 2008). Recently, we have reported AM-induced alterations in defence gene expression in the root system of mycorrhizal rice plants (Campos-Soriano *et al.*, 2010). In this work, our research objectives were twofold: (i) to determine whether the AM symbiosis results in systemic alterations in the expression of defence-related genes, namely in the leaves of mycorrhizal rice plants; and (ii) to investigate whether the mycorrhizal symbiosis has an impact on resistance to infection by the rice blast fungus *M. oryzae*. To this end, a rice defensome containing *M. oryzae*-regulated genes was initially used to identify genes that are differentially expressed in the leaves of mycorrhizal relative to nonmycorrhizal rice plants. The expression of genes for which a role in the plant defence response is well established was also monitored in the leaves of mycorrhizal plants. Evidence is presented that the AM symbiosis triggers systemic alterations in gene expression and is accompanied by enhanced disease resistance to the foliar pathogen *M. oryzae* in rice.

RESULTS

Systemic alterations of gene expression in leaves of mycorrhizal rice plants

Rice (*O. sativa* L. cv. Senia) plants were inoculated with *Glomus intraradices* (Campos-Soriano *et al.*, 2010). At 42 days post-inoculation (dpi), all the events related to fungal development, namely intraradical hyphae, arbuscules at different morphological stages of formation and vesicles, were present in *G. intraradices*-inoculated roots, thus confirming the establishment of the symbiotic association. Using microscopic observations of trypan blue-stained roots, the overall colonization of rice roots ranged from

25% to 30% (results not shown; similar results have been reported previously; Campos-Soriano *et al.*, 2010).

We investigated whether root colonization by the AM fungus *G. intraradices* has an effect on the expression of defence-related responses in leaves of mycorrhizal rice plants. Firstly, gene expression studies were carried out using a rice defensome contained in a macroarray (Campo *et al.*, 2008). The macroarray comprised a total of 6144 expressed sequence tags representing genes whose expression is induced in leaves by *M. oryzae* infection (at 3, 6, 9, 24 and 48 h post-inoculation). Differential screening of the rice macroarray was carried out with ^{32}P -labelled cDNAs obtained from either mycorrhizal plants or mock-inoculated plants. Only genes showing a statistically significant ($P < 0.05$) fold change ≥ 2.0 (leaves from mycorrhizal plants vs. leaves from nonmycorrhizal plants) were considered.

Genes up-regulated in leaves of mycorrhizal plants were grouped into different functional categories (Table 1). Among them were genes involved in defence and stress responses, transcriptional control, signal transduction and protein synthesis. The defence category comprised an important number of genes involved in protection against oxidative stress and the detoxification of ROS. Peroxidases (three genes), catalase, ascorbate peroxidase and thioredoxins were all expressed at higher levels in leaves of mycorrhizal relative to nonmycorrhizal plants. The expression of these genes is typically activated in rice leaves during infection with the rice blast fungus *M. oryzae* (Campo *et al.*, 2008; Kim *et al.*, 2001; Ramalingam *et al.*, 2003). Three distinct PR genes, namely a PR1, a chitinase and a lipid transfer protein gene, were also included in the defence category (Table 1).

Of interest, the macroarray data revealed the up-regulation of genes encoding distinct transcription factors, such as members of the AP2/EREBP (APETALA2/ethylene-responsive element-binding protein) family of transcription factors (*OsAP2*, Os01g04020 and *OsEREBP*, Os09g26420) and a basic helix—loop—helix (bHLH)-type transcription factor (*OsbHLH*, Os01g72370) (Table 1). The functional category of signal transduction comprised genes encoding the receptor-like protein kinase *DUF26* (domain unknown function 26) (*OsDUF26*, Os03g16950), a remorin1 (Os04g45070), a calcium-binding protein (*OsCBP*, Os12g04240) and several protein kinases. The *MPK6* (mitogen-activated protein kinase 6, *OsMPK6*, Os10g38950) gene was also found to be up-regulated in the leaves of mycorrhizal plants.

Several genes identified by macroarray analysis were randomly selected, and their expression was analysed further by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). This analysis confirmed the up-regulation of transcription factor genes, namely *OsAP2*, *OsEREBP* and *OsbHLH* (Fig. 1A), as well as genes involved in signal transduction processes, namely *OsDUF26* and *OsMPK6* (Fig. 1B) ($*P \leq 0.05$ and $**P \leq 0.001$). The induction of various *OsDUF26* genes in the rice—*M. oryzae* interaction

has been described previously (Campo *et al.*, 2008; Han *et al.*, 2004; Kim *et al.*, 2004, 2009). A role for *OsMPK6* in the rice defence response to pathogen infection has been reported (Shen *et al.*, 2010).

Overall, gene expression studies revealed that the AM symbiosis is accompanied by systemic alterations in gene expression in rice leaves. Among the mycorrhiza-induced genes were genes functioning in protection against oxidative stress, transcriptional control of gene expression and signal transduction processes.

Systemic activation of regulatory genes of the host defence response in mycorrhizal rice

The expression of genes playing a regulatory role in the plant defence response, whose expression is also known to be activated by *M. oryzae* infection in rice leaves, was monitored in leaves of mycorrhizal and nonmycorrhizal plants. These included: *OsNPR1* (Os01g09800), *OsJAmyb* (JA-regulated myb transcription factor, Os11g45740) and *OsMPK7* (mitogen-activated protein kinase 7, Os05g49140) (Gómez-Ariza *et al.*, 2007; Lee *et al.*, 2001; Reyna and Yang, 2006). Whereas *OsNPR1* functions in the SA-mediated signalling pathway for the activation of defence responses, *OsJAmyb* is involved in the JA-mediated signalling pathway (Lee *et al.*, 2001). *OsMPK7* was also found to be highly induced by JA treatment in rice leaves (Reyna and Yang, 2006). RT-qPCR analysis demonstrated that all three genes, *OsNPR1*, *OsJAmyb* and *OsMPK7*, are expressed at significantly higher levels in leaves of mycorrhizal relative to nonmycorrhizal plants (Fig. 2A; $*P \leq 0.05$ and $**P \leq 0.001$). Equally, RT-qPCR revealed that the allene oxide cyclase (*OsAOC*, Os03g32314) gene is highly expressed in leaves of mycorrhizal relative to nonmycorrhizal plants (Fig. 2B; $P \leq 0.001$). The AOC enzyme is involved in the synthesis of 12-oxo-phytodienoic acid (OPDA), the ultimate precursor of JA (Schaller, 2001).

From these results, it appears that genes playing a regulatory role in the rice response to pathogen infection, which function in either an SA-dependent (*OsNPR1*) or JA-dependent (*OsJAmyb* and *OsMPK7*) manner, as well as the *OsAOC* gene involved in JA biosynthesis, are all up-regulated in the leaves of mycorrhizal rice plants.

Systemic activation of genes involved in Ca^{2+} -mediated signalling processes in mycorrhizal rice

The crucial role of calcium as a second messenger in plant innate immunity is well established (Lecourieux *et al.*, 2006). Cellular Ca^{2+} signals are decoded and transmitted by CBPs that relay this information into downstream responses. In line with this, the *OsCBP* (Os12g04240) gene encoding a CBP from rice was identified among the set of genes overexpressed in the leaves of

Table 1 Genes identified as up-regulated in leaves of mycorrhizal relative to nonmycorrhizal plants, sorted according to functional category. Genes are listed in alphabetical order. Only genes showing a fold change ≥ 2.0 are listed.

Description	Accession number*	Ratio†	P value
1. Defence and stress responses			
<i>Acidic endochitinase</i>	LOC_Os05g15770	3.49	7.0E-03
<i>Ascorbate peroxidase (APX8), thylakoid-bound</i>	LOC_Os02g34810	2.51	9.4E-05
<i>Carbonic anhydrase</i>	LOC_Os01g45274	4.51	2.0E-04
<i>Catalase</i>	LOC_Os02g02400	2.24	5.8E-04
<i>Cathepsin B-like cysteine proteinase</i>	LOC_Os05g24550	2.28	6.8E-03
<i>Chitinase III</i>	LOC_Os01g47070	2.41	1.1E-04
<i>Glutathione synthetase</i>	LOC_Os12g34380	2.01	3.0E-03
<i>Glycine-rich protein (GRP0.9)</i>	LOC_Os10g31330	3.37	1.1E-04
<i>Glycine-rich RNA-binding protein (GRP1A)</i>	LOC_Os03g46770	2.00	3.4E-03
<i>Glycolate oxidase (GOX)</i>	LOC_Os03g57220	3.52	2.7E-07
<i>Glyoxalase I</i>	LOC_Os08g09250	2.56	1.9E-03
<i>Heat shock 70-kDa protein 1 (HSC70-1)</i>	LOC_Os11g47760	2.85	3.3E-05
<i>Lipid transfer protein (LTP11)</i>	LOC_Os12g02310	10.24	1.4E-04
<i>Lipid transfer protein (LTP14)</i>	LOC_Os12g02340	4.71	3.1E-06
<i>Oryzacystatin (cysteine protease inhibitor)</i>	LOC_Os01g58890	2.14	4.2E-03
<i>Oryzain α chain precursor</i>	LOC_Os04g55650	9.22	3.0E-04
<i>Oryzain γ chain precursor</i>	LOC_Os09g27030	3.73	2.4E-04
<i>Pathogenesis-related protein 1 (PR1)</i>	LOC_Os07g03730	2.05	1.9E-03
<i>Peroxidase (POX8_1)</i>	LOC_Os07g48010	2.10	5.0E-04
<i>Peroxidase 12 precursor</i>	LOC_Os01g73200	3.29	3.1E-04
<i>Peroxidase 71 precursor</i>	LOC_Os01g22249	2.37	6.6E-05
<i>Protein disulphide isomerase (PDI)</i>	LOC_Os01g23740	2.22	1.1E-02
<i>Thioredoxin</i>	LOC_Os07g29410	2.33	6.5E-05
<i>Thioredoxin F2 protein (TRX-F2)</i>	LOC_Os03g07300	5.40	1.7E-05
<i>Water stress-induced protein (WSI724)</i>	LOC_Os03g45280	2.07	7.9E-04
2. Transcription			
APETALA2 (AP2) domain-containing transcription factor protein	LOC_Os01g04020	2.74	2.4E-06
Basic helix—loop—helix (bHLH) family protein	LOC_Os01g72370	3.98	2.9E-07
<i>Myb-related transcriptional activator protein (D13F-MYBST1)</i>	LOC_Os08g04840	3.45	3.2E-03
Ethylene-responsive element-binding protein (EREBP)-type transcription factor	LOC_Os09g26420	4.70	1.7E-10
<i>Helix—loop—helix DNA-binding domain containing protein</i>	LOC_Os10g40740	5.21	1.3E-07
<i>DNA-directed RNA polymerase II subunit</i>	LOC_Os07g27930	2.00	2.0E-03
<i>RNA-binding protein</i>	LOC_Os12g23180	3.52	6.5E-08
<i>RNA helicase</i>	LOC_Os01g43120	2.04	2.3E-03
<i>RNA helicase</i>	LOC_Os09g34910	5.80	1.4E-02
<i>U1 small nuclear ribonucleoprotein 70 kDa</i>	LOC_Os10g02630	9.37	6.7E-03
<i>Zinc finger transcription factor</i>	LOC_Os01g09620	2.00	3.2E-03
3. Signal transduction			
<i>Absciscic stress-ripening</i>	LOC_Os01g73250	4.46	8.9E-12
<i>Auxin-repressed protein/auxin-associated protein</i>	LOC_Os03g22270	3.36	2.6E-07
<i>Auxin-repressed 12–15-kDa protein</i>	LOC_Os11g44810	3.26	3.4E-07
<i>Auxin-responsive protein IAA16 (indoleacetic acid-induced protein 16)</i>	LOC_Os03g53150	3.28	6.6E-03
<i>BR11-KD interacting protein 103 (proton pump interacting protein)</i>	LOC_Os09g17730	2.00	4.1E-03
<i>Brassinolide (BL)-enhanced gene (OsBLE1)</i>	LOC_Os09g38030	2.75	2.8E-06
Calcium-binding protein (OsCBP)	LOC_Os12g04240	3.40	1.4E-08
<i>Cation transport protein (chaC)</i>	LOC_Os02g26700	2.74	9.4E-04
<i>GTPase activating-like protein</i>	LOC_Os10g37410	2.00	3.0E-03
Mitogen-activated protein kinase (MPK6)	LOC_Os10g38950	2.29	2.4E-04
<i>Phosphatase-associated family protein SIT4</i>	LOC_Os01g40340	2.76	1.4E-05
<i>Phosphate transporter</i>	LOC_Os12g40340	2.15	3.2E-04
<i>Ran (Ras-related GTP-binding nuclear protein)</i>	LOC_Os01g42530	2.00	2.0E-03
Receptor-like protein kinase [domain unknown function 26 (DUF26)-like]	LOC_Os03g16950	2.59	6.1E-05
<i>Remorin 1</i>	LOC_Os04g45070	3.52	3.9E-08
<i>Serine/threonine protein kinase</i>	LOC_Os12g01140	2.09	1.6E-03
<i>Two-component response regulator ARR9 (Response reactor 4)</i>	LOC_Os01g72330	6.79	9.6E-14
<i>Vesicle transport v-SNARE</i>	LOC_Os01g51120	2.73	3.1E-06
4. Protein synthesis, folding and stabilization			
<i>30S ribosomal protein 1, chloroplast precursor</i>	LOC_Os03g63950	2.16	1.4E-03
<i>40S ribosomal protein S21</i>	LOC_Os03g22460	7.25	9.8E-13
<i>40S ribosomal protein S28</i>	LOC_Os10g27174	2.24	1.4E-03
<i>50S ribosomal protein L13, chloroplast precursor (CL13)</i>	LOC_Os01g54540	3.27	1.8E-03

Table 1 Continued.

Description	Accession number*	Ratio†	P value
<i>50S ribosomal protein L15, chloroplast precursor</i>	LOC_Os03g12020	5.60	4.5E-05
<i>Calnexin precursor</i>	LOC_Os04g32950	2.80	9.8E-06
<i>Proteasome subunit β type 5 precursor</i>	LOC_Os06g06030	2.95	2.4E-06
<i>Ribonuclease 3 precursor</i>	LOC_Os09g36680	6.28	3.5E-10
5. Growth and division			
<i>Dormancy-associated protein</i>	LOC_Os11g44810	2.47	3.4E-07
<i>Light-induced mRNA</i>	LOC_Os01g01340	2.49	6.3E-05
<i>Light-regulated protein precursor</i>	LOC_Os02g16820	2.70	7.2E-06
6. Energy and metabolism			
<i>2-Oxoisovalerate dehydrogenase α subunit</i>	LOC_Os12g08260	2.42	5.1E-03
<i>Adenyl-succinate synthetase</i>	LOC_Os03g49220	2.07	1.7E-03
<i>ADP/ATP translocase</i>	LOC_Os02g48720	3.00	1.5E-04
<i>ATP-dependent Clp protease ATP-binding subunit clpA homologue</i>	LOC_Os12g12850	7.27	4.4E-16
<i>b-Keto acyl reductase</i>	LOC_Os04g02620	8.45	8.5E-07
<i>Chlorophyll a/b-binding apoprotein CP24 precursor</i>	LOC_Os04g38410	4.11	2.3E-09
<i>Chlorophyll a/b-binding protein CP29-1(LHCI protein 4-1)</i>	LOC_Os07g37240	4.22	9.2E-10
<i>Chlorophyll a/b-binding protein I precursor</i>	LOC_Os09g17740	2.18	9.6E-03
<i>Chlorophyll a-b-binding protein 7 (LHCI type II CAB-7)</i>	LOC_Os07g38960	2.32	6.6E-05
<i>Chlorophyll a-b-binding protein of LHCII type III (CAB)</i>	LOC_Os07g37550	2.00	8.0E-03
<i>Cytochrome P450</i>	LOC_Os10g38110	2.00	3.7E-03
<i>Cytokinin dehydrogenase precursor</i>	LOC_Os01g10110	10.95	1.3E-02
<i>Dehydroascorbate reductase</i>	LOC_Os05g02530	2.11	1.6E-03
<i>Ferredoxin-dependent glutamate synthase (Fd-GOGAT)</i>	LOC_Os07g46460	2.22	1.9E-04
<i>Ferredoxin-NADP reductase</i>	LOC_Os02g01340	2.22	3.1E-04
<i>Ferredoxin-NADP reductase</i>	LOC_Os06g01850	2.04	1.6E-03
<i>Fructose-bisphosphate aldolase</i>	LOC_Os11g07020	2.00	4.0E-03
<i>Glutamine synthetase, catalytic domain</i>	LOC_Os04g56400	10.14	1.0E-02
<i>Glyceraldehyde-3-phosphate dehydrogenase</i>	LOC_Os08g03290	2.96	4.8E-04
<i>Glycine dehydrogenase</i>	LOC_Os01g51410	2.62	3.8E-05
<i>Hydrolase, α/β fold family protein</i>	LOC_Os03g10620	4.68	2.2E-03
<i>Hydroxyanthranilate hydroxycinnamoyltransferase</i>	LOC_Os04g42250	2.57	1.9E-05
<i>Magnesium-protoporphyrin IX monomethyl ester cyclase</i>	LOC_Os01g17170	5.33	1.2E-11
<i>Malate dehydrogenase, glyoxysomal precursor</i>	LOC_Os03g56280	2.27	8.1E-05
<i>NADP-dependent malic enzyme</i>	LOC_Os01g52500	2.00	3.7E-03
<i>Methyltransferase (NOL1/NOP2/sun family protein)</i>	LOC_Os09g29630	3.63	2.2E-07
<i>Oxygen-evolving enhancer protein 1 (OEE1)</i>	LOC_Os07g37240	2.41	9.2E-10
<i>Oxygen-evolving enhancer protein 1</i>	LOC_Os01g31690	2.57	3.1E-05
<i>Oxygen-evolving enhancer protein 2</i>	LOC_Os07g04840	13.82	1.9E-05
<i>Oxygen-evolving enhancer protein 3-1</i>	LOC_Os07g36080	3.46	2.7E-04
<i>Peptide transporter PTR2/nitrate transporter</i>	LOC_Os08g05910	2.22	1.2E-02
<i>Phosphoglycerate kinase</i>	LOC_Os05g41640	2.00	4.8E-03
<i>Phosphoribulokinase precursor</i>	LOC_Os02g47020	2.73	2.2E-05
<i>Photosystem I antenna protein</i>	LOC_Os09g01000	4.11	4.7E-11
<i>Photosystem I antenna protein</i>	LOC_Os07g38960	3.62	6.6E-05
<i>Photosystem I reaction centre subunit III</i>	LOC_Os03g56670	5.42	5.8E-06
<i>Photosystem I reaction centre subunit IV A</i>	LOC_Os07g25430	2.56	6.5E-03
<i>Photosystem I reaction centre subunit V</i>	LOC_Os09g30350	3.12	4.9E-07
<i>Photosystem I reaction centre subunit N</i>	LOC_Os12g08770	4.36	1.6E-04
<i>Photosystem II 10-kDa polypeptide</i>	LOC_Os08g10020	3.15	1.4E-06
<i>Photosystem II oxygen-evolving complex protein 2 precursor (PsbP)</i>	LOC_Os07g04840	3.82	1.9E-05
<i>Potassium transporter 5 (AtPOT5) (AtHAK1) (AtHAK5)</i>	LOC_Os04g53620	3.59	6.0E-04
<i>Δ-12 oleate desaturase</i>	LOC_Os02g48560	8.33	1.2E-08
<i>Pyruvate dehydrogenase E1 component β subunit, mitochondrial precursor (PDHE1-B)</i>	LOC_Os09g33500	2.30	3.7E-04
<i>Ribulose bisphosphate carboxylase small chain C</i>	LOC_Os12g19470	3.84	3.5E-09
<i>Ribulose bisphosphate carboxylase small chain</i>	LOC_Os04g07530	5.59	9.1E-12
<i>Ribulose bisphosphate carboxylase small chain</i>	LOC_Os12g19381	3.69	5.2E-09
<i>Ribulose bisphosphate carboxylase/oxygenase activase</i>	LOC_Os11g47970	3.76	2.5E-03
<i>Ribulose-phosphate 3-epimerase</i>	LOC_Os03g07300	2.42	1.7E-05
<i>S-Adenosylmethionine decarboxylase 2</i>	LOC_Os02g39795	3.96	8.5E-10
<i>Serine-glyoxylate aminotransferase</i>	LOC_Os08g39300	2.22	1.7E-02
<i>Squalene monooxygenase</i>	LOC_Os03g12910	2.35	1.7E-04

Table 1 Continued.

Description	Accession number*	Ratio†	P value
<i>Transketolase</i>	LOC_Os06g04270	2.00	6.0E-03
<i>Triosephosphate isomerase</i>	LOC_Os09g36450	2.04	1.7E-03
<i>Type I light-harvesting chlorophyll a/b-binding protein of photosystem II (LHCP1)</i>	LOC_Os12g17600	2.49	8.7E-05
<i>Ubiquinol-cytochrome c reductase complex 8.0-kDa protein</i>	LOC_Os05g33210	5.20	3.3E-12
7. Unknown function			
<i>Ankyrin repeat domain-containing protein</i>	LOC_Os09g33810	5.18	2.0E-11
<i>CP12 protein precursor</i>	LOC_Os01g19740	2.07	1.7E-03
<i>Expressed protein</i>	LOC_Os10g39900	2.00	1.7E-07
<i>Expressed protein</i>	LOC_Os08g14620	5.31	1.0E-03
<i>Expressed protein</i>	LOC_Os03g52680	5.14	5.5E-07
<i>Expressed protein</i>	LOC_Os05g40300	4.06	5.7E-09
<i>Expressed protein</i>	LOC_Os04g48860	3.39	2.9E-07
<i>Expressed protein</i>	LOC_Os02g51020	2.45	6.5E-03
<i>Expressed protein</i>	LOC_Os01g45914	2.28	1.1E-04
<i>Expressed protein</i>	LOC_Os02g45930	2.15	6.1E-04
<i>Expressed protein</i>	LOC_Os02g12480	2.12	2.9E-03
<i>Expressed protein</i>	LOC_Os06g49640	2.78	4.6E-04
<i>Expressed protein</i>	LOC_Os01g48790	4.85	1.3E-13
<i>Hypothetical protein</i>	LOC_Os04g30240	2.50	3.4E-03
<i>Hypothetical protein</i>	LOC_Os01g37790	4.73	5.0E-03
<i>Hypothetical protein</i>	LOC_Os02g42960	3.90	1.8E-10
<i>Hypothetical protein</i>	LOC_Os10g39900	3.05	1.7E-07
<i>Hypothetical protein, expressed under carbonate stress-PLN</i>	LOC_Os06g19095	3.61	3.9E-03
<i>Leucine-rich repeat protein, putative</i>	LOC_Os03g32580	2.59	5.6E-04
<i>Unknown protein</i>	LOC_Os10g39900	5.22	1.7E-07
<i>Unknown protein</i>	LOC_Os03g41080	3.04	9.9E-07
<i>Unknown protein</i>	LOC_Os08g08650	2.29	3.8E-03
<i>Unknown protein</i>	LOC_Os12g42250	2.18	3.9E-04

Genes validated by real-time quantitative polymerase chain reaction are indicated in bold type.

*Locus ID: locus name from the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu>).

†Fold change: signal intensity ratio of leaves of mycorrhizal plants relative to leaves of nonmycorrhizal plants.

mycorrhizal plants (Table 1). In other studies, the activation of *CBP* genes during the infection of rice leaves with *M. oryzae* has also been reported (Kim *et al.*, 2001; Shim *et al.*, 2004).

In this work, the AM-induced systemic alterations in the expression of genes acting in Ca^{2+} -mediated signalling processes were further explored. Initially, the up-regulation of the *OsCBP* gene identified by macroarray analysis was confirmed by RT-qPCR analysis (Fig. 2C; $P \leq 0.05$). Next, we investigated the expression of the calmodulin (*OsCaM*, Os04g36660) and calmodulin-like 4 (*OsCML4*, Os03g53200) genes in mycorrhizal rice. *OsCaM* and *OsCML4* expression is activated in response to infection by the rice blast fungus, as recorded in gene expression databases [Rice Expression Database (RED) <http://cdna02.dna.affrc.go.jp/RED/> and 'Genevestigator' <https://www.genevestigator.com/gv/>]. As shown in Fig. 2C, a tendency for a higher expression of the two genes, *OsCaM* and *OsCML4*, occurs in leaves of mycorrhizal relative to nonmycorrhizal plants.

Although not proven, the observed up-regulation of genes encoding different types of calcium sensor protein (*OsCBP*, *OsCaM* and *OsCML4*) in mycorrhizal plants points to a possible role of Ca^{2+} -mediated signalling processes in the AM-induced systemic resistance to pathogen infection in rice plants.

Effect of the AM symbiosis on resistance to the rice blast fungus *M. oryzae*

We then addressed the very important question of whether mycorrhizal rice is resistant to infection by a foliar pathogen, namely the rice blast fungus. Blast resistance assays were conducted on young leaves of rice plants at 42 dpi with *G. intraradices*. As a control, young leaves from mock-inoculated plants were assayed. Leaves were inoculated with increasing doses of *M. oryzae* spore suspension, as described previously (Coca *et al.*, 2004). Using a concentration of inoculum of 10^6 spores/mL, leaves from mock-inoculated plants developed clear symptoms of infection at 2–3 dpi (Fig. 3A, –Gi). By this time, small necrotic spots were also evident on *M. oryzae*-infected leaves of mycorrhizal plants. Lesions developed actively with time on the leaves of nonmycorrhizal plants, these leaves being severely damaged at 7 dpi with *M. oryzae* spores (Fig. 3A, –Gi). Under the same experimental conditions, however, leaves of mycorrhizal plants showed a clear reduction in disease severity (Fig. 3A, +Gi).

We further tested the ability of mycorrhizal plants to block the growth of *M. oryzae* in leaf tissues by determining the percentage of the leaf area affected by blast lesions. A direct correlation

Fig. 1 Changes in transcript abundance of transcription factor genes and genes involved in signal transduction processes in leaves of mycorrhizal rice plants. The expression of genes selected by macroarray analysis was determined by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). Leaves were collected from mycorrhizal plants at 42 days post-inoculation (dpi) with the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* (+Gi) or from mock-inoculated plants (-Gi). To normalize the RT-qPCR data, each gene was compared with the *ubiquitin 1* (*OsUbi1*, Os06g46770) transcript. Three independent biological samples were analysed with similar results. Error bars indicate the standard error (SE) from three technical replicates. Asterisks indicate a significant difference between conditions (* $P \leq 0.05$ and ** $P \leq 0.001$). (A) Expression of the transcription factor genes *OsAP2* (Os01g04020), *OsEREBP* (Os09g26420) and *OsbHLH* (Os01g72370). (B) Expression of the receptor like-protein kinase *OsDUF26* (Os03g16950) and *MPK6* (Os10g38950) genes. AP2, APETALA2; bHLH, basic helix—loop—helix; DUF26, domain unknown function 26; EREBP, ethylene-responsive element-binding protein; MPK6, mitogen-activated protein kinase 6.

between lesion area and inoculum concentration was observed in fungus-infected leaves of nonmycorrhizal plants, these lesions gradually increasing with time (Fig. 3B). In agreement with visual inspection, the percentage of lesion area was higher in *M. oryzae*-infected leaves of nonmycorrhizal plants relative to mycorrhizal plants. Differences were more evident at 7 dpi with the highest inoculum dose. Lesion development was apparently blocked in fungus-inoculated leaves of mycorrhizal rice plants. Finally, fungal sporulation did not occur in the leaves of mycorrhizal rice, not even at 7 dpi with the highest inoculum dose used in this study (10^6 spores/mL), as revealed by spore counting (results not shown).

Knowing that mycorrhizal plants exhibit a resistance phenotype to the rice blast fungus, it was of interest to examine *PR* gene expression in the leaves of these plants. *PR* genes whose expression has been reported to be activated by *M. oryzae* infection in rice leaves were assayed (Gómez-Ariza *et al.*, 2007; Quilis *et al.*, 2008). These included: *OsPR1a* (Os07g03710; a member of the *PR1* family different from that identified by macroarray hybridization, Table 1), *OsPR5* (thaumatin-like protein, Os03g46070), *OsPR10* (Os03g18850) and *OsPBZ1* (*probenazole inducible 1*, Os12g36880). The *PR1* and *PBZ1* genes are typical markers of the activation of the rice defence response to pathogen infection (Agrawal *et al.*, 2001; Midoh and Iwata, 1996). *PR* gene expression was initially analysed in noninfected leaves from both myc-

orrhizal and nonmycorrhizal plants. Intriguingly, reduced levels of transcript accumulation were observed for all four genes (*OsPR1a*, *OsPR5*, *OsPBZ1* and *OsPR10*) in the leaves of mycorrhizal relative to nonmycorrhizal plants in the absence of pathogen infection (Fig. 4, 0 dpi). In other studies in leaves of *Medicago* and tomato plants, the down-regulation of *PR* gene expression as a result of AM symbiosis has also been described (Fiorilli *et al.*, 2009; Liu *et al.*, 2007).

PR gene expression was then monitored in *M. oryzae*-infected leaves of both mycorrhizal and nonmycorrhizal plants at two different times after inoculation with *M. oryzae* spores (1 and 3 dpi) (Fig. 4, 1 dpi, 3 dpi). Under pathogen infection conditions, a potentiation of *PR* gene expression was observed. In the case of the *OsPR1a*, *OsPR5* and *OsPBZ1* genes, a significantly higher expression occurred in the leaves of mycorrhizal plants at 3 dpi with *M. oryzae* spores (Fig. 4, 3 dpi; * $P \leq 0.05$ and ** $P \leq 0.001$). With regard to *OsPR10*, a stronger activation occurred at 1 dpi with *M. oryzae* in leaves of mycorrhizal rice (Fig. 4, $P \leq 0.05$).

Overall, the results presented here suggest that the AM symbiosis confers systemic resistance to infection by the rice blast fungus *M. oryzae*. Consistent with the observed resistance phenotype, a stronger induction of *PR* gene expression occurs in leaves of mycorrhizal plants in response to infection with the rice blast fungus.

Fig. 2 Expression of regulatory defence-related genes and genes acting in Ca^{2+} -mediated signalling processes in mycorrhizal rice plants. Gene expression was determined by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) in leaves from mycorrhizal (+Gi) and nonmycorrhizal (–Gi) plants. Expression levels were normalized using the rice cyclophilin gene (*OsCyc*, Os02g02890). Similar results were obtained using the *ubiquitin 1* (*OsUbi1*) gene to normalize the expression data. Three independent biological samples were analysed with similar results. Error bars indicate the standard error from three technical replicates. Asterisks indicate a significant difference between conditions (* $P \leq 0.05$ and ** $P \leq 0.001$). (A) Analysis of *OsNPR1* (Os01g09800), *OsJAmyb* (Os11g45740) and *OsMPK7* (Os05g49140) gene expression. (B) Expression of the *OsAOC* gene (Os03g32314) involved in jasmonic acid (JA) biosynthesis. (C) Expression of rice genes acting in Ca^{2+} -mediated signalling processes: *OsCBP* (Os12g04240), *OsCaM* (Os04g36660) and *OsCML4* (Os03g53200). Amyb, JA-regulated myb transcription factor; AOC, allene oxide cyclase; CaM, calmodulin; CBP, calcium-binding protein; CML4, calmodulin-like 4; MPK7, mitogen-activated protein kinase 7; NPR1, nonexpresser of PR1.

DISCUSSION

AM fungi are mainly thought to facilitate nutrient uptake in plants, an aspect that has received great interest in AM research, although AM fungi can perform several other functions that are equally beneficial for the plant. Evidence indicates that root colonization by the AM fungus confers resistance to soil-borne pathogens (Pozo *et al.*, 2010). Priming of defence reactions has been proposed to mediate the mycorrhiza-induced resistance at the root system level (Pozo *et al.*, 2009). As an example, Cordier *et al.* (1998) demonstrated a rapid and strong activation of defence mechanisms in nonmycorrhizal parts of *Glomus mossae*-inoculated tomato roots, which was accompanied by resistance to *Phytophthora parasitica* infection in these roots.

However, the effect of the AM symbiosis during pathogen infection in above-ground tissues is variable, and mycorrhizal colonization does not always increase disease resistance to foliar pathogens. Although systemic resistance to leaf pathogens induced by root colonization has been reported (Liu *et al.*, 2007), there are also examples in which mycorrhizal colonization results in increased susceptibility to infection by biotrophic pathogens in aerial parts of the plant (Gernns *et al.*, 2001; Pozo and Azcón-Aguilar, 2007; Whipps, 2004). A differential pathogen resistance is sometimes observed depending on the AM fungus (de la Noval *et al.*, 2007). Whenever AM-induced bioprotective effects against foliar pathogens are observed, the molecular mechanisms responsible for this effect are still poorly understood.

Fig. 3 Resistance to infection by the rice blast fungus *Magnaporthe oryzae* in mycorrhizal rice plants. Leaves were collected from mycorrhizal plants at 42 days post-inoculation (dpi) with the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* (+Gi) or from mock-inoculated plants (–Gi). Results shown are from one of three experiments that produced similar results. (A) Leaves were challenged with *M. oryzae* spores using the detached leaf assay (Coca *et al.*, 2004). Increasing doses of *M. oryzae* (PR9 strain) spores were used for inoculation (10⁶, 10⁵ and 10⁴ spores/mL). Control leaves were inoculated with sterile water. Disease symptoms at 3, 5 and 7 dpi of leaves are shown. (B) Percentage of leaf area affected by blast lesions in leaves of nonmycorrhizal and mycorrhizal rice plants at 3, 5 and 7 dpi with *M. oryzae* spores. The percentage of lesion area was determined using Image Analysis Software, Assess 2.0, for plant disease quantification (Lamari, 2008). Bars represent the average of three independent experiments. For each experiment, at least four leaves were inoculated with each inoculum and five inoculations per leaf were made. Means and standard errors are indicated. Bars with different letters are significantly different by $P \leq 0.05$.

Studies in *Medicago* have shown that the AM symbiosis is accompanied by alterations in gene expression in root and shoot tissues, and that these AM-regulated transcriptional responses differ between the two tissues (Liu *et al.*, 2007). Consistent with the results presented here in the leaves of mycorrhizal rice plants, genes involved in transcriptional regulation (e.g. AP2/EREBP transcription factor) and in Ca²⁺-mediated signalling processes are also induced in shoots of mycorrhizal *Medicago* plants (Liu *et al.*,

2007). Moreover, the down-regulation of *PR* gene expression is also observed in leaves of *Medicago* mycorrhizal plants in the absence of pathogen infection (Liu *et al.*, 2007). Thus, our results on the systemic alterations in gene expression in mycorrhizal rice plants are essentially in agreement with those reported by Liu *et al.* (2007) in mycorrhizal *Medicago* plants. Root colonization by *G. intraradices* results in enhanced disease resistance in both rice and *Medicago* plants.

Fig. 4 Expression of pathogenesis-related (*PR*) genes in leaves of nonmycorrhizal and mycorrhizal rice plants (grey and black bars, respectively) in response to *Magnaporthe oryzae* infection. For inoculation, a suspension of *M. oryzae* spores at 1×10^6 spores/mL was used. Total RNA was isolated at the indicated times after inoculation with *M. oryzae* spores (0, 1 and 3 days). Changes in transcript abundance were determined by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) analysis. The relative expression of the *OsPR1a* (Os07g03710), *OsPR5* (Os03g46070), *OsPBZ1* (Os12g36880) and *OsPR10* (Os03g18850) genes is shown. The gene expression level was normalized using the rice cyclophilin gene (*OsCyc*, Os02g02890) as an internal reference. Similar results were obtained using the *ubiquitin 1* (*OsUbi1*) gene as an internal reference. Three independent biological samples were analysed with similar results. Error bars indicate the standard error from three technical replicates. Asterisks indicate a significant difference between conditions (* $P \leq 0.05$ and ** $P \leq 0.001$). PBZ1, probenazole inducible 1.

An interesting aspect of this study comes from the observation that the AM symbiosis is accompanied by systemic alterations in the expression of genes playing a regulatory role in the host immune system. It should be mentioned here that little attention has been paid to the study of defence regulatory genes during symbiotic interactions, and most studies have focused on the mycorrhiza-regulated expression of defence effector genes, such as *PR* genes. Evidence is presented here on the systemic activation of *OsNPR1*, *OsJAmyb* and *OsMPKs* in the leaves of mycorrhizal rice plants. With regard to NPR1 protein functioning, it is well known that pathogen recognition triggers alterations in the cellular redox status which, in turn, results in changes in the NPR1 protein. The inactive cytosolic oligomeric form of NPR1 is reduced to a monomeric state that translocates to the nucleus for the activation of *PR* gene expression (Kinkema *et al.*, 2000; Mou *et al.*, 2003). Although most studies on NPR1 functioning have been performed in Arabidopsis, the existence of a disease resistance pathway in rice similar to the Arabidopsis NPR1-mediated signalling pathway has been described (Chern *et al.*, 2001; Quilis *et al.*, 2008). Intriguingly, even though *OsNPR1* expression is up-regulated in leaves of mycorrhizal rice, its transcriptional activation is not accompanied by an enhanced expression of the downstream NPR1-regulated rice *PR* genes (i.e. *PBZ1*, *PR5*) (Quilis *et al.*, 2008). Instead, *PR* gene expression appears to be lower in leaves of mycorrhizal relative to nonmycorrhizal plants in the absence of pathogen challenge. Only under pathogen infection conditions are the NPR1-regulated *PR* genes expressed at higher levels in the leaves of mycorrhizal rice

plants. Assuming that the activation of *OsNPR1* expression occurs first, and that this is followed by translation of the mycorrhiza-induced *OsNPR1* transcripts, there is the possibility that the observed systemic transcriptional activation of *OsNPR1* leads to the accumulation of inactive NPR1 proteins. This would explain why the activation of *PR* gene expression does not occur in the leaves of mycorrhizal plants, even though *OsNPR1* expression is activated in these leaves. The *OsNPR1* protein will only become active under pathogen infection conditions. Together with this, the pool of latent *OsNPR1* proteins will respond more rapidly and/or more strongly to pathogen challenge which, in turn, will result in the strong expression of *PR* genes and enhanced disease resistance. In favour of these hypotheses, several transcription factors or MPK proteins have been shown to accumulate in an inactive state in a subcellular compartment different from that in which these regulatory proteins exert their activity during rhizobacteria- or chemically induced systemic resistance (Beckers *et al.*, 2009; Dahan *et al.*, 2010). Regulation by the AM symbiosis of the cellular redox status may contribute to the maintenance of the inactive oligomeric NPR1 protein in the leaves of mycorrhizal rice plants. Clearly, these strategies provide the plant with an enhanced capacity to effectively counteract pathogen infection, and are less costly than the constitutive expression and massive accumulation of a battery of defence effector proteins in systemic tissues. Further studies are, however, needed to determine whether there is a pool of inactive NPR1 proteins in the leaves of mycorrhizal rice in the absence of pathogen infection.

Our current knowledge of the role of the defence-related hormones, SA, JA and ET, during the development of systemic responses, SAR and induced systemic resistance comes mainly from studies in dicotyledonous species. In certain species, such as *Solanum lycopersicum*, *Lotus japonicus*, *Glycine max* and *Cucumis sativus*, the defence-related JA (and its jasmonate derivatives) appears to control defence gene expression during the AM symbiosis (Hause and Schaarschmidt, 2009; López-Ráez *et al.*, 2010). In other species, however, JA does not appear to play a role during the AM symbiosis (Riedel *et al.*, 2008). Our study showed that the AM symbiosis results in the systemic activation of genes functioning in the SA-dependent (*OsNPR1*) and JA-dependent (*OsJAMYB* and *OsMPK7*) signalling pathways. The expression of the *OsAOC* gene, which is involved in JA synthesis, was also found to be activated in leaves of mycorrhizal rice. These findings raise the possibility of a possible mycorrhiza-regulated modulation of the level of the defence-related hormones SA and/or JA in rice plants, an aspect that remains to be investigated.

It is generally assumed that pathogens that require a living host (biotrophs) are more sensitive to SA-mediated responses, whereas pathogens that kill the host (necrotrophs) are generally affected by JA/ET-mediated defences (Glazebrook, 2005). The effectiveness of the mycorrhiza-induced resistance in systemic tissues may well depend, at least in part, on the lifestyle of the pathogen attacking above-ground tissues and/or the signalling pathway(s) that is activated by each particular pathogen in the host plant. The fungus *M. oryzae* is a hemibiotrophic pathogen which maintains an initial biotrophic phase with the host, and then switches to a necrotrophic lifestyle (Campos-Soriano and San Segundo, 2009; Talbot, 2003). Hence, the mycorrhiza-induced bioprotective effect to infection by this hemibiotrophic pathogen may operate in a different manner, or at a different level, depending on whether the fungus invades the host tissue in a biotrophic or necrotrophic manner. From our results, it appears that, under severe infection conditions (10^5 – 10^6 spores/mL), the fungus manages to develop small lesions in the leaves of mycorrhizal rice plants early during the infection process. However, leaf disease progression appears to be blocked at later stages of the infection process, thus suggesting a bioprotective effect of the AM symbiosis during the necrotrophic period of leaf colonization by *M. oryzae*. A better knowledge of the roles of the SA and JA defence hormones in the rice–*M. oryzae* pathosystem is, however, needed to better understand the involvement of these defence signalling pathways in the mycorrhiza-induced resistance to *M. oryzae* infection in rice. It would also be of interest to determine whether the mycorrhiza-induced systemic alterations in host gene expression have an impact on diseases caused by other types of pathogens in rice plants, both necrotrophic and biotrophic pathogens.

Finally, it is well known that Ca^{2+} signalling mediates the recognition of both pathogenic and beneficial microbes in plants. Thus, rapid and transient elevations in cellular Ca^{2+} concentration

occur during PAMP-induced defence responses (Blume *et al.*, 2000), as well as during the AM symbiosis in roots (Bonfante and Genre, 2010). Specificity in the Ca^{2+} signalling system and downstream responses most probably depends on the availability of a specific set of Ca^{2+} sensors (and their target proteins) in each type of interaction. Our results show distinct calmodulin, calmodulin-like protein and calmodulin-binding protein genes whose expression is up-regulated in the leaves of mycorrhizal rice plants; these genes are also up-regulated by *M. oryzae* infection. As calmodulin and calmodulin-related proteins are known to function as sensors of Ca^{2+} signals in pathogenic interactions, these particular genes may be involved in the decoding of Ca^{2+} signals induced by *M. oryzae* infection in the leaves of mycorrhizal rice.

To summarize, the results presented here suggest that the AM symbiosis confers resistance to infection by the rice blast fungus in rice plants. The protective effect of the AM symbiosis is accompanied by the systemic activation of genes playing a regulatory role in host immunity. Priming for a faster and/or stronger *PR* gene expression also occurs in leaves of mycorrhizal rice following infection by the rice blast fungus *M. oryzae*. Overall, this study provides new insights into the molecular mechanisms involved in the mycorrhiza-induced resistance to *M. oryzae* infection in rice plants, which might be useful to better exploit the benefits of the AM symbiosis in rice protection under field conditions.

EXPERIMENTAL PROCEDURES

Plant and fungal material

The elite *japonica* rice cultivar Senia was used in this study. Rice plants were soil grown at 27 ± 2 °C under an 18-h/6-h light/dark photoperiod. The AM fungus *G. intraradices* (DAOM197198) was aseptically grown on Petri dishes containing modified minimal medium agar at 24 °C until the fungus sporulated (approximately 3 months). The monoxenic culture (*G. intraradices* and carrot roots) was grown as described previously (Campos-Soriano *et al.*, 2010).

The *M. oryzae* isolate PR9, which establishes a compatible interaction with the commercial rice cultivar Senia, was used for blast disease resistance assays (Coca *et al.*, 2004). The fungus was grown for 15 days at 28 °C on oat agar (Oatmeal Agar, Difco, France) Petri dishes. Spores were collected from fungal mycelium by adding sterile water to the surface of the mycelium. After filtration and microscopic observation, spores were adjusted to the appropriate concentration with sterile water with a Bürker counting chamber.

Inoculation of rice plants with the AM fungus *G. intraradices*

Each plant was grown in a separate pot on a sand–vermiculite mixture (50:50, v/v) and inoculated with a piece of monoxenic culture of *G. intraradices* (Campos-Soriano *et al.*, 2010). Control plants were mock-inoculated with uninfected carrot roots. Plants were watered every 2 days

with a modified Hoagland nutrient solution containing 25% of the standard phosphorus concentration to induce the establishment of symbiosis. Plants were harvested at 42 dpi; their roots were extensively washed with sterile water and analysed for AM colonization. Microscopic analysis of trypan blue-stained roots was performed to confirm the establishment of the AM symbiosis, as described previously (Campos-Soriano *et al.*, 2010). Three separate experiments using at least three plants and conditions (noninoculated and *G. intraradices*-inoculated) were carried out.

RNA isolation and analysis of gene expression by RT-qPCR

Total RNA was isolated from rice leaves using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). For gene expression studies, young leaves from mycorrhizal rice plants were collected and immediately frozen in liquid nitrogen. Leaf material was also harvested from nonmycorrhizal plants. At least six leaves from three individual plants were analysed. RT-qPCR analyses were carried out in 96-well optical plates in a LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Germany) according to the following programme: 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s, and an additional cycle of dissociation curves to ensure a unique amplification. The reaction mixture contained 10 µL of 2 × SYBR Green Master mix reagent (Roche), 2 µL of cDNA sample and 300 µM of each gene-specific primer in a final volume of 20 µL. Primers used for RT-qPCR are shown in Table S1 (see Supporting Information). Three replicate reactions were used for each sample. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta C_t)}$ method (Livak and Schmittgen, 2001). As internal controls, *ubiquitin 1* (*OsUbi1*) and *cyclophilin* (*OsCyc*) were routinely used. Three independent biological samples were analysed. One-way analyses of variance (ANOVAS) were used to evaluate differences in gene expression between mycorrhizal and nonmycorrhizal plants. For ANOVAS in which the *F* test was significant at 0.05 or a lower probability level, the least-significant difference test was applied to detect differences.

Macroarray hybridization, scanning and data analysis

A macroarray containing genes that are up-regulated during *M. oryzae* infection in rice leaves was used (Campo *et al.*, 2008). For hybridization experiments, cDNAs were obtained by reverse transcription from 40 µg of total RNA and labelled with ^{32}P - α -deoxycytidine triphosphate (dCTP) using SuperScript II Reverse Transcriptase (Invitrogen). The labelled cDNA probes were denatured at 100 °C for 5 min, followed by 5 min on ice, and then used for hybridization. Before hybridization, the membranes were washed in 0.5% sodium dodecylsulphate (SDS) for 30 min at 80 °C. The membranes were prehybridized in 5 × standard saline citrate (SSC), 0.5% SDS and 5 × Denhardt's solution at 65 °C for at least 1 h. Hybridization was carried out for 40–64 h at 60 °C. The membranes were washed at 65 °C in 2 × SSC, 0.1% SDS for 20 min, and twice in 0.2 × SSC, 0.1% SDS for 30 min. Hybridization signals were recorded by phosphor imaging (FujiFilm FLA3000 Phosphorimager, Berlin, Germany). The image data obtained were imported into the software program ArrayVision 7.0 (Imaging Research, St. Catharines, ON, Canada) for spot detection and quantification of hybridization signals. Backgrounds were subtracted using

ArrayVision 7.0 to obtain raw signal intensities. To control against biological errors, hybridizations were performed with two independently labelled cDNAs from RNA samples obtained from two independently pooled leaf samples. To assess the reproducibility of the macroarray analysis, two independent hybridizations were conducted for each sample and each biological replicate was hybridized in a different membrane. Normalization and statistical validation were carried out using ArrayStat 1.0 software (Imaging Research). In this software, normalization is performed in two steps: first, an evaluation of the repeatability of the replicates (replicate normalization) and the removal of outliers are performed; second, normalization is performed between conditions and the fold change for every gene is estimated. To establish the statistical significance of each fold change, a *z* test was performed and a *P* value (*P* < 0.05) was calculated for each spot ratio using the false discovery rate (FDR) method for false-positive error correction. Only clones with a two-fold change or greater, and statistically significant from the *z* test, were considered for further analysis. The nucleotide sequences of differentially expressed sequence tags contained in the rice macroarray were determined and compared with nucleotide and protein sequence databases (GenBank, <http://www.ncbi.nlm.nih.gov>; KOME, <http://cdna01.dna.affrc.go.jp>; Rice Genome Annotation Project, <http://rice.plantbiology.msu.edu>; MGOS, <http://www.mgosdb.org>).

Blast resistance assays

Resistance to infection by the rice blast fungus *M. oryzae* (PR9 isolate) was determined using the detached leaf assay, as described previously (Coca *et al.*, 2004). For this, young leaves of either mycorrhizal or nonmycorrhizal soil-grown plants were placed into Petri dishes with 1% w/v water—agar containing 2 mg/L kinetin. Whatman filter papers saturated with an *M. oryzae* PR9 spore suspension at the appropriate concentration were placed onto the upper face of the leaf for 48 h and then removed. The inoculated leaves were maintained in the dark in a chamber under high-humidity conditions for 48 h and the filter papers were removed. Leaves were maintained at 28 °C and 90% relative humidity under a 16-h/8-h light/dark photoperiod for the required period of time. The ability of the fungus to produce spores was estimated by counting the number of spores collected from infected leaves at 7 dpi with the highest spore concentrations (10^6 spores/mL) (Campos-Soriano and San Segundo, 2009). Lesion areas were quantified by Image Analysis Software, Assess 2.0, for plant disease quantification (Lamari, 2008). One-way ANOVAS were used to evaluate differences in the leaf area affected by blast lesions between mycorrhizal and nonmycorrhizal plants. For ANOVAS in which the *F* test was significant at the probability level of 0.05 or lower, the least-significant difference test was applied to detect differences.

Total RNA was also obtained from *M. oryzae*-infected leaves at different times after inoculation with fungal spores and used for gene expression analysis.

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SUPPORTING INFORMATION

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

Table S1 Primers used for quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) analysis.

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