Running head: *OsMADS26*, a multiple hormone regulator

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Further characterization of a rice AGL12-group MADS-box gene, OsMADS26

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Plant MADS-box genes can be divided into 11 groups. Genetic analysis has revealed that most of them function in flowering-time control, reproductive organ development, and vegetative growth. Here, we elucidated the role of OsMADS26, a member of the AGL12 group. Transcript levels of OsMADS26 were increased, in an age-dependent manner, in the shoots and roots. Transgenic plants of both rice and Arabidopsis over-expressing this gene manifested phenotypes related to stress responses, such as chlorosis, cell death, pigment accumulation, and defective root/shoot growth. In addition, apical hook development was significantly suppressed in Arabidopsis. Plants transformed with the OsMADS26-glucocorticoid receptor (GR) fusion construct displayed those stress-related phenotypes when treated with dexamethasone (DEX). Microarray analyses using this inducible system showed that biosynthesis genes for jasmonate, ethylene, and reactive oxygen species as well as putative downstream targets involved in the stress-related process were up-regulated in OsMADS26-overexpressing plants. These results suggest that OsMADS26 induces multiple responses that are related to various stresses.
The MADS-box gene family encodes transcription factors with a conserved DNA-binding domain, called the MADS-box. These genes, ubiquitous in living organisms, have a wide range of functions. Plant MADS-box genes can be grouped into two evolutionary lineages (Types I and II) (Alvarez-Buylla et al., 2000; Becker and Theissen, 2003). When restricted to the putative functional MADS-box genes, this list includes about 100 and 70 genes in *Arabidopsis* and rice, respectively (Nam et al., 2004). Approximately 40 Type II MADS-box genes each have been identified in *Arabidopsis* (Kofuji et al., 2003; Parenicova et al., 2003) and rice (Lee et al., 2003); these can be divided into 11 groups (Becker and Theissen, 2003; Lee et al., 2003; Arora et al., 2007).

Detailed genetic analyses have shown that, whereas some MADS-box genes are involved in reproductive organ development (being preferentially expressed in the floral organs), others are expressed in the vegetative organs, where they perform various roles in flowering-time control, vegetative growth, and root development (Becker and Theissen, 2003). Becker and Theissen (2003) have speculated that AGL12 group genes originated before the gymnosperm–angiosperm split about 300 million years ago. In northern blot analyses, *AGL12*, the sole MADS-box gene from the AGL12 group in *Arabidopsis*, shows root-specific expression (Rounsley et al., 1995). Recently, *AGL12* overexpression analyses of suspension cells from *Catharanthus roseus* have demonstrated that this gene promotes the organization of those cells into globular parenchyma-like aggregates (Montiel et al., 2007). Loss-of-function analyses have elucidated that *AGL12* regulates root meristem cell proliferation and flowering transition (Tapia-Lopez et al., 2008). In addition, *in situ* hybridization analyses have shown that this gene is also detected in leaves and floral meristems. In rice, the AGL12-group *OsMADS26* is expressed not only in the roots but also in the shoots and panicles (Shinozuka et al., 1999). Pelucci et al. (2002) have also observed that *OsMADS26* is highly expressed in the leaves and inflorescences. Furthermore, Arora et al. (2007) have shown the expression of this gene in panicles and seeds. These results imply that this gene functions in a broad
range of rice organs.

Some MADS-box genes are active in aging-related processes. For example, transgenic plants expressing sense AGAMOUS-like 15 (AGL15) inhibit senescence programs in the perianth organs and developing fruits (Fernandez et al., 2000; Fang and Fernandez, 2002). FRUITFULL (FUL), SHATTERPROOF1 (SHF1), and SHATTERPROOF2 (SHF2) are involved in differentiation within the fruit dehiscence zone (Gu et al., 1998; Liljegren et al., 2000). When a tomato MADS-box gene that is most homologous to Short Vegetative Phase (SVP) is mutated, plants fail to develop abscission zones on their pedicels (Mao et al., 2000). Mutation in another tomato MADS-box gene, LeMADS-RIN, causes failure of fruit ripening (Vrebalov et al., 2002). Recently, the roles of rice SVP-group MADS-box genes in senescence responses have been reported as well (Lee et al., 2008). Here, we report the functional roles of OsMADS26 that is up-regulated in older tissues, and causes stress-related abnormalities when ectopically expressed.

RESULTS

Expression patterns of OsMADS26

We used semi-quantitative RT-PCR to study the expression patterns of OsMADS26 at various developmental stages (Fig. 1). In 5-day-old seedlings, this gene was more strongly expressed in the roots than in the shoots. Transcript levels rose as the plants aged (Fig. 1A). In the roots, transcription reached a maximum at Day 10 and remained at that level in older plants. However, in the leaves, transcripts continued to increase as the plants matured. Detailed analyses at broader developmental stages showed a dramatic rise in OsMADS26 transcripts in the roots between Days 6
and 9 (Fig. 1B). In contrast, transcript levels continuously increased in leaves up to Day 70 (Fig. 1C). Within individual plants, expression was much stronger in the mature leaves than in young, still-developing leaves (Fig. 1D). Therefore, these results indicate that *OsMADS26* is more active in older tissues.

**Phenotypes of *ubi:OsMADS26S* plants**

To elucidate *in vivo* functioning, we regenerated transgenic rice plants that expressed either sense or anti-sense constructs of the full-length *OsMADS26* cDNA. Plants that ectopically expressed the anti-sense *OsMADS26* showed no visible phenotypic changes (data not shown). We previously identified an *OsMADS26* knockout (KO) line (1A-16632) from a T-DNA tagging population via reverse-screening (Lee et al., 2003; Ryu et al., 2004). In that line, T-DNA is inserted into the first intron and the *OsMADS26* transcript is not present. As observed with our antisense plants in the current study, the T-DNA insertional knockout plants exhibited no abnormality in their growth habit (Supplementary Fig. 1).

In contrast, primary T1 transgenic plants expressing the sense *OsMADS26* transcript (*ubi:OsMADS26S* plants) showed several abnormal phenotypes (Fig. 2). Among our 50 regenerated plants, 40 died at the young stage, after they manifested such traits as defective root/shoot growth (Fig. 2A, B), chlorosis and cell death (Fig. 2A, B), screw-like root curling (Fig. 2C, D), and pigment accumulation in their roots (Fig. 2B, D). The remaining 10 plants showed less severe phenotypes and survived to maturity, with the adults displaying semi-dwarfism (Fig. 2E), pale-green coloration (Fig. 2E), spotted leaves (Fig. 2F), and shrunken seeds (Fig. 2G). Except for three lines, most of the plants were sterile. The T2 seedlings from those fertile lines had phenotypes similar to those observed from the primary transgenic plants, including retarded root/shoot growth, screw-like root...
curling, and pigment accumulation (Fig. 2H).

To check whether the phenotypes were induced by OsMADS26, we performed RNA-gel blot analysis of four representative lines: one that survived to maturity (Fig. 2E), two that developed roots and shoots but died at the seedling stage (Fig. 2B), and one that manifested more serious growth defects (Fig. 2A). In both roots and shoots, the levels of transgene expression were correlated with the severity of the phenotype (Fig. 2I), thereby suggesting that the phenotypes were due to OsMADS26 expression.

Because ubi:OsMADS26S plants developed phenotypes of severe growth retardation that is associated with various stresses, we tested whether this gene is regulated by signaling mediators. Wild-type (WT) plants were treated with 1 µM ACC, 10 µM MJ, or 1 mM H2O2 beginning at the germination stage; their shoots and roots were sampled 9 d after germination. Expression levels of OsMADS26 mRNA were not significantly changed (Supplementary Fig. 2). Moreover, no alterations were noted when 10-d-old rice seedlings were exposed to these molecules for 1, 3, or 24 h. Therefore, our results suggest that this gene is not regulated by such signaling molecules at the transcriptional level.

Furthermore, we examined behavior of the OsMADS26 knockout plants under various stresses. When 3-week-old plants were grown under water-deficit or high-salt (200 mM NaCl) conditions, they showed a degree of stress response similar to that of our WT controls (data not shown). We also investigated but found no visible phenotypic changes after treatment with 1 µM ACC, 1 mM H2O2, or 10 µM MJ (Supplementary Fig. 3).

**Phenotypes of plants expressing the OsMADS26 - GR fusion protein**

The phenotypes displayed by the ubi:OsMADS26 plants suggested that this gene might be involved...
in various stress-related processes. However, some of those characteristics may have been due to indirect effects caused by ectopic overexpression at the regeneration stage. To observe the more direct effects, we generated transgenic plants carrying the OsMADS26-GR fusion construct (ubi:OsMADS26GR plants).

Among the 32 T1 primary transgenics, 11 independent lines were examined to test whether this inducible system would be successful when plants were treated with dexamethasone (DEX). Six lines clearly showed abnormal phenotypes (Supplementary Fig. 4). For example, Line #33 developed curled and shorter roots while Line #17 had severe growth retardation. The six confirmed lines were followed through the next generations and genotyped to obtain homozygous (HO) plants from each line. For genotyping, at least 50 T2 seedlings were tested for hygromycin resistance. If all plants survived, the lines were regarded as HO; if all died, they were considered to be of the WT.

For further study, Line #33 was selected and its seedlings were treated with DEX in a dose-dependent manner to determine the effective concentration. In the WT segregants, DEX did not induce growth defects at up to 1 μM (Fig. 3A) while the transgenic plants showed growth limitations at the lowest concentration (10 nM) and severe retardation at 1 μM (Fig. 3B). Their shoots and roots were significantly shorter (Fig. 3A, 3B; Supplementary Fig. 5A, B), and purple pigments were accumulated in the transgenic roots (Fig. 3B, inset). We obtained the same results with Line #17 (data not shown). To understand the nature of these shortened roots, we sectioned their maturation zones. Histological analysis showed that cell elongation was significantly inhibited in the DEX-treated plants (Fig. 3E, F).

The numbers of emerged roots and leaves were also reduced in DEX-treated transgenic plants in both line #33 and #17. For example, by Day 9, WT segregants of Line #33 had developed their 4th leaves and had an average total of 8 to 10 roots (seminal plus nodal roots; Table I). When
HO plants of Line #33 were treated with DEX, root numbers were reduced at the lowest concentration (10 nM) while leaf numbers were reduced in response to 100 nM DEX. However, by Day 12, both WT and HO plants grown at 100 nM DEX had developed a similar number of leaves (about 4) and roots (about 10). These results suggest that the production of fewer roots and leaves associated with OsMADS26 overexpression was caused by a slower growth rate rather than because of defective primordia development.

To see the direct effects of this overexpression, we applied 1 µM DEX to six-day-old seedlings. When treated for three consecutive days, the transgenics manifested phenotypes of retarded growth, pigment accumulation by their roots, and wilting, chlorosis, and senescence in their shoots (Fig. 3C). To further understand the role of this gene in these processes, we measured chlorophyll amounts after DEX treatment. Whereas WT control plants did not exhibit any difference, HO plants showed a 41% decrease in chlorophyll a and a 12% decrease in chlorophyll b (Fig. 3G). We examined expression levels of two previously reported senescence-related genes, Osl2 (aminotransferase) and Osl55 (β-methylcrotonyl-CoA carboxylase) (Lee et al., 2001). Our ubi:OsMADS26GR plants treated with DEX expressed higher levels of these genes, suggesting that the senescence process had been triggered (Fig. 3H, I).

To study the role of OsMADS26 in mature plants, we treated 80-day-old ubi:OsMADS26GR glasshouse-grown plants with 10 µM DEX. After 7 d of treatment, abnormal phenotypes were revealed only in the transgenic plants, and included curled leaves, lesions, and chlorosis (Fig. 3D). In comparison, those transgenics treated with 1 µM DEX did not show any significant abnormality. Therefore, we can conclude that the phenotypes observed in the ubi:OsMADS26 plants were clearly re-enacted in our GR-inducible system, suggesting that OsMADS26 may causes plant stress.
Identification of putative *OsMADS26* downstream genes

To identify the *OsMADS26* downstream genes, we compared genome-wide RNA expression levels between the *ubi:OsMADS26GR* plants and their WT segregants, using a 60 K-oligo chip. Total RNAs were prepared from the roots of 7-day-old seedlings treated with 1 µM DEX for 3 or 9 h. Two independent lines (#17 and #33) were tested, which entailed four sets of microarray analyses: #17 (3 h), #17 (9 h), #33 (3 h), and #33 (9 h).

Supplementary Table I list the genes that were up- (146) or down-regulated (155) at least once and by a minimum of 1.5-fold in the *ubi:OsMADS26GR* plants. Pearson correlation co-efficiencies between the two replicates for these 301 selected target genes were 0.745, 0.735, 0.928, and 0.923 for #33 (3 h), #33 (9 h), #17 (3 h), and #17 (9 h), respectively (Supplementary Fig. 6), indicating that Line #17 generated more consistent results. When we applied a 2-fold difference as our cut-off criterion, 48 genes were identified, with respective Pearson correlation co-efficiencies of 0.806, 0.880, 0.937, and 0.861 for #33 (3 h), #33 (9 h), #17 (3 h), and #17 (9 h). Interestingly, this standard allowed us to identify only 13 down-regulated genes compared with the isolation of 35 up-regulated genes, which implies that results fluctuated more with the former type. Our KMC clustering analyses showed global expression patterns for these 48 genes (Fig. 4). All were induced or suppressed more strongly at 9 h than at 3 h. Moreover, 5 were induced dramatically at both 3 and 9 h (Fig. 4A) while 30 were weakly induced (Fig. 4B). Among the up-regulated genes, *OsMADS26* was the most highly expressed (Supplementary Table II and Fig. 4A).

The 301 genes with at least 1.5-fold differences in expression were classified according to their functioning via the Clusters of Orthologous Groups (COG; http://www.ncbi.nlm.nih.gov/COG/) analysis (Table II). Major up-regulated or down-regulated genes included those involved in defense mechanisms, signal-transduction, post-translational
modification/protein turnover/chaperones, carbohydrate transport/metabolism, and secondary metabolite transport/metabolism. Genes belonging to three groups were changed more frequently, i.e., defense mechanisms (2.40%), inorganic ion transport and metabolism (1.02%), and secondary metabolite transport/metabolism (1.08%). Genes related to secondary metabolites were more abundant in the up-regulated group whereas those involved in defense were more abundant in the down-regulated group.

**Transcript analyses of putative OsMADS26 downstream genes**

We chose eight genes to examine the reliability of our microarray data (Table III). Four iron/ascorbate family oxidoreductase genes were found in the up-regulated group, and could be divided into two groups: 1-aminocyclopropane-1-carboxylate (ACC) oxidase genes involved in ethylene biosynthesis (A09021902 and A05041211), and putative flavanone 3-hydroxylase genes (A05011009 and B10022103). From these, we selected one ACC oxidase gene (A09021902) and one flavanone 3-hydroxylase gene (A05011009) for further confirmation. The A05011009 protein showed high homology to gibberellin β-hydroxylase. We also identified a lipoxygenase gene (A09032318), an NADPH oxidase gene (B03011909), and the S-adenosylmethionine decarboxylase (SAMDC) gene (A10031622), which function in the biosyntheses of JA, reactive oxygen species (ROS), and polyamine, respectively. In addition, a MAP kinase gene (A05011217) involved in hormone signaling/biosynthesis and two harpin-induced protein genes (B05032110 and A03011404) were examined.

For semi-quantitative RT-PCR analyses of these eight selected genes, seven-day-old OsMADS26GR plants and their WT segregants were treated with 1 µM DEX for up to 9 h, and RNAs were prepared from their roots. The OsMADS26 and actin genes were included as controls.
As expected, OsMADS26 transcript was highly expressed in the transgenic roots, with that level increasing after DEX treatment (Fig. 5). Transcripts of these eight genes were induced post-treatment, and showed expression patterns similar to those obtained from the microarray analyses. Therefore, these data support the reliability of the microarray results.

The relationship between OsMADS26 and biosynthesis genes associated with stress responses

The phenotypes observed in our ubi:OsMADS26 and ubi:OsMADS26GR plants were broadly correlated with stress responses. Microarray analyses demonstrated the up-regulation of several genes for the biosynthesis of stress-inducing molecules such as ET, JA, ROS, and polyamine (Supplementary Table II).

In ET biosynthesis, ACC synthase and oxidase are the most important genes in mediating the final two steps. While none of ACC synthase genes was changed significantly, four ACC oxidase genes were up-regulated in three experimental sets. The JA biosynthesis genes include lipoxygenase, allene oxide synthase (AOS), allene oxide cyclase (AOC), oxo-phytodienoic acid reductase (OPR), and JA carboxyl methyltransferase (JMT) (Agrawal et al., 2004). Our microarray analyses showed that the following JA biosynthesis genes were up-regulated: OsLOX3, OsAOS1, OsAOS4, OsAOS5, OsOPR2, OsOPR12, and OsOPR13. Among the genes involved in ROS production, NADPH oxidase mRNA was clearly up-regulated, while a gene homologous to aldehyde oxidase was down-regulated. Regarding polyamine biosynthesis, only SAM decarboxylase was up-regulated while genes encoding ornithine decarboxylase, arginine decarboxylase, and spermidine synthase were not changed.

Phenotypes of 35S:OsMADS26 Arabidopsis plants
To further elucidate the role of OsMADS26, we utilized the Arabidopsis system, in which expression is under the control of the CaMV35S promoter. Of our 105 kanamycin-resistant T1 transgenic plants (35S:OsMADS26 plants), 11 developed severe dwarfism, chlorosis, and tilted leaves (Fig. 6A). Their growth was halted and they eventually died without developing reproductive organs. The rest of the T1 plants, which produced fertile seeds, were used for further analyses.

T2 segregants of these transgenic lines were analyzed phenotypically. Among the 11 independent lines examined, those from Lines #1, #8, and #11 displayed a wide range of abnormal phenotypes (Fig. 6B, C). Generally, their plants were smaller but had more lateral roots. Furthermore, Line 1 accumulated red pigments while Lines 8 and 11 developed twisted leaves. Plants from Line #11 were only about one-third as large as the WT, and they showed a delayed rate of leaf emergence. OsMADS26 transcripts were detected in all the plants with abnormal phenotypes.

To determine whether the abnormal phenotypes were induced by JA, we checked the expression levels of AtMyc2, VSP, and PDF1.2 (Bell and Mullet, 1993; Benedetti et al., 1995; Boter et al., 2004; Lorenzo et al., 2004; Penninckx et al., 1998) whose expressions generally are induced by that compound. Our analysis showed that transcript levels for VSP were increased in the transgenic plants with medium or strong phenotypes (Supplementary Fig. 7). In contrast, PDF1.2 transcripts were down-regulated in the transgenic plants in proportion to their phenotypic severity, whereas AtMyc2 expression was unaffected. Therefore, these results suggest that OsMADS26 controls the subsets of JA-inducible genes.

**Inhibition of apical hook development in 35S:OsMADS26 Arabidopsis plants.**

Ellis and Turner (2001) have reported that methyl jasmonate blocks apical hook development in a
dose-dependent manner while ethylene promotes such formations. Therefore, we employed this physiology to study any possible relationship between OsMADS26 and those hormones. As previously reported, MJ induced shorter roots and hypocotyls, and inhibited apical hooks, while ACC induced exaggerated development of the latter tissue (Fig. 7A). Proper hooks are defined as those where the angle between hypocotyl and cotyledon is <90°. When homozygous plants were grown in the dark, 86% of the transgenics did not have properly formed hooks (Fig. 7B). Moreover, when treated with 1 µM MJ, all transgenic plants failed to develop apical hooks; ACC also did not induce drastic apical hook development. Therefore, these results suggest that some of the phenotypes observed in our OsMADS26-overproducing Arabidopsis plants are associated with MJ.

Discussion

OsMADS26 transcript is more abundant in old tissues

OsMADS26 was the first of four rice genes identified in the AGL12 group. Its expression patterns have now been elucidated, with transcripts being detected in the roots, shoots, panicles, and inflorescences throughout all developmental stages (Shinozuka et al., 1999, Pelucchi et al., 2002). In this study, we showed that the OsMADS26 transcript level was elevated in older leaves and roots, implying that this gene may be involved in senescence or maturation processes.

Suppression of OsMADS26 expression does not cause phenotype alterations

Transgenic plants over-expressing the anti-sense OsMADS26 or T-DNA insertional mutant showed
no visible alterations in their phenotypes. We examined the KO plants under various stress conditions such as drought, high salt, and stress mediators such as ACC, MJ, and H$_2$O$_2$; however, there were no differences between KO and segregant WT plants. This indicates that the gene may function under specific conditions. Alternatively, other MADS-box genes may complement the loss of its functioning. The rice genome contains three AGL12-group proteins that are closely related to OsMADS26: OsMADS33, OsMADS35, and OsMADS36 – these share 52 to 53% overall amino acid identity with OsMADS26 (Lee et al., 2003). Potentially, OsMADS33 can be the candidate because it is expressed in a similar pattern to OsMADS26 (Lee et al., 2003). Interestingly, whereas four AGL12-group MADS-box genes have now been isolated from rice, only one from this group has been identified in other species, such as Arabidopsis, tomato, and Magnolia praecocissima. The loss-of-function mutant in Arabidopsis agl12 showed defects in root meristem development on vertical plates as well as a phenotype of late flowering under long days (Tapia-Lopez et al., 2008). This suggests that the rice AGL12-group genes are functionally redundant.

**Overexpression of OsMADS26 causes multiple stress responses in rice and Arabidopsis**

To elucidate the role of OsMADS26, we regenerated transgenic rice plants over-expressing that gene. Various phenotypes were displayed, such as defective growth, chlorosis, cell death, pigment accumulation, spotted leaves, and senescence. These were almost re-enacted in OsMADS26 over-expressing Arabidopsis plants, demonstrating the conserved role of this MADS-box gene in both model systems. We think that these phenotypes reflect the actual function of OsMADS26 because we employed an inducible system that showed the similar phenotypes to be independent of developmental stage. Therefore, the induced phenotypes are likely related to the action of OsMADS26. If the abnormalities had, instead, been artifacts due to disturbing the action of other
proteins, we would have expected the influence to be linked with a particular growth stage. A
number of overexpression analyses have been conducted previously to study gene function,
especially when loss-of-function mutants do not provide any clues.

The phenotypes observed from the transgenics were similar to those previously reported for
plants exposed to various stresses. In Arabidopsis, stresses mediated by heavy metals, nutrient
deficiencies, and hypoxia induce the development of characteristic traits that include diminished
leaf, shoot, and root elongation, as well as enhanced formation of lateral roots (see review by
Potters et al., 2007). Similar abnormalities, e.g., chlorosis and cell death, are commonly observed in
rice grown under extremely harsh conditions. Pigments are also accumulated in stressed plants
(Harvaux and Kloppstech, 2001; Jordan et al., 1998). Although genes related to cell death and
pigment accumulation were identified in our microarray analyses, no gene directly associated with
chlorosis was detected. Therefore, the chlorosis phenotype seems to be more of an indirect effect
compared with other abnormalities.

OsMADS26 may generate various stress mediators

Because stress phenomena are connected with various factors, including phytohormones and ROS,
the OsMADS26-mediated response described here might be related to hormonal activity. We
speculated that jasmonate is the most probable candidate because the phenotypes observed from our
transformants were similar to ones from plants that over-express JA-inducible genes. Using a
genetics screening system to isolate mutants that constitutively express the thionin (Thi) 2.1 gene,
Hilpert et al. (2001) have identified at least five different constitutive expression of thionin (cet)
mutants. These show phenotypes of retarded growth, whitish rosettes, downward-bending leaves,
and spontaneous lesions. Two other mutants, constant expression of JA inducible (cex) 1 and
constitutive expression of VSP (cev) 1, also manifest slower growth and the accumulation of anthocyanin (Ellis et al., 2002; Xu et al., 2001)

Our microarray analyses revealed that OsMADS26 overexpression indeed induced JA biosynthesis genes, such as lipoxygenase (A09032318), OsLOX3, OsAOS1, OsAOS4, OsAOS5, OsOPR2, OsOPR12, OsOPR13, and OsJMT4. Except for OsAOS1, OsAOS5, OsACO2, and OsJMT4, at least one CArG box existed within the 2-kb promoter regions of the putative target genes (data not shown), which furthers the possibility that OsMADS26 directly binds to these promoters. Furthermore, MJ-treated rice seedlings partially resembled those with OsMADS26-induced abnormal phenotypes, including reduced root/shoot growth and pigment accumulation in the roots (Supplementary Fig. 8A, 8B). The suppression of apical hook development seen in our 35S:OsMADS26 Arabidopsis plants also supports the idea that OsMADS26 activates JA-signaling.

However, treatments with JA-biosynthesis inhibitors (10 µM ibuprofen, 1 mM SA, or 100 µM diethylthiocarbamic acid (DIECA)) did not recover the abnormal phenotypes induced earlier by DEX treatment (data not shown). DIECA inhibits the octadecanoid pathway by reducing the intermediate 13-S-hydroperoxylinolenic acid to 13-hydroxylinolenic acid (Farmer et al., 1994). Ibuprofen and acetylsalicylic acid work as lipoxygenase inhibitors (Doares et al., 1995; Nojiri et al., 1996). This might be because total oxylipin contents were increased. Recently, Vellosillo et al. (2007) have reported on the diverse roles of oxylipin compounds formed by the oxygenation of fatty acids. These molecules prompt not only JA-induced development overall, but also are associated with root waving and the loss of apical dominance. Actually, 35S:OsMADS26 Arabidopsis plants show both a general retardation of growth and no apical dominance. Therefore, it is possible that OsMADS26 induces complex phenotypes by elevating oxylipin contents. Alternatively, OsMADS26 may regulate JA, SA, and ethylene pathways simultaneously. While antagonistic interactions between SA and ET/JA signalings have been documented, overlap in gene
induction among JA, SA, and ethylene treatments also has been reported (Cheong et al., 2003; Sasaki et al., 2004). For example, BWMK1, up-regulated in our microarray analyses, was increased in response to SA, JA, and ethephon. Therefore, OsMADS26 may act as a common positive regulator for a subset of genes that respond to these hormones.

Ethylene is another possible candidate because some of our phenotypes were similar to those from plants treated with ET, in which four ACC oxidase genes were up-regulated. However, ACC synthase transcript levels did not change here. Because ACC synthesis is the rate-limiting step in ET production, the effect of OsMADS26 in the ET-mediated response is restricted to the regions where ACC synthase activity is high. In the apical hook, ET-mediated signaling seemed not to be activated because we did not find any ET-induced exaggeration of a hook. However, the leaf-curling phenotype observed in the 35S:OsMADS26 seedlings was similar to that of WT plants treated with ET.

Finally, the third candidate is ROS – this may be possible based on our data showing that NADPH oxidase transcript levels were up-regulated in the OsMADS26-overexpressing plants. ROS induces morphogenic responses that include defective growth and a relatively large number of lateral roots (Olmos et al., 2006). Enhanced ROS production is associated with a broad range of biotic and abiotic stresses, e.g., heat, UV-radiation, heavy metal, anoxia, and pathogen attacks (Apel and Hirt, 2004). Unexpectedly, a gene with high homology to gibberellin β-hydroxylase was up-regulated. However, we did not study a relationship between gibberellin and OsMADS26 because this hormone is rarely involved in stress-related responses observed in the OsMADS26-overexpressing plants.

OsMADS26 may directly bind to the promoter regions of these biosynthesis genes. Alternatively, it might regulate these genes via cross talk between stress-mediators or by positive feedback mechanisms (Sasaki et al., 2001; Zhong and Burns, 2003; Chung and Choi, 2007). For
example, three JA biosynthesis genes -- \textit{OsAOS1}, \textit{OsAOC1}, and \textit{OsOPR1} -- are induced not only by JA itself but also by treatment with ET, abscisic acid, salicylic acid (SA), or hydrogen peroxide (Agrawal et al., 2002, 2003a, b). Likewise, \textit{OsACO2} transcript levels are elevated in IAA-treated etiolated rice seedlings whereas \textit{OsACO3} mRNA is greatly accumulated following ET exposure (Chae et al., 2000).

\textit{OsMADS26} regulates various stress-induced genes

Microarray analyses have produced a global spectrum for the genes regulated by JA, ET, and ROS. MJ differentially controls the transcription of genes involved in oxidative bursts and programmed cell death, such as those for catalase, glutathione S-transferase, and cysteine protease (Schenk et al., 2000). Numerous genes associated with cell rescue, disease, and defense mechanisms have been identified as early ET-regulated genes (de Paepe et al., 2004). Extensive comparisons have demonstrated redundant and specific roles for ROS in connection with stresses (Gadjev et al., 2006). Furthermore, considerable cross talk occurs among these signaling pathways. Schenk et al. (2000) have reported that 50\% of the genes induced by ET are also induced by MJ. Transcriptome analysis of Col;35S: \textit{ERF1} transgenic plants and ET/JA-treated WT plants has further revealed a large number of genes responsive to both ET and JA (Lorenzo et al., 2003). In the \textit{flu} mutant, which over-produces \textsuperscript{1}O\textsubscript{2}, the ethylene-responsive element-binding proteins are highly induced, indicating cross talk between \textsuperscript{1}O\textsubscript{2} and ethylene-signaling (Gadjev et al., 2006).

Our microarray analyses showed that genes inducible by JA, ET, or ROS were up-regulated in transgenic plants over-expressing \textit{OsMADS26}. These include not only the biosynthesis genes already discussed here, but also many putative downstream genes, such as \textit{cysteine proteinase}, \textit{S-adenosylmethionine decarboxylase}, \textit{protease inhibitor}, \textit{peroxidase}, and \textit{MAP-kinase} genes.
Expression profiles for 22 rice *peroxidase* genes have revealed that many of them respond to disease, wounding, SA, JA, and ACC (Sasaki et al., 2004). A *MAP kinase* gene (A05011217), designated as *BWMK1* (Genbank Accession Number AF177392), is induced not only by blast, wounding, and H$_2$O$_2$, but also by the phytohormones SA, JA, and ethephon (Cheong et al., 2003; He et al., 1999). The two putative *flavanone 3-hydroxylase* genes found from our microarrays may also cause pigment accumulation. In other species, MJ induces the accumulation of anthocyanin in soybean and *Arabidopsis* (Franceschi and Grimes, 1991; Jung, 2004). Furthermore, transcripts involved in anthocyanin production are co-regulated in response to O$_2^-$, whereas H$_2$O$_2$ negatively impinges on their expression (Gadjev et al., 2006).

Our analyses also showed the activity of HR-related genes that encode a harpin-induced protein or a cell death-associated protein. Harpin from *Erwinia amylovora* causes the HR response (Wei et al., 1992). A putative cell death-associated gene has close homology with *hsr203J*, which is expressed in the leaves of *Nicotiana tabacum* cv. Samsun NN infected with *Ralstonia solanacearum* 8107 (Kiba et al., 2003). An *aldo/keto reductase* family gene also was induced here. Members of the *aldo/keto reductase* superfamily can detoxify a major lipid peroxide degradation product, 4-hydroxynon-2enal (HNE) (Vander Jagt et al., 1995), and the rice *aldo/keto reductase* gene is induced in vegetative tissues in response to PEG-mediated water stress and salinity (Karuna Sree et al., 2000).

Three members belonging to the protease inhibitor family were down-regulated, suggesting their negative roles in stress-related responses. These proteins contain a domain commonly found in trypsin-alpha amylase inhibitors, seed storage proteins, and lipid transfer proteins (Rico et al., 1996). Some peroxidase genes were also down-regulated, perhaps causing the cell death signal to be amplified by reducing H$_2$O$_2$ scavenging. Down-regulation of peroxidase
genes by ethylene has previously been reported (de Paepe et al., 2004). Altogether, our results indicate that OsMADS26 controls various stress responses.

Materials and Methods

Plant materials and chemical treatments

*Oryza sativa* var. japonica cv. Dongjin and the Columbia ecotype of *Arabidopsis thaliana* were used. Rice seeds were surface-sterilized and seedlings were grown at 28°C on gauze embedded in sterile Murashige and Skoog (MS) media containing 0.2% agar, 3% sucrose, and 0.01% myo-inositol. Plants were grown to maturity in a greenhouse supplemented with artificial lighting during the winter period. DEXamethasone was dissolved in 95% alcohol at 1 mM and an appropriate amount was added to the growth media to arrive at the desired final concentration. Methyl jasmonate and aminocyclopropane-1-carboxylate (ACC) were dissolved in 95% alcohol and sterilized water, respectively, at 10 mM, before a suitable amount was added to MS solid media containing 0.2% agar, 3% sucrose, and 0.01% myo-inositol. For *Arabidopsis*, MJ and ACC were added to a 1/2 Gamborg B5 agar (0.8%) medium supplemented with 1% sucrose.

For the treatments with JA-biosynthesis inhibitors, plants were grown for 7 d in DEX-free MS solid media. Healthy plants were selected and incubated in tap water for 1 d. Ibuprofen, salicylic acid, and DIECA were added at their final concentrations of 10 µM, 1 mM, and 100 µM, respectively. After 1 h, 1 µM DEX was added and phenotypes were observed for 3 consecutive days.

Vector construction
The full-length cDNA clone of *OsMADS26* (GenBank Accession Number AB003326) was isolated by nested PCR, using the following four primers: forward 1, 5’-atcaagcttgagctatcgatcatcaagc-3’; forward 2, 5’-atcaagcttgagacttatcttgatcgatgg-3’; reverse 1, 5’-ttgggtaccaataaggtacatcagaatgec-3’; and reverse 2, 5’-ttgggtaccgtagaaggaatagccatctcc-3’. These primers contained the Hind III and Asp718 restriction enzyme sites for subsequent cloning. The PCR product was first cloned into pBluescript SK- (Stratagene, La Jolla, CA). Afterward, the cDNA was sub-cloned into the pGA1611 binary vector between the maize *ubi* promoter and the *nos* terminator for the sense construct (Lee et al., 1999; Kim et al., 2003). For the anti-sense construct, we used the region between 404 and 900 of *OsMADS26*. For the DEX-inducible system, the *OsMADS26* stop codon was changed to the Asp718 site by using the reverse primer (5’-ttgggtaccgaaggaatagccatctcc-3’). The rat *GR* gene (AY066016) was inserted into that Asp718 site, generating an in-frame fusion between the two molecules. For *Arabidopsis* transformation, the pGA1535 binary vector with the *CaMV35S* promoter and a kanamycin selectable marker was used to sub-clone *OsMADS26*, with Hind III and Asp718.

**Transformation**

Rice transformation was performed according to the *Agrobacterium*-mediated methods described by Jeon et al. (1999) and Lee et al. (1999). All transgenic plants were grown in glass tubes, and then transferred to a confined paddy field. The Columbia ecotype was used for *Arabidopsis* transformation using floral dip method (Clough and Bent, 1998).

**Microarray analyses**

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Microarray analyses were conducted as described previously (Jung et al., 2005). Total RNAs (100 mg) were prepared from two independent lines of the *ubi:OsMADS26GR* plants and WT segregants. KMC clustering analyses were performed with TIGR MeV software (Saeed et al., 2003).

**RT-PCR, real time PCR and northern blot analysis**

Total RNAs were isolated from fresh tissues with an RNA isolation kit (Tri Reagent; MRC Inc., Cincinnati, OH). First-strand cDNA was synthesized from 4 µg of total RNAs, using M-MLV reverse transcriptase (Promega, Madison, WI). Synthesized cDNAs were used for semi-quantitative RT-PCR and real-time PCR. The latter was performed with Roche Lightcyler II. *Actin* primers, GCACAGGAAATGGCTTCTAATTCTT and AATCACAAGTGAGAACCACAGGTA, were used for normalizing the cDNA quantity. The primers used in real-time PCR experiments were CTGATCATGTGAAGCAAAATTTTCTC and ACGCTAAGAACACGCTTATTAC for *Osl2* (AF251073), and AAGCATCAGCATCATTACGAGCA and CTAATTTCACACAGATGAACC for *Osl55* (AF251074). For RT-PCR, the primers for the *actin* genes were designed as reported previously (Takakura et al., 2000). Gene-specific primers were designed for each target gene (Supplementary Table III). After PCR-amplification, the products were separated on a 1.2% agarose gel and photographed. In some cases, PCR products were blotted onto a nylon membrane, and hybridized with a 32P-labeled probe. For northern blot analyses, total RNAs were fractionated on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with a 32P-labeled probe. PCR primers F and R indicated in Supplementary Fig 1 were used to generate the probe.
Chlorophyll content measurement

Five d-old seedlings were treated with 1μM DEX for 3 days. Shoots were harvested, weighed, and ground into fine powder in liquid nitrogen. Chlorophylls were extracted in 80% acetone and diluted to 1/100 for spectrophotometer measurements. Chlorophyll a and b concentrations were determined according to the method of Lichtenthaler (1987).

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Table I. Root and leaf numbers for *ubi:OsMADS26GR* plants treated with DEX.

| DEX   | Root number | leaf number |
|-------|-------------|-------------|
|       | WT          | HO          | WT          | HO          |
| **Line #33** |             |             |             |             |
| 0 M   | 8.1 ± 1.8   | 8.0 ± 2.2   | 4.0 ± 0.0   | 4.0 ± 0.0   |
| 10 nM | 7.9 ± 1.1   | 6.5 ± 1.9   | 4.0 ± 0.0   | 4.0 ± 0.0   |
| 100 nM| 8.8 ± 1.0   | 5.4 ± 2.6   | 4.0 ± 0.0   | 2.0 ± 0.0   |
| 1 µM  | 9.2 ± 2.1   | 2.3 ± 3.1   | 4.0 ± 0.0   | 2.0 ± 0.0   |
| 10 µM | 2.1 ± 1.2   | 0.0 ± 0.0   | 2.0 ± 0.0   | 2.0 ± 0.0   |
| **Line #17** |             |             |             |             |
| 0 M   | 10.0 ± 1.8  | 8.3 ± 1.1   | 4.0 ± 0.0   | 3.8 ± 0.5   |
| 10 nM | 9.2 ± 2.1   | 6.1 ± 0.7   | 3.8 ± 0.4   | 3.0 ± 0.0   |
| 100 nM| 9.0 ± 2.0   | 6.2 ± 0.9   | 3.9 ± 0.4   | 3.0 ± 0.0   |
| 1 µM  | 10.6 ± 2.1  | 0.0 ± 0.0   | 3.9 ± 0.4   | 3.0 ± 0.0   |
Table II. Functional classification of genes that are up- or down-regulated by at least two-fold in *ubi:OsMADS26GR* plants treated with DEX. Relative numbers indicate the percentage of up- and down-regulated genes relative to the total genes within a category.

| Functional category                                      | Up  | Down | Total | Relative number (%) |
|----------------------------------------------------------|-----|------|-------|---------------------|
| INFORMATION STORAGE AND PROCESSING                        |     |      |       |                     |
| Translation, ribosomal structure and biogenesis           | 0   | 0    | 0     | 0.00               |
| RNA processing and modification                          | 0   | 1    | 1     | 0.17               |
| Transcription                                            | 4   | 1    | 5     | 0.34               |
| Replication, recombination and repair                     | 0   | 0    | 0     | 0.00               |
| Chromatin structure and dynamics                         | 0   | 0    | 0     | 0.00               |
| CELLULAR PROCESSING AND SIGNALING                         |     |      |       |                     |
| Cell cycle control, cell division, chromosome partitioning| 1   | 0    | 1     | 0.19               |
| Nuclear structure                                         | 0   | 0    | 0     | 0.00               |
| Defense mechanisms                                       | 4   | 7    | 11    | 2.40               |
| Signal transduction mechanisms                           | 16  | 4    | 20    | 0.58               |
| Cell wall/membrane/envelope biogenesis                   | 3   | 2    | 5     | 0.99               |
| Cell motility                                            | 0   | 0    | 0     | 0.00               |
| Cytoskeleton                                             | 1   | 0    | 1     | 0.34               |
| Extracellular structure                                  | 0   | 0    | 0     | 0.00               |
| Intracellular trafficking, secretion and vesicular transport| 1  | 0    | 1     | 0.13               |
| Posttranslational modification, protein turnover, chaperones | 3  | 9    | 12    | 0.66               |
| METABOLISM                                               |     |      |       |                     |
| Energy production and conversion                         | 0   | 3    | 3     | 0.37               |
| Carbohydrate transport and metabolism                    | 2   | 8    | 10    | 0.82               |
| Amino acid transport and metabolism                      | 4   | 2    | 6     | 0.74               |
| Nucleotide transport and metabolism                      | 2   | 0    | 2     | 0.99               |
| Coenzyme transport and metabolism                        | 0   | 0    | 0     | 0.00               |
| Lipid transport and metabolism                           | 4   | 0    | 4     | 0.41               |
| Inorganic ion transport and metabolism                   | 2   | 3    | 5     | 1.02               |
| Secondary metabolites biosynthesis transport and metabolism| 11 | 1    | 12    | 1.08               |
| POORLY CHARACTERIZED                                     |     |      |       |                     |
| General function prediction only                         | 17  | 16   | 33    | 0.55               |
| Function unknown                                         | 3   | 5    | 8     | 0.48               |
| Unnamed protein                                          | 6   | 6    | 12    | 0.71               |
Table III. A partial list of putative target genes that are up- or down-regulated by at least 1.5-fold in *ubi:OsMADS26GR* plants treated with DEX.

| Spot_ID   | CHR_Locus | Function                                | Fold change ± SD | 3 hr | 9 hr  |
|-----------|-----------|-----------------------------------------|------------------|------|-------|
| A09021902 | LOC_Os11g08380 | Iron/ascorbate family oxidoreductases | 1.969 ± 0.465    | 2.209 ± 0.376 |
| A05041211 | LOC_Os04g10350 | Iron/ascorbate family oxidoreductases | 1.437 ± 0.400    | 2.314 ± 0.622 |
| A05011009 | LOC_Os09g39720 | Iron/ascorbate family oxidoreductases | 1.379 ± 0.166    | 2.503 ± 0.501 |
| B10022103 | LOC_Os10g39140 | Iron/ascorbate family oxidoreductases | 2.119 ± 0.998    | 3.426 ± 1.785 |
| A09032318 | LOC_Os04g37430 | Lipoxygenase                             | 1.379 ± 0.166    | 2.503 ± 0.501 |
| A05011009 | LOC_Os06g49430 | Mitogen-activated protein kinase         | 1.794 ± 0.502    | 3.209 ± 1.329 |
| B04022213 | LOC_Os02g04230 | Mitogen-activated protein kinase         | 1.660 ± 0.802    | 2.233 ± 0.563 |
| B05032110 | LOC_Os01g64470 | A Harpin-induced protein                 | 0.600 ± 1.126    | 5.256 ± 2.566 |
| A03011404 | LOC_Os04g58850 | A Harpin-induced protein                 | 1.272 ± 0.209    | 2.299 ± 0.616 |
| A04030908 | LOC_Os12g04150 | Cell death associated protein           | 0.144 ± 1.538    | 3.154 ± 1.697 |
| A07022114 | LOC_Os04g57440 | Cysteine proteinase Cathepsin L         | 1.318 ± 0.130    | 2.009 ± 0.375 |
| A05031002 | LOC_Os05g04490 | Peroxidase                              | 0.634 ± 1.117    | 3.912 ± 2.174 |
| B10321214 | LOC_Os07g48020 | Peroxidase                              | 1.055 ± 1.487    | 2.586 ± 1.162 |
| A10030822 | LOC_Os03g55410 | Peroxidase                              | -2.026 ± 0.896   | -2.230 ± 0.727 |
| B11022306 | LOC_Os01g6450 | Peroxidase                              | -1.713 ± 0.586   | -2.508 ± 1.003 |
| B03042101 | LOC_Os02g41910 | Gamma-thionins family                   | -1.386 ± 0.286   | -1.767 ± 0.123 |
| A12031901 | LOC_Os01g62890 | Protease inhibitor/seed storage/LTP family | -0.120 ± 1.503  | -1.767 ± 0.282 |
| B09020319 | LOC_Os04g33930 | Protease inhibitor/seed storage/LTP family | -0.052 ± 1.492  | -1.899 ± 0.289 |
| B01042103 | LOC_Os05g06780 | Protease inhibitor/seed storage/LTP family | -0.056 ± 1.438  | -2.062 ± 0.267 |
| A02031602 | LOC_Os07g07930 | Protease inhibitor/seed storage/LTP family | 1.514 ± 0.384   | 2.283 ± 0.728 |

Spot_ID, oligomers selected by microarray; CHR_locus, chromosomal locus given in TIGR database; KOME, Knowledge-based *Oryza* Molecular Biological Encyclopedia Accession number; SD, standard deviation.
FIGURE LEGENDS

Figure 1. Expression patterns for OsMADS26 at various developmental stages.
A, Transcript levels in roots and leaf blades from 5-, 10-, 20-, and 40-day-old plants.
B, Transcript levels of OsMADS26 in total roots from 3-, 6-, 9-, 25-, and 50-day-old plants.
C, Transcript levels of OsMADS26 in leaf blades from 15-, 30-, 50-, 70-, and 90-day-old plants.
D, Transcript levels of OsMADS26 at 1st, 2nd, 5th, and 6th positions within 35-day-old plants, counted from newly emerging leaf.

Figure 2. Phenotypes of ubi:OsMADS26 plants.
A, Cell death phenotype (arrows). B, Defective growth, chlorosis, and accumulation of purple pigment in roots. C, Regenerating root showing screw-like curling phenotype. D, Regenerating roots accumulating purple pigments. E, Mature transgenic plant displaying pale-green and dwarf phenotypes (right), compared with wild-type (WT) control (left). F, Transgenic leaves with pigmented spots (right), compared with WT control (left). G, Shrunken transgenic seed (lower) and WT control (upper). H, T2 ubi:OsMADS26 plants displaying defective growth, cell death (arrow), and pigment accumulation. I, Northern blot analyses of T1 transgenic plants. R, roots; S, shoots.

Figure 3. Abnormal phenotypes of ubi:OsMADS26GR plants.
A and B, Effects of OsMADS26 on germinating rice seedlings. WT segregants (A) and HO plants (B) were treated with DEXamethasone. Six to nine T3 plants were analyzed after growing for 9 d in MS solid media containing various concentrations of DEX. (B, inset) Purple pigments accumulated only in roots of HO plants treated with 100 nM DEX.
C, Effects of OsMADS26 on post-germinated rice seedlings. After growth in MS liquid media for 6
d, 1 µM DEX was treated for 2 consecutive days.

D, Effects of OsMADS26 on mature rice. After growing for 80 d in glasshouse, plants were treated with 10 µM DEX for 7 d.

E and F, Longitudinal sections of the root maturation zones of a WT segregant (E) and HO plant (F).

G, Chlorophyll a/b contents per gram fresh weight. Each data point is average of 4 or 5 replicates.

H and I, Real-time PCR analyses of senescence-related genes OsI2 (H) and OsI52 (I) in ubi:OsMADS26GR plants. Y-axis represents relative values between transcript levels of target genes and actin. Each data point is average of 4 or 5 replicates.

**Figure 4.** KMC clustering analyses for 48 target genes showing more than 2-fold change in expression.

A, Cluster containing genes up-regulated by >4-fold on average at both 3 and 9 h. OsMADS26 is included in this cluster.

B, Cluster containing genes up-regulated by <4-fold on average at both 3 and 9 h.

C, Cluster containing down-regulated genes.

**Figure 5.** RT-PCR analyses to confirm microarray results. After 0, 3, or 9 h of DEX treatment, roots were sampled from wild-type and ubi:OsMADD26GR plants. gDNA controls were used to detect contamination with genomic DNA. Putative functions are indicated in right panel.

**Figure 6.** Phenotypes of 35S:OsMADS26 Arabidopsis. All plants were grown on ½ MS media.

A, 28-day-old T1 35S:OsMADS26 plant.

B, 10-day-old T2 35S:OsMADS26 and WT plants.

C, 20-day-old T2 35S:OsMADS26 and WT plants.
Figure 7. Apical hook development. Etiolated seedlings of WT (A) and 35S: OsMADS26 (B) were grown on ½ MS media, then treated with MJ and ACC at 0, 1, or 10 μM concentrations.
Figure 1. Expression patterns for *OsMADS26* at various developmental stages.

A, Transcript levels in roots and leaf blades from 5-, 10-, 20-, and 40-day-old plants.
B, Transcript levels of *OsMADS26* in total roots from 3-, 6-, 9-, 25-, and 50-day-old plants.
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A, Cell death phenotype (arrows). B, Defective growth, chlorosis, and accumulation of purple pigment in roots. C, Regenerating root showing scrotal-like nodals from one end. D, Regenerating roots accumulating purple pigments. E, Mature transgenic plant displaying pale-green and dwarf phenotypes (right), compared with wild-type (WT) control (left). F, Transgenic leaves with pigmented spots (right), compared with WT control (left). G,
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Figure 6. Phenotypes of 35S:OsMADS26 Arabidopsis. All plants were grown on 1/2 MS media.
A, 28-day-old T1 35S:OsMADS26 plant.
B, 10-day-old T2 35S:OsMADS26 and WT plants.
C, 20-day-old T2 35S:OsMADS26 and WT plants.
Figure 7. Apical hook development. Etiolated seedlings of WT (A) and 35S:OsMADS26 (B) were grown on 1/2 MS media, then treated with MJ and ACC at 0, 1, or 10 μM concentration.