

OsMADS26 Negatively Regulates Resistance to Pathogens and Drought Tolerance in Rice^{1[OPEN]}

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Functional analyses of MADS-box transcription factors in plants have unraveled their role in major developmental programs (e.g. flowering and floral organ identity) as well as stress-related developmental processes, such as abscission, fruit ripening, and senescence. Overexpression of the rice (*Oryza sativa*) *MADS26* gene in rice has revealed a possible function related to stress response. Here, we show that *OsMADS26*-down-regulated plants exhibit enhanced resistance against two major rice pathogens: *Magnaporthe oryzae* and *Xanthomonas oryzae*. Despite this enhanced resistance to biotic stresses, *OsMADS26*-down-regulated plants also displayed enhanced tolerance to water deficit. These phenotypes were observed in both controlled and field conditions. Interestingly, alteration of *OsMADS26* expression does not have a strong impact on plant development. Gene expression profiling revealed that a majority of genes misregulated in overexpresser and down-regulated *OsMADS26* lines compared with control plants are associated to biotic or abiotic stress response. Altogether, our data indicate that *OsMADS26* acts as an upstream regulator of stress-associated genes and thereby, a hub to modulate the response to various stresses in the rice plant.

MADS-box transcription factors belong to a multi-genic family and have been identified in yeasts, plants, insects, nematodes and lower vertebrates, and mammals, where they control different aspects of development and cell differentiation (Shore and Sharrocks, 1995). For example, the yeast (*Saccharomyces cerevisiae*) MINICHROMOSOME MAINTENANCE1 MADS-box transcription factor is involved in diverse regulatory mechanisms underlying cell viability, cell cycle control, mating, minichromosome maintenance, and recombination but also, osmotolerance (Messenguy and Dubois, 2003). The MADS-BOX PROTEIN REQUIRED FOR INFECTIOUS GROWTH1/RESISTANCE TO LEPTOSPHAERIA MACULANS1 MADS-box transcription factor is required for pathogenicity of the causal fungal agent of the rice (*Oryza sativa*) blast disease, *Magnaporthe oryzae* (Mehrabi et al., 2008). In

plants, analyses of MADS-box transcription factors have mainly revealed a function in flower development, flowering induction, or fruit development (Theissen et al., 2000; Arora et al., 2007; Smaczniak et al., 2012). Expression of other MADS genes in pollen, endosperm, guard cells, roots, and trichomes suggests a function in the differentiation of these organs and tissues (Alvarez-Buylla et al., 2000; Parenicová et al., 2003; Puig et al., 2013). Some plant MADS-box transcription factors are involved in the control of stress-related developmental programs, such as abscission, fruit ripening, and senescence. For example, in *Arabidopsis thaliana*, overexpression (OX) of *AGAMOUS-LIKE15* (*AGL15*) was found to delay flowering, senescence, fruit ripening, and floral organ abscission, suggesting that this MADS-box transcription factor is a negative regulator of these processes

(Fernandez et al., 2000; Fang and Fernandez, 2002). Similarly, FOREVER YOUNG FLOWER represses floral organ senescence and abscission in Arabidopsis (Chen et al., 2011). SHATTERPROOF1 (SHP1) and SHP2 are involved in the cell specification of the dehiscence zone in Arabidopsis fruits, where they promote the lignification of cells adjacent to this zone (Liljegren et al., 2000). In tomato (*Solanum lycopersicum*), the MADS domain protein JOINTLESS is necessary to specify pedicel abscission zone MADS-RIPENING, and TOMATO AGL1 controls fruit ripening (Mao et al., 2000; Vrebalov et al., 2002, 2009; Itkin et al., 2009). Nevertheless, no MADS-box gene has been yet identified in plants to have a function related to biotic or abiotic stress response regulation.

The rice genome contains 75 genes encoding MADS-box transcription factors, but the function of only few of them has been determined. Most of the studied genes are involved in the control of development, including tillering, flower development, and flowering time (Arora et al., 2007; Guo et al., 2013). Some of them are involved in development by controlling stress-related processes, such as *OsMADS3*, which is involved in reactive oxygen species homeostasis during anther development, and *OsMADS29*, which controls cell degeneration during seed development (Hu et al., 2011; Yang et al., 2012). A possible specific involvement of rice MADS genes in stress response has been reported only for *OsMADS26*, the rice ortholog of *AGL12* (Lee

et al., 2008b, 2011). In Arabidopsis, *AGL12* regulates cell proliferation in the root apical meristem as well as flowering transition and was suggested to control root secondary cell wall synthesis (Tapia-López et al., 2008; Chávez Montes et al., 2014). When overexpressed in *Catharanthus roseus* cell suspension, *AGL12* promotes cell aggregation and stimulates expression of genes involved in the biosynthesis of terpene indole alkaloids (Montiel et al., 2007). In rice, *OsMADS26* OX causes a severe stress phenotype that generally leads to plant death. Expression of *OsMADS26* under the control of a dexamethasone-inducible promoter provokes the differential regulation of genes involved in jasmonic acid biosynthesis and reactive oxygen species production (Lee et al., 2008b).

To precisely study the involvement of *OsMADS26* in stress response in rice, we succeeded in generating viable plants overexpressing *OsMADS26* and plants where *OsMADS26* expression was down-regulated through RNA interference (RNAi). Our data showed that *OsMADS26*-down-regulated plants have no dramatic alteration of their development and were more resistant to *M. oryzae* and *Xanthomonas oryzae* pv *oryzae*, the main fungal and bacterial pathogens of rice. However, *OsMADS26* OX increased moderately their susceptibility to these pathogens. Enhancement of recovery capacity after a severe water stress was also observed in *OsMADS26*-down-regulated plants. These phenotypes were further confirmed in the field, with *OsMADS26* OX increasing *M. oryzae* susceptibility and *OsMADS26* down-regulation (DR) promoting resistance against water deficit. A transcriptome analysis revealed that genes differentially regulated between control and overexpressing or down-regulated *OsMADS26* plants were enriched with already known biotic and abiotic stress-related genes. Altogether, these results indicate that *OsMADS26* is a major negative regulator of both biotic and abiotic stress responses in rice.

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J.-B.M. and P.G. conceived the original research plans and supervised the experiments; G.N.K. performed most of the experiments; P.K.P. cloned *OsMADS26* in different binary vectors and started the rice transformation; F.R. selected the transgenic T1 and T2 lines; B.P., A.-M.G., J.-B.M., T.B., and P.G. analyzed the microarray data; P.B. participated in the gene expression analysis; C.D.M. challenged the transgenic lines with *Magnaporthe oryzae* in Vietnam; M.B., I.B., and D.M. contributed to the plant transformation and selection; M.G.S. and I.M. performed the drought field experiments; C.B. supervised the virus infection tests; C.B., V.N.D., E.G., J.-B.M., and P.G. supervised the project and wrote the article with contributions of all the authors.

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RESULTS

OsMADS26 Is Preferentially Expressed in Peripheral Tissues and Regulated by Biotic and Abiotic Stresses

Accumulation of *OsMADS26* transcripts in roots, leaves, and panicles has been previously reported (Shinozuka et al., 1999; Pelucchi et al., 2002; Arora et al., 2007) and was found to increase with organ aging (Lee et al., 2008b). To further precisely study the expression pattern of *OsMADS26*, we carried out quantitative reverse transcription (qRT)-PCR and in situ hybridization assays in the organs of 7-d-old rice seedlings. *OsMADS26* was found to be expressed in all of the investigated organs (i.e. leaf blade, stem bases, and seminal and crown roots; Fig. 1A) in a consistent manner with regards to the available expression data (www.genevestigator.com with Os.4174.1.S1_at). In seminal roots, the expression of *OsMADS26* in the 0.5-cm

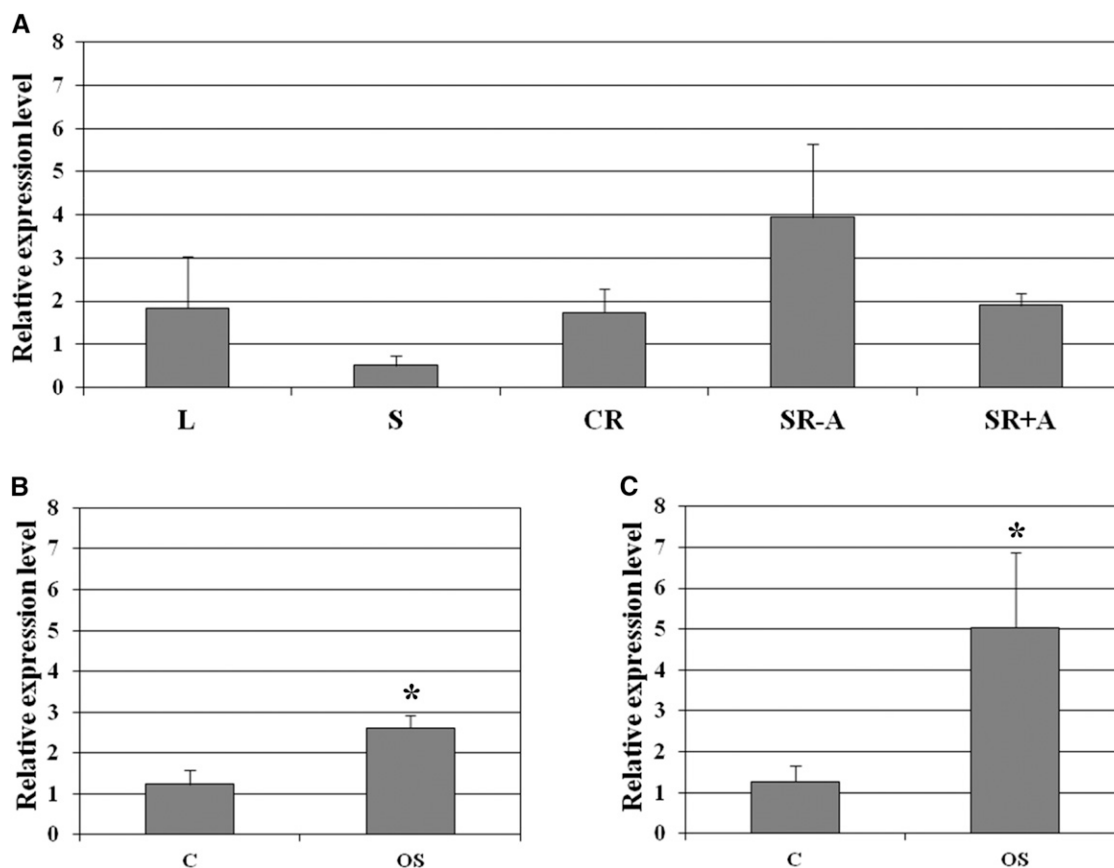


Figure 1. *OsMADS26* is expressed in shoots and roots and induced by osmotic stress. A, Expression of *OsMADS26* in different organs of 7-d-old rice seedlings cultivated in standard condition (MS). CR, Crown root; L, leaf; S, stem base; SR+A, seminal root apex; SR-A, seminal root without apex. B and C, Expression patterns of *OsMADS26* in root (B) and shoot (C) in standard condition (C) or under osmotic stress (OS; MS + 100 mM mannitol). Means and se were calculated from two independent experiments consisting of three technical replicates each. A Student's *t* test was used to compare the relative expression level observed in standard and stress conditions. *, Significant difference with $P < 0.05$.

segment above the root tip was 2-fold higher than in the root tip itself (the 0.5-cm apical part of the seminal root; Fig. 1A). In situ hybridization specified qRT-PCR data, showing that *OsMADS26* transcripts accumulate in the differentiated epidermis, exodermis, sclerenchyma, and cortical aerenchyma layers but do not accumulate in the meristematic zone of the root or the root cap (Fig. 2, A–H). *OsMADS26* mRNA was not detected in the stele tissues (Fig. 2, A and E). In leaves, *OsMADS26* was expressed in the epidermal cells, bulliform cells, phloem, and xylem-associated parenchyma cells (Fig. 2, I–L).

To determine whether *OsMADS26* expression is influenced by osmotic stress, rice seedlings were grown on culture media supplemented with 100 mM mannitol. Under these conditions, the seedling growth is reduced but not abolished (data not shown). Mannitol treatment induced the expression level of *OsMADS26* in both shoot and root tissues (Fig. 1, B and C).

Because available microarray data indicate that *OsMADS26* is slightly down-regulated late after infection (48 h postinoculation [hpi]) by the FR13 virulent

isolate of the blast fungus *M. oryzae* (Ribot et al., 2008; Gene Expression Omnibus accession no. GSE7256), we further investigated its expression time course after inoculation with virulent and avirulent isolates (FR13 and CL3.6.7, respectively; Delteil et al., 2012) of *M. oryzae* (Fig. 3). We confirmed that *OsMADS26* transcription is slightly repressed late after inoculation (72 hpi) with the virulent isolate FR13 but not the avirulent isolate CL3.6.7. More strikingly, *OsMADS26* was strongly repressed in an early phase of infection by both isolates (4 and 8 hpi) before the fungus had penetrated into the leaf (Fig. 3).

***OsMADS26* Misregulation Does Not Strongly Affect Plant Development**

To precisely study the function of *OsMADS26*, we investigated the effect of its OX and its RNAi-mediated DR in rice plants. For OX, the *OsMADS26* complementary DNA (cDNA) was placed under the control of the maize (*Zea mays*) ubiquitin1 promoter that allows

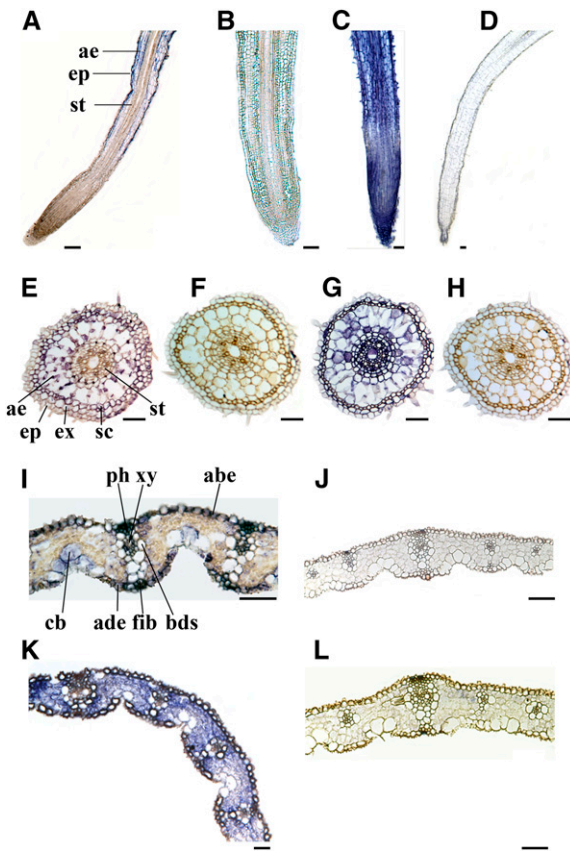


Figure 2. *OsMADS26* is expressed in differentiated peripheral tissues. In situ hybridizations were revealed with the VectorBlue Kit III. Antisense (A, E, and I) and sense (B, F, and J) *OsMADS26* probe hybridizations on a longitudinal section of the root tip (A and B), a transverse section in the seminal root (E and F), and a transverse section in the third leaf (I and J) of 7-d-old rice seedling. Hybridization with antisense (C, G, and K) and sense (D, H, and L) 18S ribonucleic RNA probes, which were used as positive and negative controls, respectively. abe, Abaxial epidermis; ade, adaxial epidermis; ae, aerenchyma; bds, bundle sheath; cb, bulliform cells; ep, epidermis; ex, exodermis; fib, fiber; ph, phloem; sc, sclerenchyma; st, stele; xy, xylem. Bars = 70 μ m.

high-level, constitutive expression in rice (Cornejo et al., 1993). We selected two independent, homozygous single-transfer DNA (T-DNA) copy events, OX1 and OX2, accumulating *OsMADS26* transcripts at 30- and 20-fold higher levels than the control, respectively (Fig. 4A). *OsMADS26* OX remained stable in further generations (Supplemental Fig. S1A). For constitutive RNAi-mediated DR of *OsMADS26*, two constructs specifically targeting either its 5' untranslated region (UTR; DR5) or the 3' UTR (DR3) region were prepared. Two independent, homozygous, single-T-DNA copy events were randomly selected for each construct (DR5-1 and DR5-2 and DR3-1 and DR3-2). A wild-type line regenerated from untransformed callus used for the transformation experiment was kept as control (the wild type). In addition, one line transformed with the empty OX T-DNA (OX0) and one line obtained by transformation with the empty RNAi T-DNA (DR0) were used

as additional controls. Plantlets of these three control lines accumulated *OsMADS26* transcripts at a similar level (Fig. 4, A and B). In all of the RNAi lines, *OsMADS26* expression was reduced strongly and stably over the subsequent generations (Fig. 4B; Supplemental Fig. S1B) and did not respond to osmotic stress (Supplemental Fig. S1C).

To further establish the influence of *OsMADS26* on rice development, the phenology of the transformed lines was investigated. The height of 7-d-old seedlings grown in vitro was scored. All control lines (the wild type, OX0, and DR0) exhibited similar development, whereas the heights of the OX1, OX2, DR5, and DR3 lines were significantly reduced (Table I). DR5 and DR3 plantlets were the most affected. However, 2 months after transfer in pots in the greenhouse (76 d after germination [DAG]), the average heights of OX1, OX2, DR5, and DR3 lines were similar to those of control lines, except the DR5-1 line, which still exhibited a reduced size (Table I). At the same time, all of the down-regulated lines displayed a reduction in tiller number (Table I; Fig. 4C). This was particularly significant for the DR5-2 line, which displayed a 45% reduction in the number of tillers compared with its control (DR0; Table I). The dry weights of the aerial parts of the DR plants, especially the two DR5 lines, were lower than those of the control and OX plants (Table I). The two DR3 lines also exhibited significant delay of 3 to 4 d in flowering (Table I). No significant difference for these two traits was observed among the rest of the lines. Total weight and 1,000-seed weight of the main panicle were comparable in all of the lines studied (Table I). In summary, although the overexpressing and down-regulated *OsMADS26* lines exhibited a retarded growth at early stages of development after germination, further transfer and growth in the greenhouse allowed them to recover and exhibit a performance generally similar or close to that of control plants. The weak impact of constitutive *OsMADS26* OX or DR on plant development

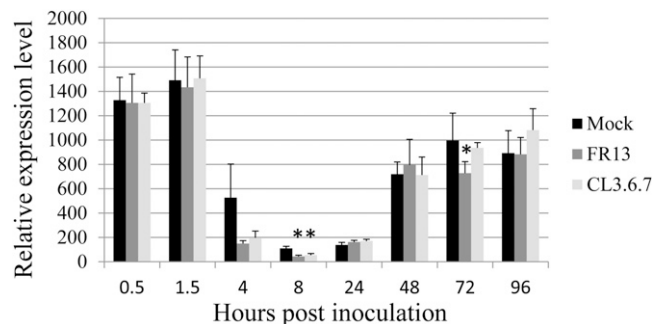


Figure 3. *OsMADS26* expression is regulated by *M. oryzae* infection. Three-week-old rice seedlings of cv Nipponbare were challenged with two isolates of *M. oryzae* virulent FR13 and avirulent CL3.6.7 or mock treated. The expression of each gene was normalized using the actin gene as control. The mean and SD were calculated from three independent experiments. A Student's *t* test was done to establish whether the relative expression level in inoculated condition was different from mock treated. *, $P < 0.05$; **, $P < 0.01$.

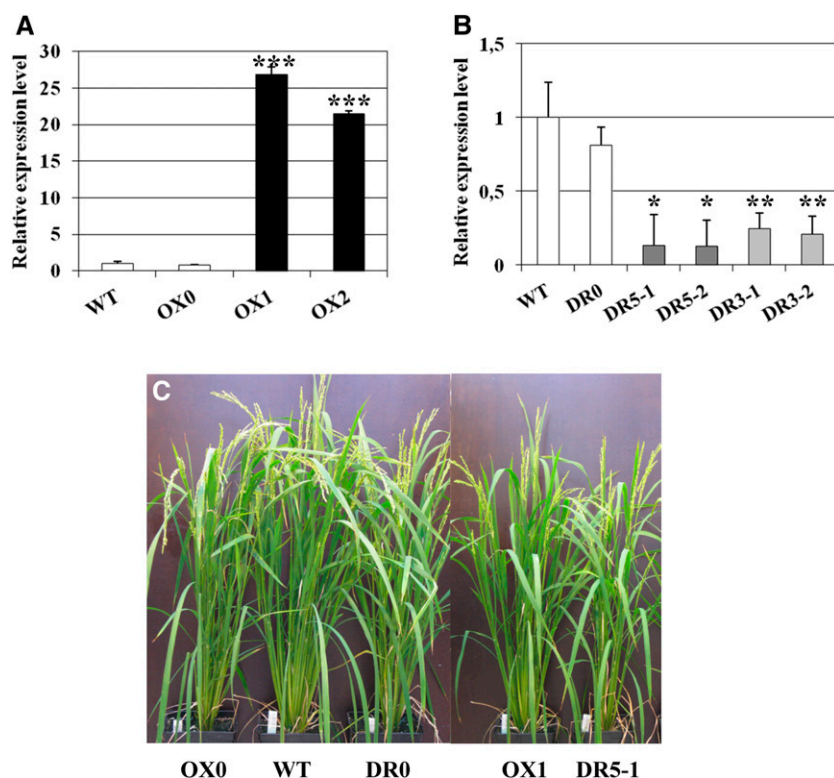


Figure 4. OX and DR of *OsMADS26* do not interfere with overall plant development. **A**, *OsMADS26* relative expression levels in 3-week-old T2 overexpressing (OX1 and OX2; black bars) and control (the wild type [WT] and OX0; white bars) plants cultivated in the greenhouse. **B**, *OsMADS26* expression levels in RNA down-regulated (DR5-1, DR5-2, DR3-1, and DR3-2; gray bars) and control (the wild type and DR0; white bars) plants cultivated in the greenhouse. Means and SE were obtained from two individual plants of each line. **C**, Control and transgenic *OsMADS26* T2 plants cultivated in the greenhouse observed at flowering stage. A Student's *t* test was done to establish whether the relative expression level in the transgenic line was different from the corresponding null segregant line. *, Significant difference with $P < 0.05$; **, significant difference with $P < 0.01$; ***, significant difference with $P < 0.001$.

was confirmed in the field, where we observed only a reduced height for the OX2 line and a higher biomass and yield for the DR3-1 line compared with their relative controls (Supplemental Fig. S2).

OsMADS26 Is Required for Resistance against Blast Fungus and Bacterial Blight

Because *OsMADS26* was found to be a stress-related gene in rice (Lee et al., 2008b, 2011), we further evaluated the response of the *OsMADS26* transgenic lines to pathogen infection.

First, plantlets of the different *OsMADS26* lines were inoculated with the moderately virulent fungal isolate GUY11 of *M. oryzae* (Delteil et al., 2012). This isolate triggers lesions in the leaf blade of cv Nipponbare consisting of an average of 50% grayish lesions surrounded by brown margins that are characteristic of successful invasion of the fungus (disease). The others are small and dark spots characteristic of unsuccessful invasion events (the wild type, OX0, and DR0 plants in Fig. 5A). Differences in the degree and development of disease symptoms caused by *M. oryzae* between transformed and untransformed plants were clearly visible at 7 d postinoculation (dpi; Fig. 5A). The two overexpressing lines (OX1 and OX2) presented more disease symptoms compared with the controls (the wild type and OX0). In contrast, all of the down-regulated lines displayed many small and dark spots characteristic of resistance and very few disease symptoms. These observations were further

confirmed by calculating the percentage of susceptible lesion versus the total number of observed lesion on each infected leaf (Fig. 5B). Thus, this suggested that *OsMADS26* negatively regulates blast resistance. In addition, the susceptibility to *M. oryzae* of OX0, OX2, and DR3-1 lines was challenged in a nethouse in Vietnam on 10-week-old plants inoculated with the VT15 Vietnamese isolate virulent on cv Nipponbare (Supplemental Fig. S3). In this experiment, the number of susceptible lesions was significantly higher in OX2 line and slightly lower in DR3-1 line than in the control (OX0), confirming the opposite phenotypes observed for overexpressing and down-regulated *OsMADS26* lines. The expression of a set of selected major defense-related genes, *PEROXIDASE22.3* (*POX22.3*; Vergne et al., 2007), *CHITINASE7* (*CHI7*; Kaku et al., 2006), *PATHOGENESIS-RELATED PROTEIN5* (*PR5*), *NONEXPRESSOR OF PATHOGENESIS-RELATED1 HOMOLOG1* (*NH1*; Chern et al., 2005), (*FLAGELLIN SENSING2*), *OsWRKY28*, and *PROBENAZOLE-INDUCIBLE1* (*PBZ1*; Delteil et al., 2012), was examined in OX2 lines 2 dpi with *M. oryzae* GY11 isolate or mock treatment (Fig. 6). This showed that, in mock-treated and inoculated plants, the expression of most of these genes (*POX223*, *CHI7*, *PR5*, *NH1*, *FLS2*, and *WRKY28*) was significantly reduced in the OX2 line compared with OX0 before and/or after infection. This result suggests that *OsMADS26* acts as a negative regulator of defense gene expression.

Second, to evaluate whether constitutive deregulation of *OsMADS26* affects the susceptibility to a

Table 1. Plant phenotype of control and transgenic *OsMADS26* lines after 7 d of *in vitro* culture (MS), 72 DAG in greenhouse, and from flowering to harvest

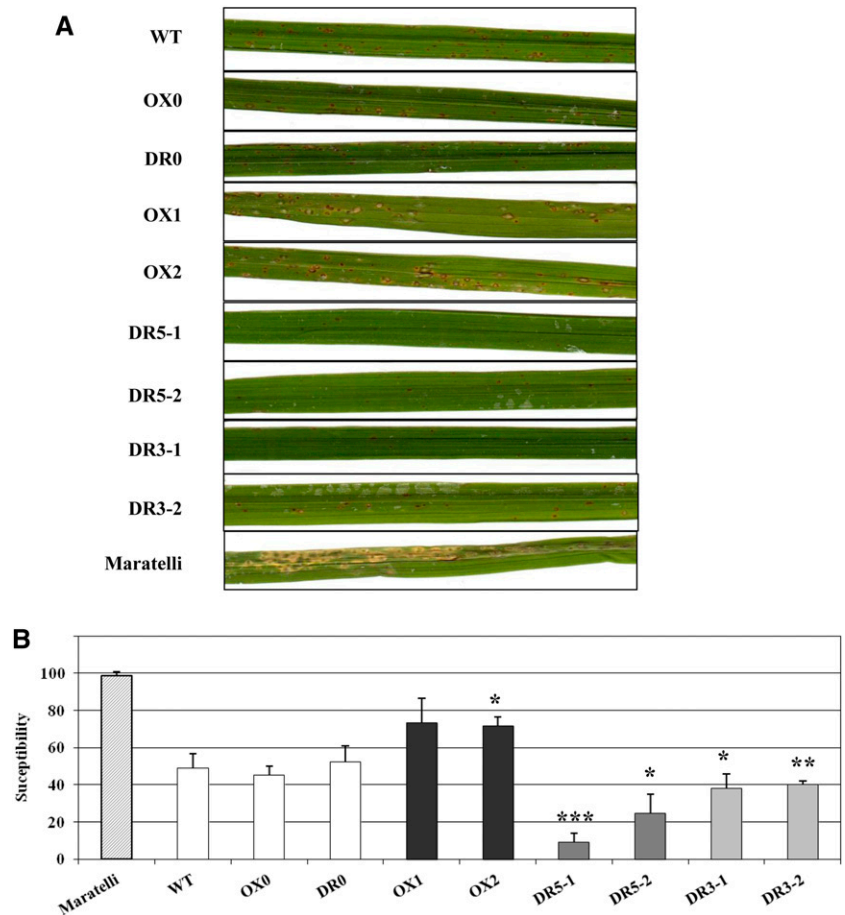
Results shown are from one of two independent biological repetitions that produced similar results. Reported values are the means and \pm SE for three individual plants of each line. A Student's *t* test was done to establish whether the parameter measured in transgenic lines was different from corresponding control lines. BEG, Flowering beginning; DW, plant dry weight after seed harvesting; FD, flowering date; HTG_7, plant height measured at 7 DAG; HTG_76, plant height measured at 76 DAG; P1,000, weight of 1,000 seeds; PW, panicle weight; TIL_76, number of tillers counted at 76 DAG. Significant difference: *, $P < 0.05$, **, $P < 0.01$; and ***, $P < 0.001$.

Line name	HTG_7	HTG_76	TIL_76	BEG	FD	DW	PW	P1,000
	<i>cm</i>			<i>DAG</i>			<i>g</i>	
Wild type	6.06 \pm 1.51	97.53 \pm 0.59	12.33 \pm 0.33	80.33 \pm 0.33	81.67 \pm 0.88	9.74 \pm 2.34	15.18 \pm 2.45	21.80 \pm 0.71
OX0	6.34 \pm 1.33	97.47 \pm 2.06	10.67 \pm 0.33	82.33 \pm 1.20	84.00 \pm 1.00	8.73 \pm 0.87	9.52 \pm 0.95	20.34 \pm 0.62
DR0	6.72 \pm 1.27	95.23 \pm 1.36	11.33 \pm 1.33	81.67 \pm 0.67	83.67 \pm 0.67	7.96 \pm 3.80	8.88 \pm 4.28	17.89 \pm 3.93
OX1	3.84 \pm 0.67**	100.60 \pm 2.17	10.33 \pm 1.45	80.00 \pm 1.53	81.67 \pm 1.86	8.00 \pm 1.42	8.78 \pm 1.50	21.39 \pm 0.30
OX2	2.41 \pm 0.92***	93.40 \pm 2.84	12.33 \pm 0.88	83.67 \pm 0.88	86.00 \pm 1.00	8.21 \pm 1.12	8.93 \pm 1.34	20.38 \pm 0.72
DR5-1	1.68 \pm 0.68***	87.90 \pm 2.51*	7.80 \pm 2.08	83.00 \pm 1.15	85.67 \pm 0.67	3.86 \pm 1.07	4.21 \pm 1.14	16.32 \pm 0.48
DR5-2	1.61 \pm 0.29***	95.37 \pm 1.84	6.67 \pm 0.67*	82.33 \pm 0.67	85.00 \pm 0.00	4.93 \pm 0.40	5.48 \pm 0.39	19.79 \pm 1.15
DR3-1	1.61 \pm 0.31***	90.53 \pm 1.79	9.67 \pm 1.33	85.00 \pm 0.00**	87.00 \pm 0.58**	6.62 \pm 1.37	7.33 \pm 1.65	21.42 \pm 0.73
DR3-2	0.84 \pm 0.18***	97.20 \pm 1.73	9.00 \pm 1.00	84.67 \pm 0.33**	86.33 \pm 0.33*	7.76 \pm 0.73	8.41 \pm 0.67	20.01 \pm 0.68

bacterial pathogen, we challenged the overexpressing and down-regulated *OsMADS26* lines with *X. oryzae* pv *oryzae*. Similar data were obtained for resistance to bacterial blight *X. oryzae* pv *oryzae* as with *M. oryzae*. In this case, the length of the necrotic and yellowing zone extending from the wounded extremity of the infected

leaves was measured 14 dpi. The symptoms had a significantly higher severity for OX1 and OX2 lines compared with the control lines (Supplemental Fig. S4, A and B). Conversely, the symptoms developed by down-regulated lines (DR5-1, DR5-2, DR3-1, and DR3-2) were limited to a short necrosis just below the

Figure 5. *OsMADS26* negatively regulates resistance against *M. oryzae*. OX (OX1 and OX2; black bars) and DR (DR5-1, DR5-2, DR3-1, and DR3-2; gray bars) *OsMADS26* lines and corresponding control lines transformed with empty vectors or untransformed lines (OX0, DR0, and the wild type [WT]; white bars) and cv Maratelli, a highly susceptible cultivar, were tested. A, Symptom severity in leaves of transgenic and control plants inoculated with the GUY11 strain of *M. oryzae*. Photographs were taken 7 dpi. B, Percentage of susceptible versus total lesions observed in *M. oryzae*-infected leaves 7 dpi. Means and \pm SE were from 10 inoculated plants for each line. Results shown are from one of two independent experiments that produced similar results. A Student's *t* test was done to establish whether one given transgenic line was different from its corresponding null segregant line. *, Significant difference with $P < 0.05$; **, significant difference with $P < 0.01$; ***, significant difference with $P < 0.001$.



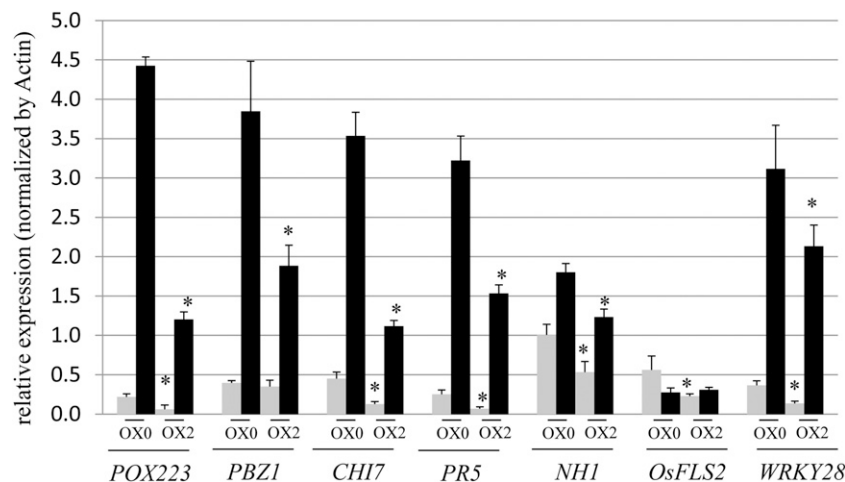


Figure 6. Expression of defense genes is down-regulated in *OsMADS26* OX before and after infection by *M. oryzae*. Three-week-old rice seedlings of *OsMADS26* overexpressing (OX2) line and control line (OX0) were challenged with the moderately virulent isolates of *M. oryzae* GY11 (black bars) or mock treated (gray bars). The RNA was extracted at postinoculation. The expression of each gene was normalized using the actin gene as control. The *POX223*, *PBZ1*, *CHI7*, and *PR5* genes are coding for pathogenesis-related proteins used as classical markers of defense. The *NH1*, *OsFLS2*, and *WRKY28* genes are coding for regulator proteins of defense in rice. The means and SD were calculated from three independent experiments. A Student's *t* test was done to establish whether the relative expression level in the OX2 lines was different with the line used as control. *, $P < 0.01$.

inoculation zone (Supplemental Fig. S4, A and B), suggesting that these lines were strongly resistant to *X. oryzae* pv *oryzae* and supporting a negative role of *OsMADS26* on blight resistance.

Third, we tested whether the response to the Rice Yellow Mottle Virus (RYMV; Kouassi et al., 2005) could be affected by *OsMADS26* OX or DR. We did not observe any difference in the development of symptoms or virus accumulation between the overexpressing lines, the down-regulated lines, and their respective controls (Supplemental Fig. S5), suggesting that misregulation of *OsMADS26* expression had no impact on the resistance against RYMV.

***OsMADS26* Inhibition Favors Plant Tolerance against Drought Stress**

Because mannitol stress induces the expression of *OsMADS26* (Fig. 1, B and C), we investigated the tolerance of overexpressing and down-regulated lines to the drought stress. After the drought stress, plants were rewatered for a period of 2 weeks to allow recovery. Although plants of all of the control and *OsMADS26*-overexpressing lines were mostly wilted and died, *OsMADS26*-down-regulated plants fully recovered from the water stress (Fig. 7A).

All of the lines exhibited at the beginning of the experiment a similar relative water content (RWC; nearly 95%) that decreased to approximately 85% after 11 d of water deficit (Fig. 7B). However, 15 d after water deprivation, the leaf RWCs of all of the control and *OsMADS26*-overexpressing lines dropped to a 47% to 62% range, whereas the two *OsMADS26*-down-regulated lines maintained significantly higher RWCs,

falling within an 81% to 84% range. This suggests that the inhibition of *OsMADS26* expression enhances the capacity of the rice plant to maintain its water content under water deficit.

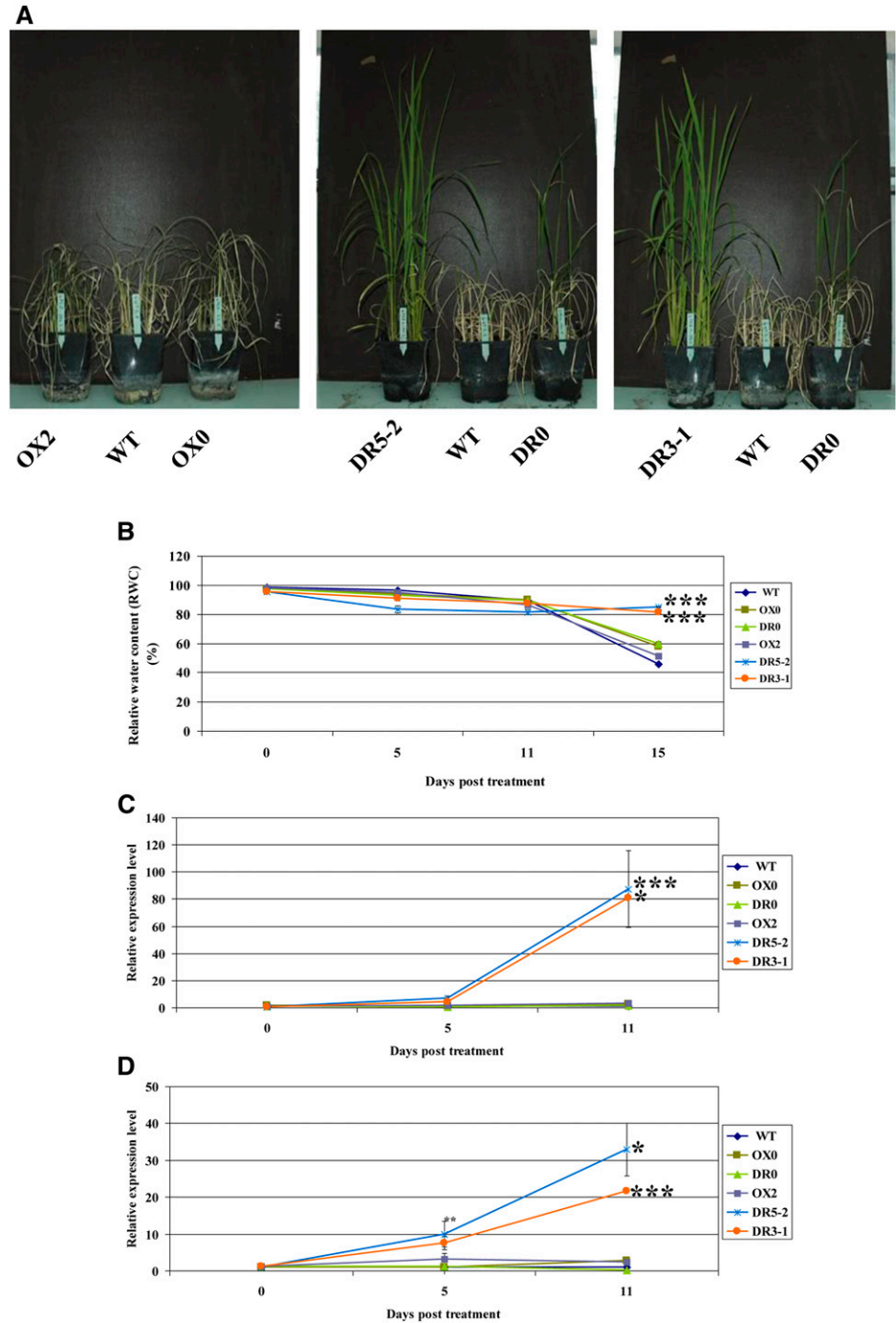
The expression of two drought-responsive genes was analyzed: *RESPONSIVE TO ABA21 (RAB21)*, a rice dehydrin and *SALT STRESS-INDUCED PROTEIN (SALT; Claes et al., 1990; Oh et al., 2005)*. Their expression levels were similar in all lines before or 5 d after the water stress. After 11 d of water stress, however, their expression was significantly higher in the two *OsMADS26*-down-regulated lines compared with control and *OsMADS26* OX lines (Fig. 7, C and D). This suggests that *OsMADS26* may play a negative role in the regulation of some drought stress-responsive genes in response to water deficit.

In addition, we challenged in the field the capacity of OX0, OX2, DR0, and DR3-1 lines to tolerate water deficit. The DR3-1 line presented a much better tolerance to water deficit conditions associated with a slower decrease of chlorophyll-a content and a better capacity to maintain yield under drought than the other lines (Fig. 8). Other measurements (leaf rolling, chlorophyll content, and biomass) confirmed that DR3-1 plants had an increased capacity to sustain drought stress (Supplemental Fig. S6). This confirmed that a constitutive DR of *OsMADS26* increases the capacity of the plant to tolerate water deficit.

Transcriptome Profiling of *OsMADS26*-Overexpressing and -Down-Regulated Lines

Preliminary evidence of altered expression of stress-related genes in *OsMADS26*-overexpressing and

Figure 7. *OsMADS26* negatively regulates water stress tolerance. Six independent lines: overexpressing (OX2) or down-regulated (DR5-2 and DR3-1) *OsMADS26* and corresponding control lines transformed with empty vectors (OX0 and DR0) or the wild type (WT) were used for this experiment. A, Drought stress was applied on 20-d-old plants growing in the greenhouse in pots by stopping watering during 18 d followed by 15 d of rewatering. The pictures were taken 15 d after rewatering. B, RWC of plants was measured on the last expanded leaf before and at 5, 11, and 15 d after watering stopping. Mean values and SE were calculated from five individual plants for each line. C and D, qRT-PCR expression analysis of drought- and salt-responsive rice genes *RAB21* (C) and *SALT* (D) in control and transgenic plants before and during drought stress. RNA were extracted from leaves of two plants of each line that had closest RWCs. We did not measure gene expression 15 d after the water deficit period, because the control and *MADS26*-overexpressing plants were already highly damaged. Means and SE were from two individual plants for each line. A Student's *t* test was done to establish whether the RWC or the gene expression level in transgenic lines was different from corresponding control line. *, Significant difference with $P < 0.05$; **, significant difference with $P < 0.01$; ***, significant difference with $P < 0.001$.



down-regulated lines led us to further identify the pathways potentially regulated by *OsMADS26* through transcriptome profiling. Transcriptome profiles were established from two independent biological replicates per line. Genes significantly and reproducibly induced or repressed (fold change > 2 and P value ≤ 0.05) across lines and replicates compared with their values in the appropriate controls were selected for further analysis (for more information, see "Materials and Methods"). We finally selected genes at least one time inversely

regulated in OX compared with DR lines or reproducibly overexpressed or repressed in OX or control lines. To compare our results with other available data, we converted the rice probes into Michigan State University's Rice Genome Annotation Project transcriptional units (Supplemental Table S1). This represented a total of 400 nonredundant genes. A total of 71 nonredundant genes presented an inverted regulation profile in OX and DR lines (Fig. 9; Supplemental Table S1). Overall, 212 genes were down-regulated in DR lines

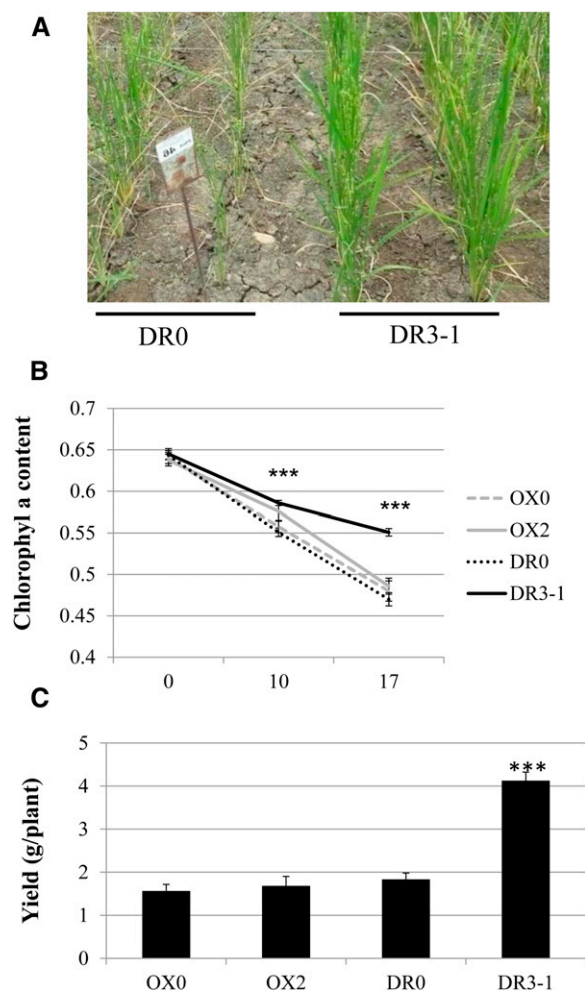


Figure 8. *OsMADS26* DR confers tolerance to water deficit under field conditions. Plants were grown in the field at the International Center for Tropical Agriculture, and a drought stress was applied ("Materials and Methods"). The shape of the plant 17 d after stress is shown (A), and the chlorophyll fluorescence (B) was measured at the indicated times after stress in three independent blocks on three plants. Yield was measured at the end of the experiment (C). The means and SD are shown, and a Student's *t* test ($n = 9$) was used to evaluate statistical difference between the overexpressing OX2 and down-regulated DR3-1 transgenic lines with their respective controls OX0 and DR0. ***, $P < 0.001$.

and/or up-regulated in OX lines. These genes should belong to pathways induced by *OsMADS26*. On the contrary, 200 genes were up-regulated in DR lines and/or down-regulated in OX lines. These genes should belong to pathways inhibited by *OsMADS26*.

We then looked for overlaps between a set of >6,800 probes that were known to be transcriptionally regulated upon pathogen infection (Vergne et al., 2008) and the 400 genes that were significantly misregulated in DR and/or OX lines (Supplemental Table S1). We found that 53% of the 200 genes up-regulated in DR and/or down-regulated in OX lines are known to be transcriptionally regulated during pathogen challenge, whereas only 30% were expected by chance in a

random selection of 2,000 genes ($P < 0.001$ as evaluated with a χ^2 test; Vergne et al., 2008). In contrast, there was no such enrichment in the 212 genes up-regulated in DR lines and/or down-regulated in OX lines. Thus, *OsMADS26* seems to down-regulate the transcription of a large number of genes known to be involved in disease resistance. Similarly, a large proportion (41%) of genes misregulated in *OsMADS26* lines was found in a previously published drought data set (Minh-Thu et al., 2013). The extent of this overlap is proportional to the one observed with genes found to be deregulated in dexamethasone-inducible *OsMADS26* lines (39%; Lee et al., 2008b). Our analysis thus resulted in a list of putative *OsMADS26* target genes that may be involved in the regulation of biotic or abiotic stress resistance.

DISCUSSION

Alteration of *OsMADS26* Expression Does Not Deeply Affect cv Nipponbare Plant Development

The *OsMADS26*-overexpressing lines presented a delayed development at the seedling stage, but their development in the greenhouse and field was almost similar to the development of control plants aside from a slight reduction in tiller number (Table I). This contrasts with the previous study by Lee et al. (2008b), which reported that OX of *OsMADS26* driven by the same constitutive promoter triggered several dramatically abnormal developmental phenotypes, including anthocyanin accumulation or lethality. A tentative explanation might be in the use of different genetic backgrounds (cv Nipponbare versus cv Dongjin) for expressing *OsMADS26*. To our knowledge, there is at least one report where OX in different rice genetic backgrounds resulted in the opposite effects (Tao et al., 2009). Alternatively, it is possible that our

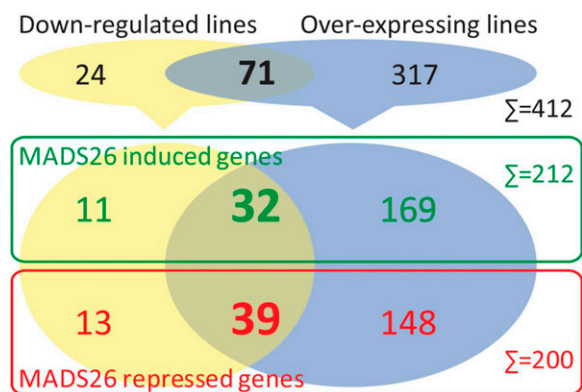


Figure 9. Genome-wide gene expression regulations in *OsMADS26*-overexpressing or -down-regulated lines. Number of genes significantly differentially expressed in the microarray experiment; 71 (32 and 39, respectively) genes presented an inverted regulation profile in OX and DR lines. Green and red colors depict genes induced or repressed, respectively, by *OsMADS26* expression.

transformation procedure (Sallaud et al., 2003) that differs from that used by Lee et al. (2008b) has counterselected plants presenting a severe reduction of their development or lethality because of very high levels of expression. Although we cannot explain the strong phenotypic differences between our overexpressing lines and the lines analyzed by Lee et al. (2008b), these differences may explain at least in part why we found little overlap between our experiments and their microarray experiments (16 genes in total; see below). Similarly, except for a delay in development observed at early stages, the overall development of the down-regulated lines was not strongly modified (Table I).

OsMADS26 Is a Negative Regulator of Both Biotic and Abiotic Stresses

Our data showed that *OsMADS26*-down-regulated lines displayed decreased susceptibility to two major pathogens of rice (Fig. 5; Supplemental Figs. S3 and S4) as well as an increased water deficit tolerance and a better recovery capacity after a drought stress (Figs. 7 and 8; Supplemental Fig. S2). The observation of consistent phenotypes in the *OsMADS26*-down-regulated lines obtained with two independent constructs targeting 5' or 3' UTR reduces the risk of misinterpretation related to transinterference with transcripts of other genes. Because the observed phenotypes are similar between the different down-regulated lines, we can assume that they are the consequence of a specific degradation of *OsMADS26* mRNAs.

Up to 60% and 40% average disease symptom reductions were observed in down-regulated lines inoculated with *X. oryzae* pv *oryzae* and *M. oryzae*, respectively (Fig. 5; Supplemental Fig. S4). This corresponds to a high level of disease reduction compared with the range attained in transgenic lines obtained through misregulation of a set of defense-associated genes (Delteil et al., 2010). Consistently, an increased susceptibility of *OsMADS26* OX lines to *M. oryzae* was also observed in the nethouse experiments, whereas the tested *OsMADS26*-down-regulated lines presented a reduction of susceptible lesions compared with the DR0 control (Supplemental Fig. S3). This shows that the negative regulation of *OsMADS26* on the resistance mechanisms to *M. oryzae* can be observed at different developmental stages, with different virulent isolates and independent of the growth conditions. It is interesting to stress that there is a coincidence between the tissue localization of *OsMADS26* transcripts and the cell barriers that pathogens have to cross in the plant (Fig. 2). For instance, *OsMADS26* is expressed in the epidermis, a barrier that *M. oryzae* has to cross to perform its lifecycle. Transcripts of *OsMADS26* also accumulated in cells around the vessels where *X. oryzae* pv *oryzae* develops. To our knowledge, this is the first report of the involvement of a *MADS* gene in disease resistance in plants. The resistance of rice against RYMV was not affected by *OsMADS26* DR. Resistance

against bacteria and fungi on the one hand and virus on the other hand involves different mechanisms, such as RNA silencing for the latter and pathways producing antimicrobial molecules for the former. Thus, *OsMADS26* negatively participates in resistance to a wide range of rice pathogens but not to RYMV.

Other than this strong effect on biotic stress resistance, the *OsMADS26*-down-regulated lines showed an increased ability to maintain their RWCs under soil water deficit and recover from a severe drought stress as well as a better capacity to maintain yield in drought conditions in the field (Figs. 7 and 8; Supplemental Fig. S6). The preferential localization of *OsMADS26* transcripts (Fig. 2) in peripheral tissues, such as epidermis and bulliform cells in leaves and exodermis in roots, supports a role for this transcription factor in the response mechanism to environmental clues. To our knowledge, *OsNAC6* (for NAM [no apical meristem], ATAF [Arabidopsis transcription activation factor], CUC [cup-shaped cotyledon]) and *OsNAC10* are the only transcription factors for which the deregulation had a joint benefit on both biotic and abiotic stress tolerances (Nakashima et al., 2007; Sun et al., 2013). *OsNAC6*-overexpressing rice plants showed an improved tolerance to dehydration and high-salt stresses as well as increased tolerance to blast disease. However, constitutive overexpressers also exhibit growth retardation and low reproductive yields in contrast to *OsMADS26*-down-regulated lines that presented only discrete developmental changes.

OsMADS26 Alters the Transcription of a Wide Range of Biotic and Abiotic Stress-Related Genes

We showed that the expression of a set of defense genes is lower in OX *OsMADS26* lines than in the control before and after inoculation with a virulent isolate of *M. oryzae* (Fig. 6). This was confirmed by microarray analysis (Supplemental Table S1), where several other genes coding for pathogenesis-related proteins were down-regulated in OX *OsMADS26* lines. Similarly, the expression of a set of drought resistance-related genes is higher in *OsMADS26* DR lines after the application of a water deficit (Fig. 7). This suggests a direct or indirect involvement of *OsMADS26* as a repressor of stress-responsive genes.

By using transcriptome analysis, we investigated whether the modified response to biotic and abiotic stresses was associated with a more global differential expression of stress-related genes before application of the stress itself. Using the Archipelago database referencing genes in rice involved in disease resistance (Vergne et al., 2008) or the drought-responsive genes data set (Minh-Thu et al., 2013), we could establish that a large proportion of the genes differentially regulated in down-regulated and overexpressing lines is known to be regulated by biotic (53%) or abiotic (41%) stresses. This was similar (49% and 39%, respectively) to what was found by Lee et al. (2008b)

after dexamethasone-induced OX of *OsMADS26*. Thus, these transcriptome analyses show that *OsMADS26* participates in the transcriptional regulation of defense-related genes. The low overlap with the data set obtained by Lee et al. (2008b) probably reflects the fact that we determined the genes regulated at steady-state levels after constitutive OX or DR of *OsMADS26* expression, whereas Lee et al. (2008b) identified the genes deregulated upon a sudden increase of *OsMADS26* transcription triggered by the dexamethasone induction treatment. Based on their transcriptome analysis, Lee et al. (2008b) stressed that *OsMADS26* may be involved in the regulation of genes involved in jasmonate and ethylene stress hormone biosynthesis. Here, we found that rice *LIPOXYGENASE8* (*Os08g39840*) is consistently up-regulated in DR lines and down-regulated in both OX *OsMADS26* lines and dexamethasone-induced *OsMADS26* lines (Lee et al., 2008b). This gene was reported to be regulated during the early stage of *M. oryzae* infection (Peng et al., 1994; Agrawal et al., 2004), by wounding (Marla and Singh, 2012), and during the senescence process (Kong et al., 2006). Two genes involved in ethylene biosynthesis, rice *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE3* (*Os09g27750*) and *ACIREDUCTONE DIOXYGENASE1* (*Os10g28350*), are down-regulated in OX *OsMADS26* lines. Rice *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE3* and *ACIREDUCTONE DIOXYGENASE1* are strongly up-regulated by ethylene and contribute to maintain elevated ethylene rate in stressed plants (Rzewuski and Sauter, 2008). Similarly, the ethylene-responsive *ETHYLENE RESPONSE FACTOR063* transcription factor (*Os09g11480*; Ma et al., 2013) was found to be down-regulated in OX *OsMADS26* lines, suggesting that these lines are impaired for ethylene biosynthesis and response.

Other stress-related transcription factors were found to be differentially regulated in OX and/or DR *OsMADS26* lines. *OsNAC103* (*Os07g48450*), known to be up-regulated by water deficit treatment, salt stress, and jasmonate (Fang et al., 2008; Nuruzzaman et al., 2012) was found to be up- and down-regulated in DR and OX lines, respectively. *OsNAC045* (*Os11g03370*) down-regulated in OX lines is up-regulated in response to salt or cold stress (Fang et al., 2008). *OsWRKY24* (*Os01g61080*) represses abscisic acid and GA signaling in aleurone cells (Xie et al., 2005; Zhang et al., 2009) and is induced by chilling stress (Yun et al., 2010). It is up-regulated in DR lines and down-regulated in OX lines. *OsWRKY53* (*Os05g39720*), down-regulated in OX lines, is induced by elicitors, jasmonate, and *M. oryzae* infection and during the *Xa21*-mediated resistance to *X. oryzae* pv *oryzae*. Its OX enhances rice resistance to *M. oryzae* (Chujo et al., 2007, 2014). Interestingly, we identified that *RH1* (*Os05g30500*) is up-regulated in the OX line. RH1 is a Negative Regulator of Resistance homolog that can interact with and inhibit NH1/*OsNONEXPRESSOR OF PATHOGENESIS-RELATED1*, which is a master regulator of defense genes and systemic acquired resistance (Chern et al.,

2012). The *Wall-Associated Kinase25* (*Os03g12470*) was down-regulated in OX plants. This is consistent with the published function of this gene as a positive regulator of *X. oryzae* pv *oryzae* resistance (Seo et al., 2011). Finally, the rice *ROOT MEANDER CURLING* (*Os04g56430*) receptor-like kinase known to be highly induced by salt treatment (Serra et al., 2013) was up-regulated in DR plants and down-regulated in OX plants. Whether OX or DR *OsMADS26* plants are more resistant to salt stress remains to be established.

Taken together, this shows that *OsMADS26* contributes to the regulation of several stress-related transcriptional and regulatory pathways and that its OX or DR impacts on the expression of a wide range of biotic and abiotic defense-related genes, which is consistent with the observed phenotypes of DR and OX lines.

Is *OsMADS26* a Hub for Stress Resistance Regulation in Plants?

Our data indicate that *OsMADS26* probably mainly acts as a negative regulator of stress response. This has also been reported for *OsMADS22* and *OsMADS55*, which act as negative regulators of the brassinosteroid response (Lee et al., 2008a). Whereas the DR of *OsMADS26* transcription upon rice blast infection (Fig. 3), irrespective of the virulence of the isolate, can constitute a basal defense response, its up-regulation during osmotic stress (Fig. 1) is more difficult to interpret. We propose that this up-regulation of *OsMADS26* could be part of a negative feedback loop that would dampen abiotic stress response.

Nevertheless, it cannot be excluded that *OsMADS26* might have both activating and inhibiting activity on stress response genes depending on posttranslational modifications or interaction with other regulatory proteins. Indeed, MADS-box proteins are combinatorial transcription factors, and their regulatory specificity is affected by the interaction with other DNA binding or accessory factors (Messenguy and Dubois, 2003). In this context, *OsMADS26* could be a hub that integrates different signals, contributes to a short-term activation of defense mechanisms, and becomes, afterward, partly responsible for their cancellation. In this respect, it will be interesting to identify the proteins that can interact in vivo with *OsMADS26*.

CONCLUSION

Our data show that *OsMADS26* is a negative regulator of different stresses of major agronomical importance in rice. They also represent the description of a new range of functions for *MADS* genes in plants and open the door toward the achievement of drought-tolerant and disease-resistant plants. To reach this goal, it will be very interesting to identify in rice tilling population plants with *OsMADS26* null alleles and test their resistance against stresses. These alleles could be introduced in future breeding programs.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Dehulled and surface-sterilized seeds of rice (*Oryza sativa*) 'Nipponbare' were incubated in sterile distilled water in a growth chamber (16 h of light per day; $500 \mu\text{E m}^{-2} \text{s}^{-1}$; $28^\circ\text{C}/25^\circ\text{C}$ day-night cycle) for 2 d at 25°C . Imbibed seeds were transferred in square petri dishes ($245 \times 245 \text{ mm}$; CORNING; seven seeds per dish) containing 250 mL of one-half-strength Murashige and Skoog (DUCHEFA) medium (MS) solidified with 8 g L^{-1} agarose type II (SIGMA). These dishes were transferred and placed vertically in a growth chamber at 28°C under 16 h of light. Roots and shoots of 7-d-old seedlings were collected and used for in situ hybridization and RNA isolation for qRT-PCR or transcriptome analyses. Salt and osmotic stresses were applied by supplementing the culture medium with 150 mM NaCl (DUCHEFA) or 100 mM mannitol (DUCHEFA), respectively.

Plants were grown in 3-L pots filled with EGO 140 Soil Substrate (TREF; www.trefgroup.com) in a containment greenhouse (16-h-light/8-h-dark cycles at 28°C – 30°C). For plant phenotyping, the plants belonging to the different lines were randomly distributed in the greenhouse. Twenty DAG, plant height and tiller number were measured once a week until the early flowering stage. The latter stage was defined as the date when the first spike emerges from the flag leaf sheath on a plant. The flowering date corresponds to the date when spikes are observed on 50% of the tillers of a plant. After harvesting, the dry weight of the aerial part of the plant part was determined after drying the plant tissues at 70°C for 96 h. Panicles of each plant were also individually weighted after a drying treatment at 37°C for 3 d. The 1,000-seed weight was evaluated using seeds borne by the master tiller panicle. This experiment was repeated twice using three plants per line.

Specific culture conditions used for evaluation of pathogen and drought tolerance are detailed in the corresponding sections.

Plasmid Construction for Plant Transformation

The isolation of *OsMADS26* (*Os08g02070*) cDNA from rice 'Nipponbare' was achieved by qRT-PCR. Total RNA was extracted from 100 mg of leaf tissue of 7-d-old seedlings grounded in liquid nitrogen using 1 mL of TRIzol (INVITROGEN) following the recommendation of the supplier. A PCR amplification was performed with a couple of specific primers designed in the 5' and 3' UTR of *OsMADS26* (Supplemental Fig. S7). The amplified cDNA was cloned using the pGEM-T Easy Cloning Kit (Promega). From the cDNA, further PCR reactions were done using specific primers to amplify a 215-bp fragment located in the 5' UTR of *OsMADS26* named gene sequence tag1 (GST1) and a 321-bp fragment comprising the end of the last exon and the major part of the 3' UTR region named GST2 (Supplemental Fig. S7). PCR cycling conditions were 94°C for 4 min (1 cycle) and 94°C for 1 min, an annealing step at various temperatures depending on the melting temperature of the primers used (typically melting temperature of -5°C) for 1.5 min, and 72°C for 1 min (35 cycles) with a 5-min final extension step at 72°C . PCR was performed in a final volume of 25 μL with 0.25 units of *Taq* polymerase in MgCl_2 -free buffer (PROMEGA), 2 mM MgCl_2 , 200 nM each dNTP, appropriate oligonucleotides (1 μM), and cDNA (2 μL) or pGEM-T-PC8 plasmid (10 ng). The BP-tailed *OsMADS26*-amplified cDNA was cloned with the Gateway BP recombinase (INVITROGEN) in a modified pCAMBIA 1300 binary vector for OX named PC5300.OE, where the *Cdb* gene surrounded by the BP recombination sites was cloned between the constitutive promoter of ubiquitin gene from maize (*Zea mays*) and the terminator of the nopaline synthase gene from *Agrobacterium tumefaciens* (J.C. Breitler, unpublished data). After cloning, the presence of the *OsMADS26* cDNA in frame was ascertained by sequencing. The plasmid named PC5300.OE-PC8 was transferred into *A. tumefaciens* strain EHA105. For RNAi, the BP-tailed amplified GST1 or GST2 was cloned by BP recombination in the pDON207 Entry Plasmid (INVITROGEN) and transferred with the LR Recombinase (INVITROGEN) in the small interfering RNA binary plasmid pANDA (Miki and Shimamoto, 2004). The insertion of the GSTs in pANDA was controlled by sequencing. The resulting plasmids, named pANDA-DR5 and pANDA-DR3, were mobilized into *A. tumefaciens* strain EHA105 for plant transformation.

Transgenic plants were obtained by coculture of seed embryo-derived callus with *A. tumefaciens* strain EHA105 carrying the adequate binary plasmids following the procedure detailed in Sallaud et al. (2003). Single-locus and homozygous T2 lines were selected on the basis of the segregation of the antibiotic resistance gene carried by the T-DNA and Southern-blot analysis.

The expression of *OsMADS26* in selected transgenic lines was analyzed by qRT-PCR using specific primers (Supplemental Table S1).

Real-Time qRT-PCR Analysis

Total RNA was extracted from 100 mg of grounded leaf tissues with 1 mL of TRIzol (INVITROGEN) following the recommendation of the supplier; 2 μg of RNA was treated by RQ1 DNase (PROMEGA) to remove residual genomic DNA. The first strand cDNA synthesis was performed in 20 μL of final volume using the kit Superscripts III (INVITROGEN) following the manufacturer's instructions.

For qRT-PCR analysis, specific forward and reverse primers were designed to amplify a fragment of 200 to 400 bp in the 3' UTR of each studied gene using the Vector NTI (version 10.1) software with default parameters. Primer sequences are given in Supplemental Table S2. qRT-PCR was performed with a LighCycler 480 (ROCHE) using the SYBR Green Master Mix (ROCHE). The reaction was carried out in 96-well optical reaction plates (ROCHE). The reaction mix contained 7.5 μL of SYBR Green QPCR Master Mix (ROCHE), 250 nM each primer (forward and reverse), and 3 μL of 10-fold diluted cDNA template. All reactions were heated to 95°C for 5 min followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. Melt curve analysis and gel electrophoresis of the PCR products were used to confirm the absence of nonspecific amplification products. The primer efficiencies observed for the couples of primers used ranged between 1.86 and 2.05. Transcripts from *Expressed Protein (EXP; Os06g11070)* or actin (*Os03g50890*) genes were also detected and used as an endogenous control to normalize expression of the other genes. *EXP* or actin was chosen as the reference gene, because their expression seemed to be the most stable in different tissues and physiological conditions (Caldana et al., 2007). We verified that, in all of our experiments, the threshold cycle (C_t) value of the *EXP* and actin genes remained stable, irrespective of the treatment applied to the plants, and ranges between 26 and 28. Relative expression level was calculated by subtracting the C_t values for *EXP* or actin from those of the target gene (to give ΔC_t) and then $\Delta\Delta C_t$, and calculating $2^{-\Delta\Delta C_t}$ (Giulietti et al., 2001). Reactions were performed on technical triplicates from duplicated biological experiments.

In Situ Hybridization

For *OsMADS26* probe preparation, we used the same primers designed for *OsMADS26* qRT-PCR amplification (Supplemental Table S1). A 18S ribosome coding sequence was used as positive hybridization control and PCR amplified from cDNA using the primer couple Rib-Up (5'-CCGACCCTGATCTCTGTGAAGGG-3') and Rib-Down (5'-CAAGTCAGACGAACGATTTGCACG-3'). Primers containing the above specific sequences but extended at their 5' ends with the T7 RNA polymerase promoter sequence (5'-GCGAAATT AATACGACTCACTATAGGGAGA-3') were also designed and named *OsMADS26-T7-Up*, *OsMADS26-T7-Down*, RibT7-Up, and RibT7-Down. Finally, one primer corresponding to the T7 end was also designed and named E-T7 (5'-GCGAAATTAATACGACTCAC-3'). To generate sense and antisense probes, specific cDNAs were amplified by PCR with one primer Up and one primer T7-Down or with one primer Down and one primer T7-Up, respectively. These cDNAs were used to generate sense or antisense digoxigenin-labeled RNA probes by in vitro transcription using the T7 primer (T7 MAXIScript Kit; AMBION). Plant samples were fixed in 4% (v/v) paraformaldehyde in phosphate buffer (0.2 M, pH 7.5); inclusion, section preparation, and hybridization were done as previously described (Jabnoun et al., 2009). Sections were observed with a DM6000 (LEICA) Microscope under white light. Photographs were taken with a Retiga 2000R Camera (QIMAGING), and images were processed through Volocity 4.0.1 (IMPROVISION). In situ hybridization experiments have been conducted on the Plate-Forme d'Histocytologie et d'Imagerie Cellulaire Végétale (<http://phiv.cirad.fr/>) using microscopes of the Montpellier Rio Imaging platform (www.mri.cnrs.fr).

Microarray Hybridization and Analysis

For microarray hybridization experiments, total RNA was extracted from 100 mg of frozen leaves and roots after removal of the remaining seeds from 7-d-old seedlings using a RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. Residual genomic DNA was removed with the RNase-Free DNase Set (QIAGEN) during RNA purification. Two independent biological experiments were used for each studied plant line.

Microarray hybridization and data processing were carried out with Affymetrix Custom Service (AFFYMETRIX) by following the standard protocol for Affymetrix DNA chip as previously described (Coudert et al., 2011). The complete transcriptome data are accessible through Gene Expression Omnibus Series accession number GSE52640 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52640>). Expression values were normalized with the robust multiarray average method (Irizarry et al., 2003). Differential analysis and extraction of Affymetrix microarray analysis suite 5 call flags were done with linear models and empirical Bayes and Student's *t* tests relative to a threshold methods within affy and limma R packages (www.r-project.org; Gautier et al., 2004; Smyth, 2004; Smyth et al., 2005; McCarthy and Smyth, 2009). Raw *P* values were adjusted with the Benjamini-Hochberg method to control the false discovery rate (Benjamini and Hochberg, 1995). Empirical Bayes method with the Benjamini-Hochberg correction was kept for further analysis, because it allowed us to confirm the DRs and up-regulations of *OsMADS26* in the two replicates in the DR and OX lines, respectively. Orygenes DataBase (<http://orygenesdb.cirad.fr/>; Droc et al., 2006) was used to retrieve gene annotation corresponding to selected Affymetrix probes. Microarray control probe sets and probe sets without annotation were discarded for further analysis. Only probe sets with present detection call were kept for subsequent analysis. The two biological repetitions for each type of DR or OX transgenic lines were compared with the corresponding controls. A gene was considered significantly regulated if it presented a fold change ≥ 2 and a Benjamini-Hochberg-corrected $P \leq 0.05$ in at least two of the four different contrasts. Genes showing inconsistent regulations, such as (1) inverse regulation in two biological repeats of the same type of DR or OX line or (2) similar regulation in the two different types of DR and OX line, were discarded. A set of up-regulated genes from DNA chip analysis was confirmed by qRT-PCR analysis as previously described using specific primers (Supplemental Table S1).

Disease Resistance Assays

The GUY11 (Centre de Coopération Internationale en Recherche Agronomique pour le Développement Collection) or VT15 (Laboratoire Mixte International Rice Functional Genomics and Plant Biotechnology Collection) isolates of *Magnaporthe oryzae* were used for inoculation. GUY11 and VT15 isolates are compatible with rice 'Nipponbare' and generate moderate susceptibility symptoms. For the gene expression studies (Fig. 3), we used the fully virulent FR13 isolate and the avirulent isolate CL3.6.7 (Delteil et al., 2012). In laboratory, inoculations were performed on four- to five-leaf stage plantlets as described in Berruyer et al., 2003; rice japonica 'Maratelli' was used as a susceptible control in the experiments in addition to the studied transgenic lines. The data presented are representative of data obtained from three independent replicated experiments. For gene expression studies (Fig. 3), we used the fully virulent FR13 isolate and the avirulent isolate CL3.6.7 (Delteil et al., 2012). Leaves were collected before and after inoculation in liquid nitrogen and used for RNA extraction and qRT-PCR analysis to measure the expression level of different defense genes using specific primers (Supplemental Table S2).

For nethouse experiments in Vietnam, plants were grown in pots (28 L) filled with organic soil (10 kg by pots) and supplemented with nitrogen (2 g by pots) 3 and 9 weeks after planting. After germination in water, plants were planted (five plants by pots and one pot by line) following a randomized design, where OX, DR, and control lines were interspersed with cv Maratelli and cv Saricelick susceptible lines. Plants were grown in a nethouse and natural conditions and irrigated permanently to saturation. After 6 weeks of growth, plants were sprayed twice a week during 6 weeks using a fresh *M. oryzae* VT15 isolate spore solution (500,000 spores mL⁻¹ and 1% [w/v] gelatin). Symptoms were observed 15 weeks after sowing. Leaves were collected and scanned, and the number of susceptible lesions was numbered according to Berruyer et al., 2003.

Resistance assays against *Xanthomonas oryzae* pv *oryzae* were carried out on 8-week-old rice plants. The *X. oryzae* pv *oryzae* strain PXO99A (Salzberg et al., 2008) was inoculated using the leaf-clipping method as previously described (Kauffman et al., 1973). The data presented are representative of two independent experiments. Before inoculation and after symptom development, infected leaves were collected in liquid nitrogen and used for RNA extraction and qRT-PCR analysis to measure the expression level of different defense genes using specific primers (Supplemental Table S2).

For resistance assay against RYMV, 10 plants per line were inoculated by finger rubbing the leaves in presence of Carborundum (600 mesh) with purified RYMV particles at a concentration of 100 μ g mL⁻¹ as previously described (Quilis et al., 2008). Virus accumulation in tissues was measured by ELISA

analysis using an antibody against the RYMV coat protein (N'Guessan et al., 2000). Presented data are representative of two independent replicated experiments.

Resistance Assay to Water Deficit

Plants were germinated directly in soil and grown in the greenhouse. Each pot was filled with EGO 140 soil Substrate (TREF; www.Trefgroup.com), planted with five seedlings, and watered with the same volume of water. After 1 month, plants were subjected to 18 d of withholding water followed by 15 d of rewatering. Drought tolerance was evaluated by determining the percentage of plants that survived or continued to grow after the period of recovery. This experiment was performed using 20 plants per line and repeated three times.

During the water stress period, the RWCs of plants were monitored using a 7-cm-long segment of the last expanded leaf in a random set of five plants per line according to Barr and Weatherley, 1962. The other leaves were also harvested, frozen in liquid nitrogen, and stored at -80°C for RNA extraction and qRT-PCR analysis of stress-related genes expression using two plants per line exhibiting closest RWCs. qRT-PCR analysis was conducted as described earlier with specific primers of genes identified as drought and high salinity stress markers in rice: *RAB21*, a rice dehydrin (AK109096), and *SALT* (AF001395) genes (Claes et al., 1990; Oh et al., 2005). The primer sequences used are given in Supplemental Table S1.

Upland field experiments were carried out under a confined rain-out shelter field facility at the International Center for Tropical Agriculture. This field trial was laid out in a randomly complete block design with three replicates. Drought stress was imposed from panicle initiation (56 d after direct seeding) and continued approximately 3 weeks or until severe leaf rolling and wilting appeared in nontransgenic controls. Then, the plants were rewatered until physiological maturity. The intensity of drought was monitored through volumetric soil water. Leaf rolling scores were recorded on a one to nine International Rice Research Institute (IRRI) scale standardized for rice. The following agronomic traits were scored according to the criteria established in the Standard Evaluation System for Rice (IRRI, 2002): plant height (centimeters), single-plant dry biomass (grams), and single-plant yield were recorded. The degree of relative chlorophyll content in the fully expanded flag leaf was determined using an SPAD-502 Chlorophyll Meter (Minolta Co.) under stress at different stages of crop development. Chlorophyll-a fluorescence parameters were also measured using a Fluorpen FP100 Chlorophyll Fluorometer. Variable PSII fluorescence in the dark-adapted state (F_v)/maximum PSII fluorescence in the dark-adapted state (F_m) represented the maximal photochemical efficiency. Leaves were kept in the dark for 20 min before measurement. F_v/F_m was calculated with the following formula: $F_v/F_m = (F_m - \text{initial [minimum] PSII fluorescence in the dark-adapted state})/F_m$.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number OsMADS26 (Os08g02070).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Stability of *OsMADS26* overexpression or under-expression.

Supplemental Figure S2. Phenotype of *OsMADS26* overexpressing and down-regulated lines.

Supplemental Figure S3. Blast resistance evaluation of *OsMADS26* lines in field conditions.

Supplemental Figure S4. Blight resistance evaluation of *OsMADS26* lines.

Supplemental Figure S5. Rice Yellow Mottle Virus resistance evaluation of *OsMADS26* lines.

Supplemental Figure S6. Drought resistance evaluation of *OsMADS26* lines in field condition.

Supplemental Figure S7. *OsMADS26* cDNA, GST1, and GST2 position and primer sequences used for PCR amplification.

Supplemental Table S1. Microarray values and analysis.

Supplemental Table S2. Primers used for RT-qPCR.

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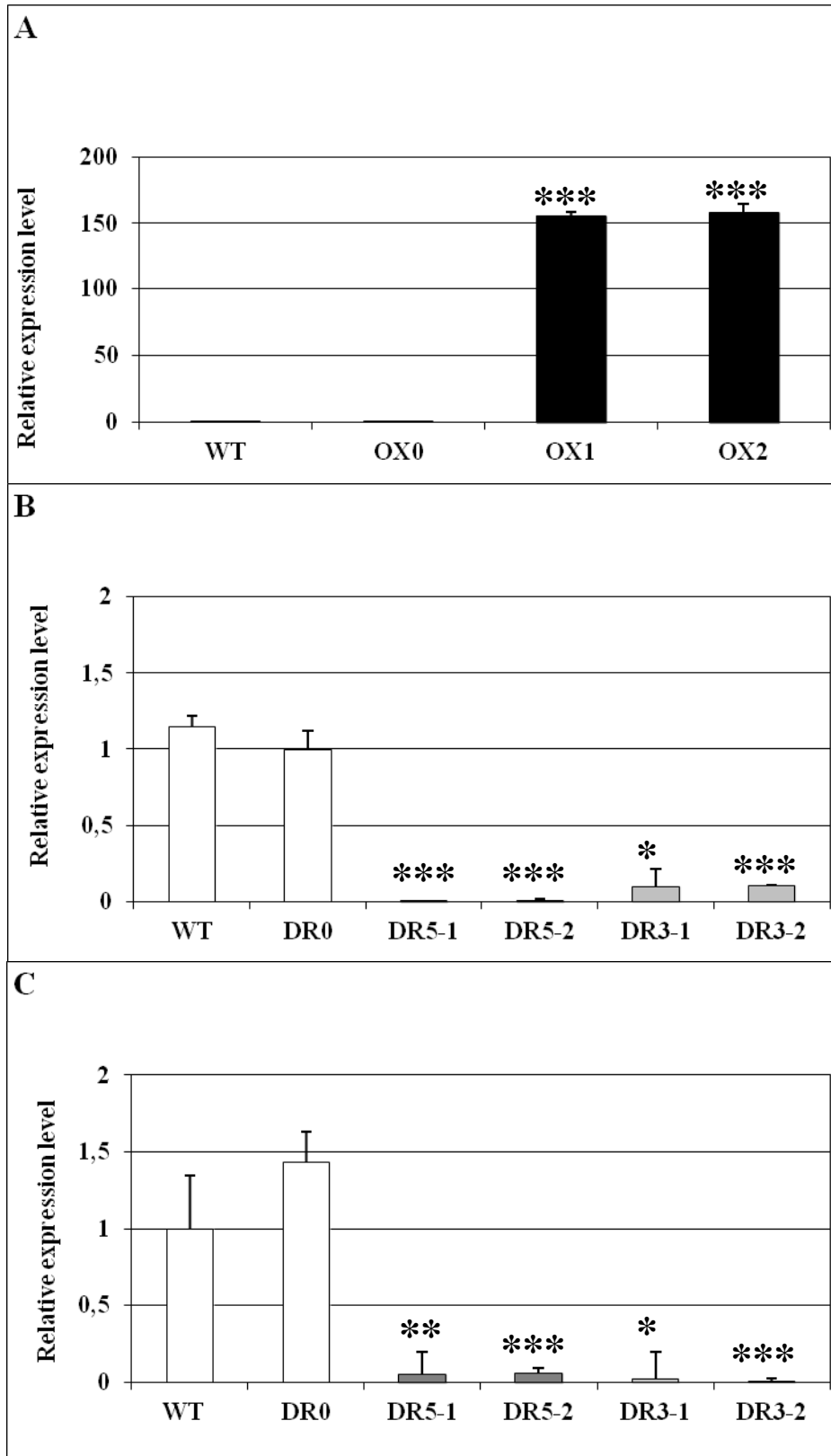


Figure S1: *OsMADS26* over- or down-expression is stable across generations

A, *OsMADS26* expression in overexpressing (OX1, OX2, dark bars) and corresponding control (OX0, WT, white bars) T4 plants. B, *OsMADS26* expression in interfered (DR5-1, DR5-2, DR3-1, DR3-2, grey bars) and corresponding control (PDP, WT, white bars) T4 plants. Mean value and standard error were obtained from two independent experiments. C, *OsMADS26* expression levels in RNA interfered (grey bars) and control (white bars) of 7-day-old T2 seedlings cultivated on MS/2 medium added with 125 mM of Mannitol. Mean and standard error were obtained from 14 individual plants of each line. A Student t-test was done to establish whether the RWC or the gene expression level in transgenic lines was different from corresponding control line; *: significant difference with $p < 0.05$; **: significant difference with $p < 0.01$; ***: significant difference with $p < 0.001$.

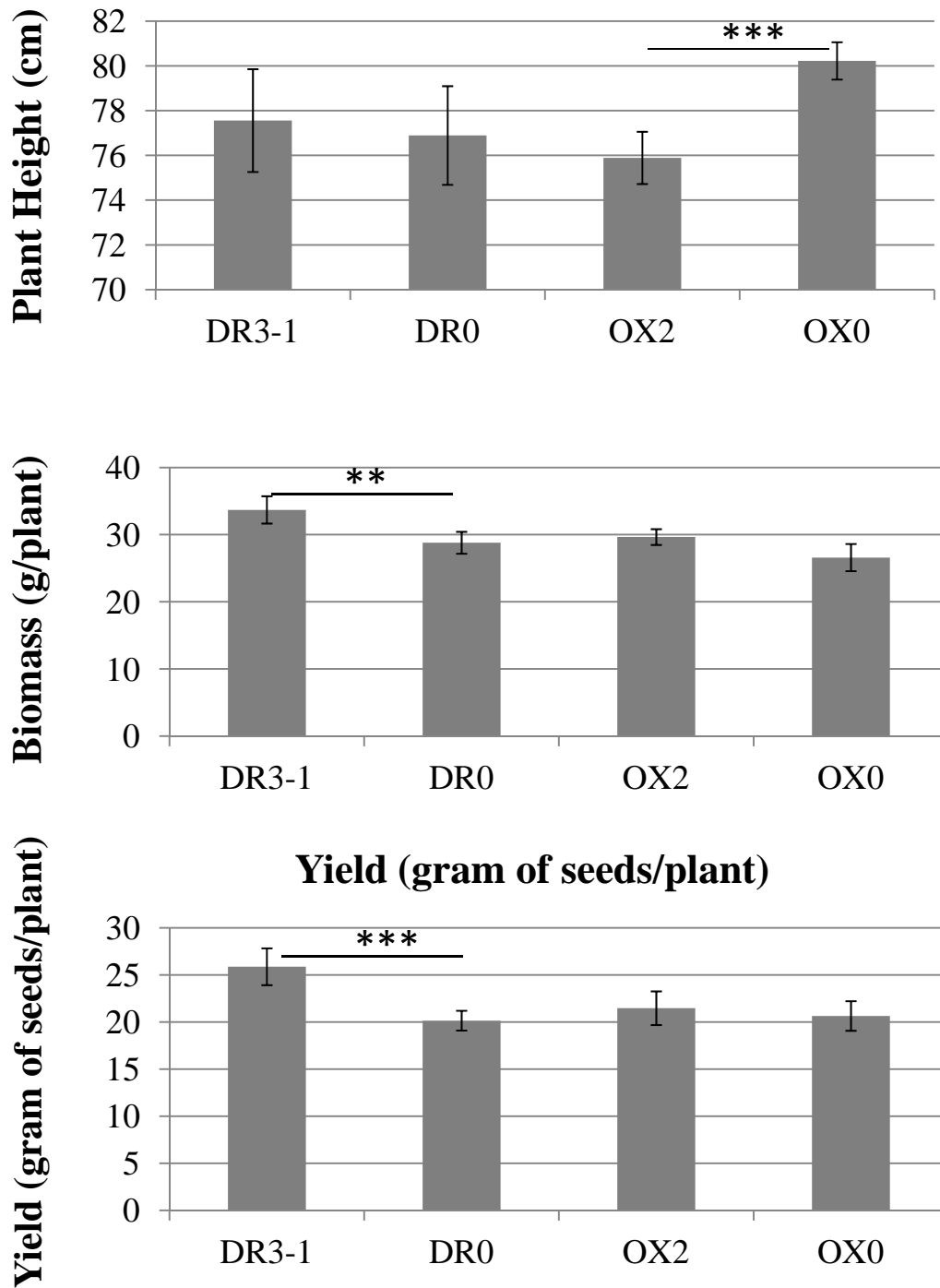


Figure S2: *OsMADS26* over-expressing and down-regulated lines growth under normal watering condition in the field.

Plants were grown under normal water condition in the field in CIAT (Colombia). The height, biomass and yield were measured at the end of the experiment. The mean and SD are shown and a T-test (n=9; **: P<0.01; ***: P<0.001) was used to evaluate statistical difference between the over-expressing OX2 and down-regulated DR3-1 transgenic lines with their respective controls OX0 and DR0.

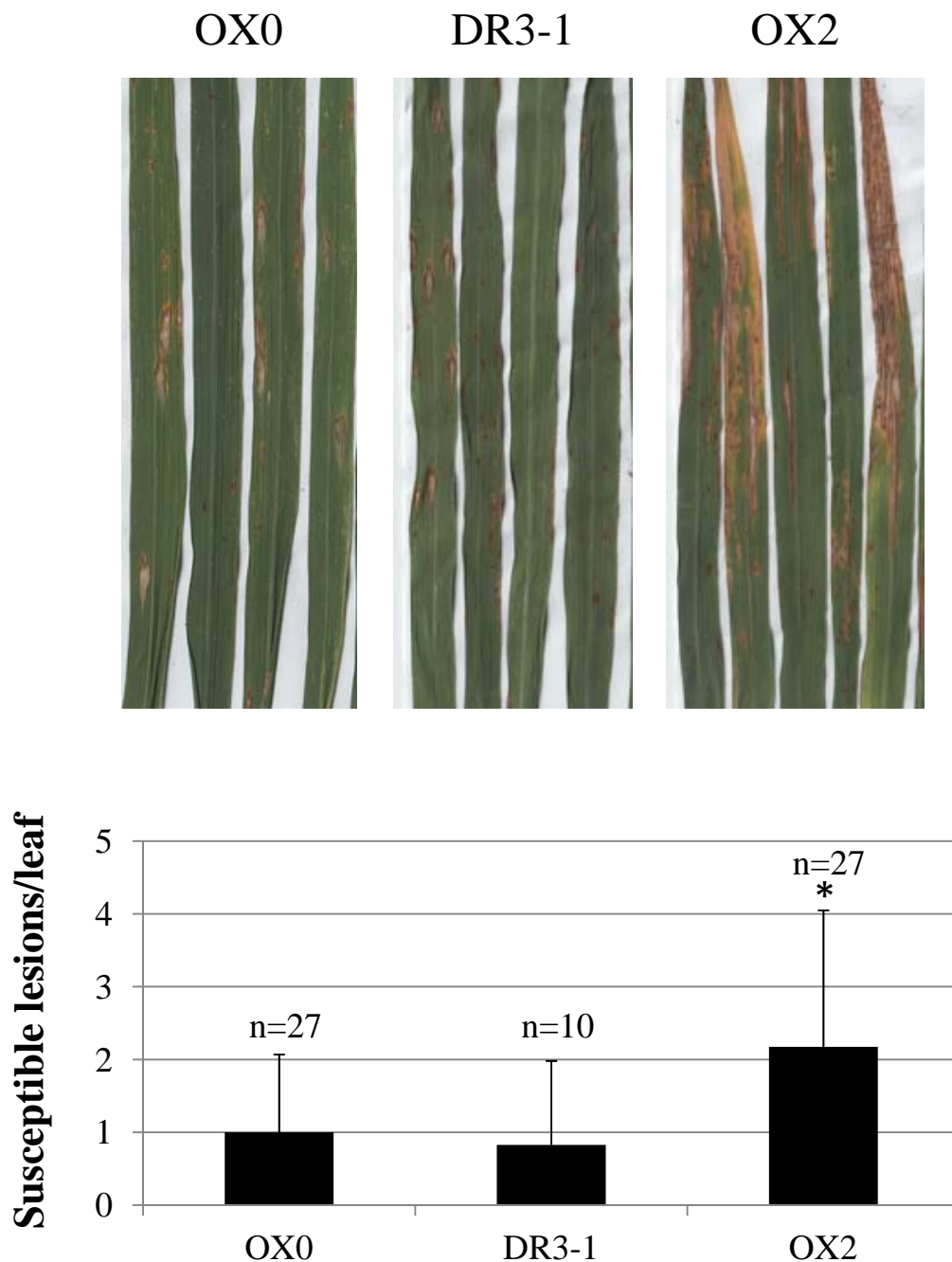


Figure S3: Rice blast resistance evaluation of over-expressing or down-regulated *OsMADS26* lines under semi-controlled field conditions.

Plants were grown in nethouses in LMI-RICE (Hanoi, Vietnam) and inoculated each week for four weeks with spores of the virulent *M. oryzae* isolate VT15. Symptoms were measured every week after epidemics started and one time point is provided. The greyish lesions were counted as a measure of susceptibility. The mean and SD are shown and a T-test (*: $P < 0.05$) was used to evaluate statistical difference between the *OsMADS26* over-expressing OX2 and down-regulated DR3-1 transgenic lines with their respective controls OX0 and DR0.

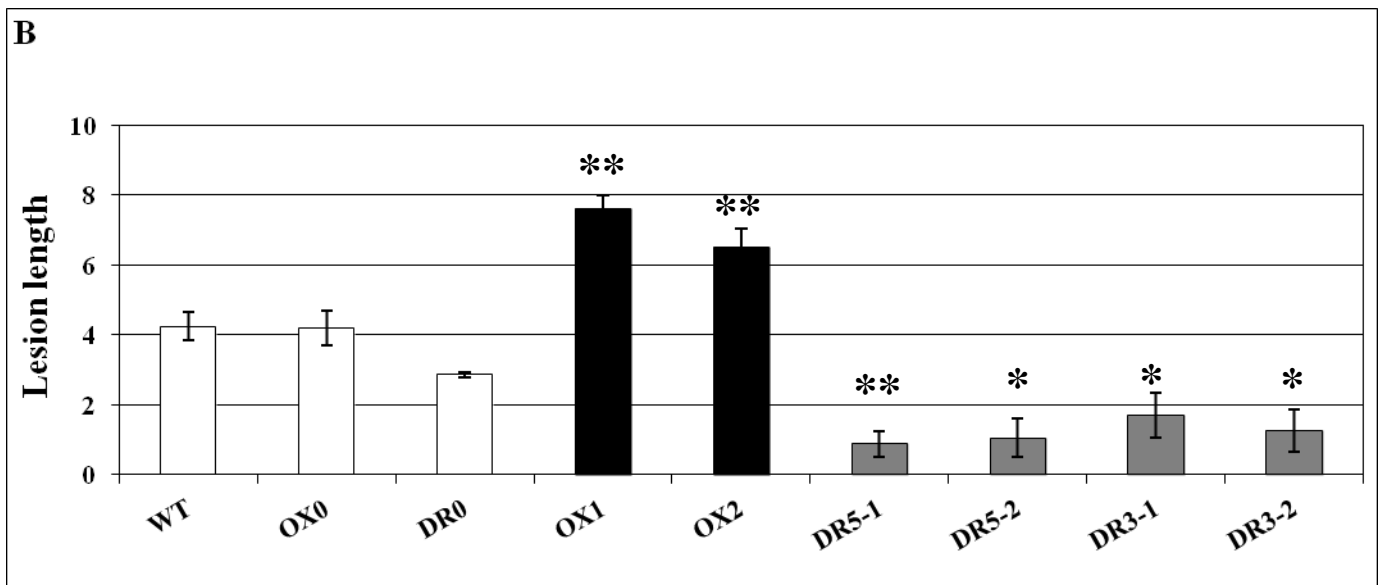


Figure S4: OsMADS26 negatively regulates resistance against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*).

Plants over-expressing (OX1, OX2) (black bars) or down-regulated (DR5-1, DR5-2, DR3-1, DR3-2) (grey bars) *OsMADS26* and corresponding control lines transformed with empty vectors (OX0, DR0) or untransformed line (WT) (white bars) were tested. A: Symptom severity in leaves of transgenic and control plants inoculated with the PXO99A strain of *Xoo*. Photographs were taken at 14 days post inoculation (dpi). B: Length of lesion produced in *Xoo*-infected leaves at 14 dpi. Mean and standard error were obtained from nine inoculated plants for each line. Results shown are from one of two independent experiments that produced similar results.

A Student t-test was done to establish whether one given mutant line was different from its corresponding control line; *: significant difference with $p < 0.05$; **: significant difference with $p < 0.01$.

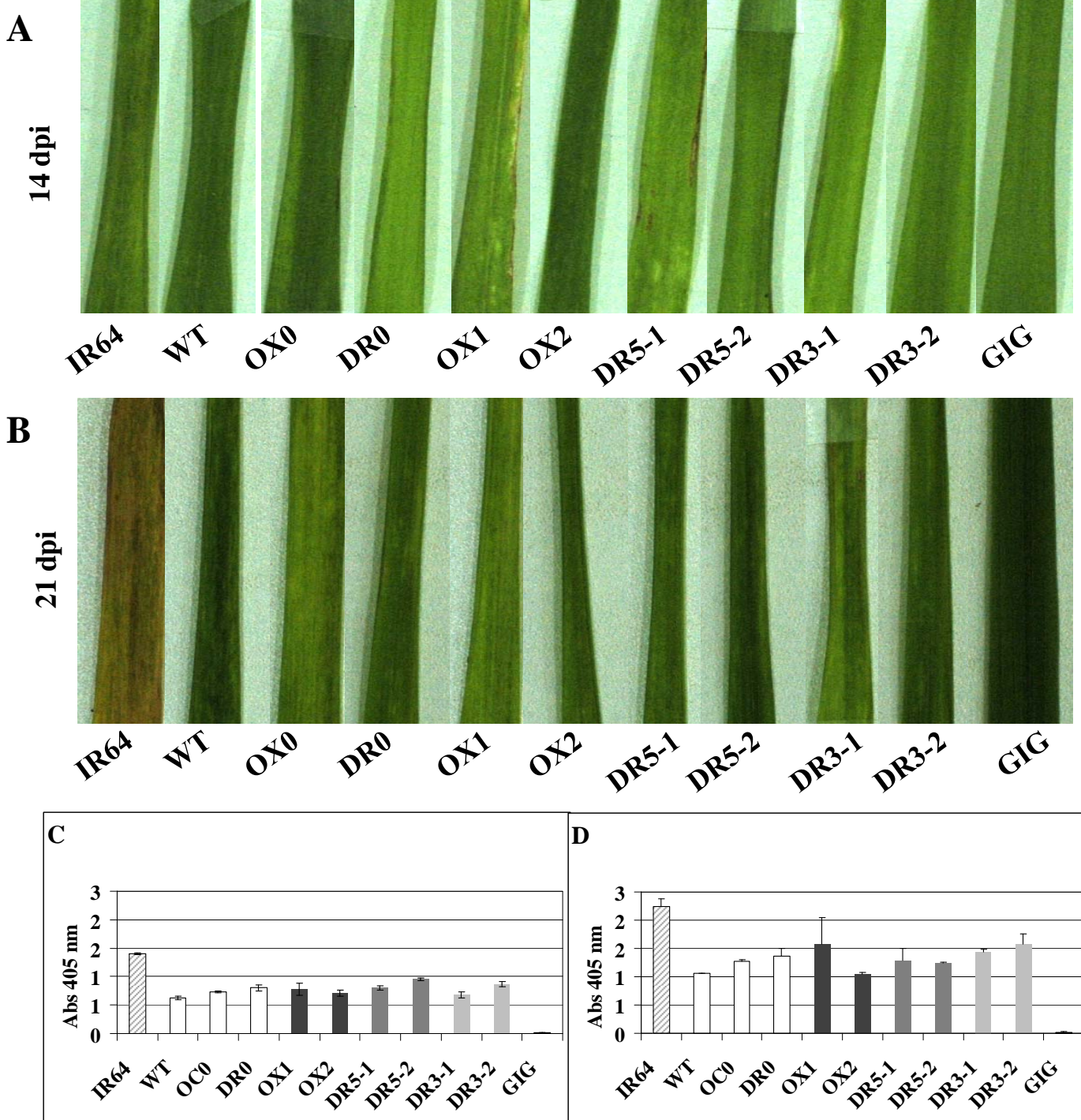


Figure S5: *OsMADS26* expression level does not affect resistance against Rice Yellow Mottle Virus (RYMV).

Nine independent lines of over-expressing (OX1, OX2, black bars), down-regulated (DR5-1, DR5-2, DR3-1, DR3-2, grey bars) *OsMADS26* lines and corresponding control lines transformed with empty vectors or untransformed line (OX0, DR0 WT, white bars), IR64 (susceptible control, dashed bar) and Gigante (resistant control) cultivars were tested. A,B, Symptom severity in leaves of transgenic and control plants inoculated with RYMV at 14, and 21 days postinoculation (dpi). C,D, ELISA virus accumulation quantification in leaves of transgenic and control plants inoculated with RYMV at 14 and 21 (dpi). WT and control transformed with empty vectors (white bars), over-expressing lines (black bars), down-regulated lines (grey bars) and reference cultivars (dashed bars) Gigante (GIG), and IR64. Leaves from ten plants for each line were pooled and the virus content determined by enzyme-linked immunosorbent assay using an antibody generated against the coat protein as described (N'Guessan et al. 2000). Mean and standard error were obtained from ten inoculated plants for each line. Results shown are representative of data obtained from two independent experiments.

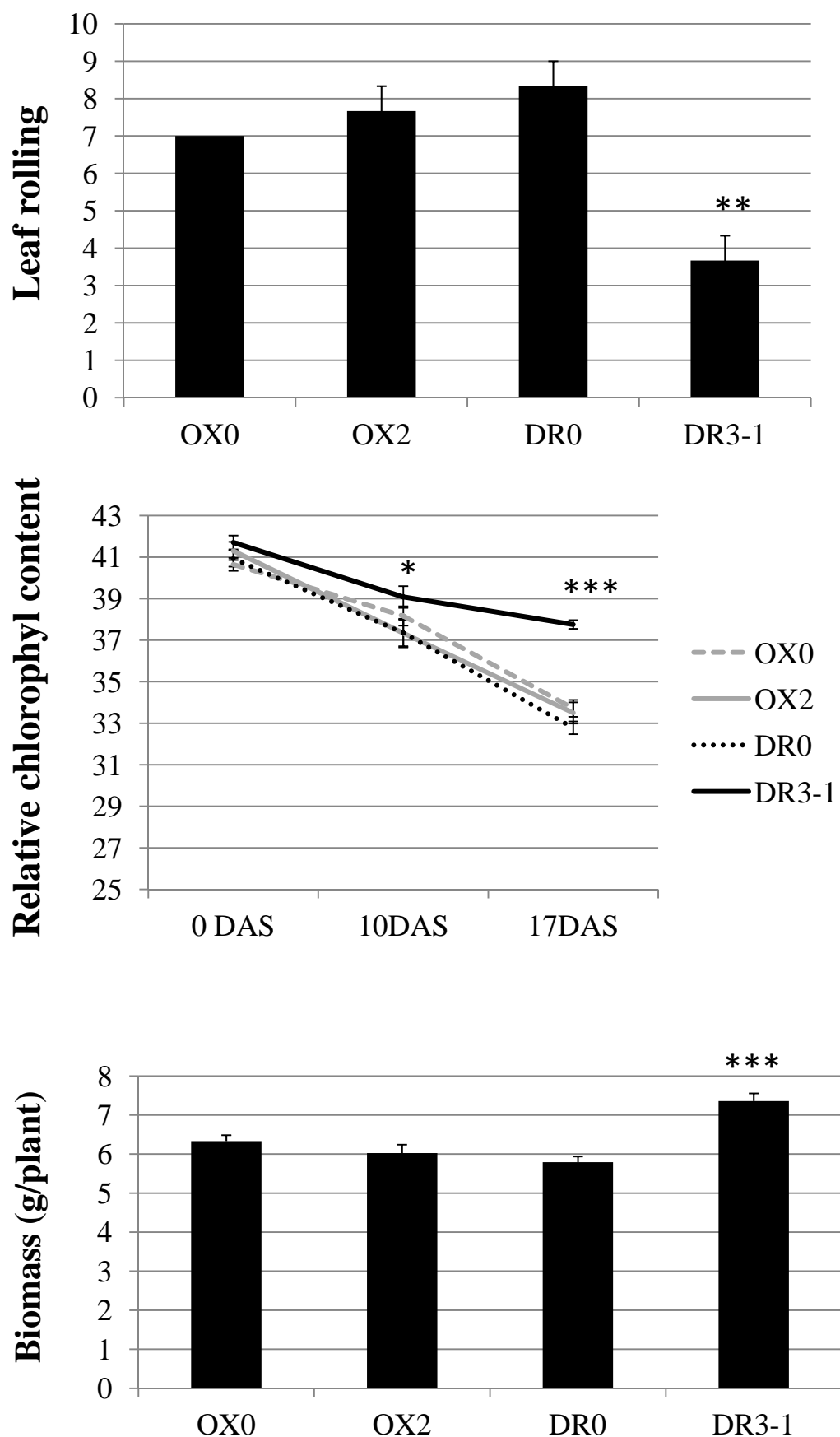


Figure S6: *OsMADS26* down regulation enhances water deficit tolerance in the field.

Plants were grown in the field in CIAT (Colombia) and a drought stress was applied (see Methods). The leaf rolling score (0-9 scale from the less to the more) of the plant 17 DAS (DAS= days after stress) is given (A) and SPAD value (B) was measured at the indicated times after stress in three independent blocks on three plants. The total biomass was measured at the end of the experiment (C). The mean and SD are shown and a T-test (n=9; *: P<0.05; **:P<0.01; ***: P<0.001) was used to evaluate statistical difference between the over-expressor OX2 and interfered DR3-1 transgenic lines with their respective controls OX0 and DR0.

GST1 { gtaagcaagagatagggataaggg**GAAGAGGAGGAAGAAGGAGG**gaggtgtagggaga
aaccggagcaacctcgaagctagtccaaactagtgaggaggtgtctttccggcaagccggagc
ccatagctaggtagacaggaggagaggaggaagaagggggagaggagacttatcttgatcg**ATG**gcg
 cgaggcaaggtgcagctccgtcgcacgcagaaccgggtc**ACCGTCAGGTCACCTTCTGCAA**
 gcgccgtgccggcctgctgaagaaggccaggagctctccatcctctgcgaggccgacatcggcacatcat
 cttctccgccacggcaagctctacacctcgcaccaccggaacatggaggagctgatcgagaggtacaa
 gagtgctagtggcgaacaggccaacgcctgcggcgaccagagaatggacccaaaacaggaggcaatggt
 gtcacaacaagaaatcaatctactgcagaaggcctgaggtacatctatgggaacagggcaaataacaca
 tgactgttgaagagctgaatgcctagagaggtacttagagatatggatgtac**AACATTCGCTCCGC**
AAAGATGCagataatgatccaagagatccaagcactaaagagcaaggaaggcatggtgaaagctgcta
 acgaaattctccaagaaaagatagtagaacagaatggctgatcgacgtagg**catgatgtagcagatcaac**
agaatgggcatttagtacagtcccactgttagaagagatcacta**accactgactatactgagtggctattcta**
ctttaggggctcggagatgggctattccttcTAAcactaataatggcctgggggatacttgtgttcattacta
 gtgtgtaatatggtaataatgcttgtgttctgtttgctttgctattctgatgtacctatttagacaagttccg
caggaagtgtcttttagtattgtattgtcttgggctgtggtgctttgttttcc**CTAAAGAACTCTT**
GAGGAGCtctgttgtgaaccatttcaagtaattgagactattgtttcc

GST2 {

Primers used for *OsMADS26* cDNA amplification

Forward: 5'-gaagaggaggaagaaggagg -3'

Reverse: 5'-gctcctcaagagttctttag -3'

Primers used for GST1 amplification and cloning

1st Amplification

Forward: 5'-aagcaagagatagggataag -3'

Reverse: 5'-cgatcaagataagtctctc -3'

2nd Amplification (with *attB* sequence)

Forward: 5'-**ggggacaagtttgtacaaaaagcaggct**gaagaggaggaagaaggagg-3'

Reverse: 5'-**ggggaccactttgtacaagaaagctgggt**ccctcttctctctctcc -3'

Primers used for GST2 amplification and cloning

1st Amplification

Forward: 5'-tagtagaacagaatggtctg -3'

Reverse: 5'-gttgaaccatttcaagtaat -3'

2nd Amplification (with *attB* sequence)

Forward: 5'-**ggggacaagtttgtacaaaaagcaggct**catgatgtagcagatcaac -3'

Reverse: 5'-**ggggaccactttgtacaagaaagctgggt**gctcctcaagagttctttag -3'

Figure S4: Sequence of *OsMADS26* cDNA, GST1 and GST2 position in 5' and 3'-UTR and primer sequences used for PCR amplification.

In bold: GST sequences cloned in pANDA vector and used for RNA interference induction; underlined: nested primers used for amplification of GST1 and GST2; Underlined capitals: primers used for the amplification of the cDNA sequence cloned in PC5300.OE vector for *OsMADS26* overexpression; Capitals: primers used for the analysis of *OsMADS26* expression by RT-qPCR in transgenic plants. In italic: Open reading frame (ORF), in italic, capital and bold: start and stop codons. In grey: BP recombination sequence (gateway cloning technology of INVITROGEN).

1 **Table SII: Primers used for RT-qPCR gene expression studies**

2

Name	Gene	Function	Forward	Reverse	Reference
<i>Actin</i>	Os03g50890	Actin	GCGTGGACAAAGTTTTCAA CCG	TCTGGTACCCTCATCAGGCAT C	-
<i>CHI7</i>	Os06g51050	chitinase	CAATGCACACGAGATTGTG A	CCGCATTGTGTTAACGTCCA	Kaku et al, 2006
<i>PR5</i>	Os08g04580	CsAtPR5, putative, expressed	TTGGCTTCTGTCTGCTTGA A	AGCTGCATCAACCATGCTAA	-
<i>EXP</i>	Os06g11070	Expressed protein	TCCATCTGCTCCCGTTGTT GTG	AAAGAGTTCGCCACCAACCGT C	(Caldana et al., 2007)
<i>NHI</i>	Os01g09800	Regulatory protein NPR1, putative, expressed	CCTGATGGTTGCCTTCTGT C	ATTCAAGCACTTGTATTACAC CTC	(Chern et al., 2005)
<i>OsFLS2</i>					
<i>OsMADS26</i>	Os08g02070	Transcription factor activity	GCTCGGAGATGGCTATTCCTTC	GACACTTCCTGCGGGAACCTG TC	(Shinozuka et al., 1999)
<i>PBZ1</i>	Os12g36880	Probenazole induced protein PBZ1/PR10	CCGGGCACCATCTACACC	CCTCGATCATCTTGAGCATGC	(Midoh and Iwata, 1996; Swarbrick et al., 2008)
<i>POX223</i>	Os07g48020	Peroxidase 2 precursor, putative, expressed	ACGACGCCCAACGCCTTC	CTTCCAGCAACGAACGCATCC	(Vergne et al., 2007)
<i>Rab21</i>	AK109096	Rice dehydrin	TGTGTGATCGGTGTTTCGA T	CCACACGCGCACTTACATAC	(Claes et al., 1990; Quilis et al., 2008)
<i>Salt</i>	AF001395	Salt-stress-induced protein	CCCCATTGTCTGTGTACGT G	GGGATTAGTTGCCCATGGAT	(Oh et al., 2005; Quilis et al., 2008)
<i>WRKY28</i>		Os06g44010	CGCCGATGAACTTTGCTC	CCACCTTGGCACGTGTAGA	Delteil et al, 2012

3

GST1 { gtaagcaagagatagggataaggg**GAAGAGGAGGAAGAAGGAGG**gaggtgtagggaga
aaccggagcaacctcgaagctagtccaaactagtgaggagttgtctttccggcaagccggagc
ccatagctaggtagacaggaggagaggaggaagaagaggggggagaggagacttatcttgatcg**ATG**gcg
 cgaggcaaggtgcagctccgtcgcacgcagaaccgggtc**ACCGTCAGGTCACCTTCTGCAA**
 gcgccgtgccggcctgctgaagaaggccaggagctctccatcctctgcgaggccgacatcggcacatcat
 cttctccgccacggcaagctctacacctcgccaccaccggaacatggaggagctgatcgagaggtacaa
 gagtgctagtggcgaacaggccaacgcctgcggcgaccagagaatggacccaaaacaggaggcaatggt
 gtcacaacaagaaatcaatctactgcagaaggcctgaggtacatctatgggaacagggcaaataacaca
 tgactgttgaagagctgaatgcctagagaggtacttagagatatggatgtac**AACATTCGCTCCGC**
AAAGATGCagataatgatccaagagatccaagcactaaagagcaaggaagggcatggtgaaagctgcta
 acgaaattctccaagaaaagatagtagaacagaatggctgatcgacgtagg**catgatggtagcagatcaac**
agaatgggcatttagtacagtcccactgttagaagagatcacta**accactgactatactgagtggctattcta**
ctttaggggctcggagatgggctattccttcTAAcactaataatggcctgggggatacttgtgttcattacta
 gtgtgtaatatggtaataatgcttgtgttctgtttgctttgctattctgatgtacctatttagacaagttccg
caggaagtgtcttttagtattgtattgtcttgggctgtggtgctttgttttcc**CTAAAGAACTCTT**
GAGGAGCtctgttgtgaaccattcaagtaattgagactattgtttcc

GST2 {

Primers used for *OsMADS26* cDNA amplification

Forward: 5'-gaagaggaggaagaaggagg -3'

Reverse: 5'-gctcctcaagagttctttag -3'

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Reverse: 5'-cgatcaagataagtctctc -3'

2nd Amplification (with *attB* sequence)

Forward: 5'-**ggggacaagtttgtacaaaaagcaggct**gaagaggaggaagaaggagg-3'

Reverse: 5'-**ggggaccactttgtacaagaaagctgggt**ccctcttctctctctcc -3'

Primers used for GST2 amplification and cloning

1st Amplification

Forward: 5'-tagtagaacagaatggtctg -3'

Reverse: 5'-gttgaaccattcaagtaat -3'

2nd Amplification (with *attB* sequence)

Forward: 5'-**ggggacaagtttgtacaaaaagcaggct**catgatggtagcagatcaac -3'

Reverse: 5'-**ggggaccactttgtacaagaaagctgggt**gctcctcaagagttctttag -3'

Figure S7 Sequence of *OsMADS26* cDNA, GST1 and GST2 position in 5' and 3'-UTR and primer sequences used for PCR amplification.

In bold: GST sequences cloned in pANDA vector and used for RNA interference induction; underlined: nested primers used for amplification of GST1 and GST2; Underlined capitals: primers used for the amplification of the cDNA sequence cloned in PC5300.OE vector for *OsMADS26* overexpression; Capitals: primers used for the analysis of *OsMADS26* expression by RT-qPCR in transgenic plants. In italic: Open reading frame (ORF), in italic, capital and bold: start and stop codons. In grey: BP recombination sequence (gateway cloning technology of INVITROGEN).