Overexpression of MoSM1, encoding for an immunity-inducing protein from *Magnaporthe oryzae*, in rice confers broad-spectrum resistance against fungal and bacterial diseases

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Potential of MoSM1, encoding for a cerato-platanin protein from *Magnaporthe oryzae*, in improvement of rice disease resistance was examined. Transient expression of MoSM1 in rice leaves initiated hypersensitive response and upregulated expression of defense genes. When transiently expressed in tobacco leaves, MoSM1 targeted to plasma membrane. The MoSM1-overexpressing (MoSM1-OE) transgenic rice lines showed an improved resistance, as revealed by the reduced disease severity and decreased *in planta* pathogen growth, against 2 strains belonging to two different races of *M. oryzae*, causing blast disease, and against 2 strains of *Xanthomonas oryzae* pv. *oryzae*, causing bacterial leaf blight disease. However, no alteration in resistance to sheath blight disease was observed in MoSM1-OE lines. The MoSM1-OE plants contained elevated levels of salicylic acid (SA) and jasmonic acid (JA) and constitutively activated the expression of SA and JA signaling-related regulatory and defense genes. Furthermore, the MoSM1-OE plants had no effect on drought and salt stress tolerance and on grain yield. We conclude that MoSM1 confers a broad-spectrum resistance against different pathogens through modulating SA- and JA-mediated signaling pathways without any penalty on abiotic stress tolerance and grain yield, providing a promising potential for application of MoSM1 in improvement of disease resistance in crops.

Plants have evolved to possess two distinct types of innate immune responses, which are referred to as pathogen (microbe)-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI, which is triggered by a large number of PAMPs, is the first layer of the innate immune responses. On the other hand, ETI, which is triggered by specific interactions between host plant R proteins and pathogen-derived avirulence proteins, is a specialized form of the innate immune responses. In addition to the innate immune responses, plants have also evolved to establish an inducible immune response system, which is activated upon pathogen infection or treatments with natural or synthetic compounds through multiple distinct signal transduction pathways. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two well-known forms of the inducible immune responses. The induced immune responses often confer durable, broad-spectrum and systemic resistance against a variety of related or unrelated pathogens at the distal tissues from the infection or treatment sites.

Plant inducible immune response can be triggered by exogenous application of a number of elicitors. During the last decade, extensive studies have identified a large number of microbe-derived compounds including polysaccharides, glycoproteins and secreted proteins that exhibit activity of inducing immune responses in different plant species. Among them, the microbe-derived proteins have been shown to possess great potentials in developing eco-friendly biopesticides or generating novel disease-resistant crop cultivars or germplasms for practical use.
application of disease control. The best example of the microbe-derived proteins with immune-inducing activity in a variety of plants is the Gram-negative phytopathogenic bacteria-secreted harpin proteins, which generally affect virulence in host plants and induce immune responses in nonhost plants11–12. Numerous studies have demonstrated that harpins, either applied to plants as a main component of formulated biopesticides or ectopically expressed in transgenic crop plants, trigger diverse beneficial effects such as induction of defense responses against diverse biotic (pathogens and insects) and abiotic stresses and enhancement of plant growth13. Elicitin and Nep1-like proteins, produced by a number of pathogenic oomycetes such as Phytophthora spp., are other examples that have been shown to be capable of triggering inducible immune response in different plants14–17. It was believed that microbe-derived proteinaceous elicitors can offer a promising strategy of durable, broad-spectrum disease control approach. During the recent years, a quite large number of novel proteinaceous elicitors have been isolated from microbes through screening and identification using a combination of molecular, biochemical and proteomic approaches18–21.

The cerato-platanin (CP) family is a newly identified small, secreted and cysteine-rich protein (~150 aa) family from filamentous fungi22–24. CP was first identified from the tree pathogen Ceratocystis platani (formerly known as Ceratocystis fimbriata f. sp. platani)25 and later was found in genomes of more than 50 fungal species with all kinds of lifestyles including biotrophic and necrotrophic plant pathogens, animal pathogens, mycoparasites, plant-beneficial fungi and saprotrophs26. Although CPs are abundantly secreted into the culture filtrate, they remain partially bound in the fungal cell wall27–29. Recent studies have shown that CPs function both as fungal virulence factors or plant defense elicitors20. For example, BcSpl1 in Botrytis cinerea and MoMSP1 in Magnaporthe oryzae were found to contribute to the fungal virulence as the knock-out mutants for BcSpl1 or MoMSP1 showed reduced virulence in their host plants30–32. Meanwhile, it has also been shown that CPs from both pathogenic and beneficial biocontrol fungi can induce locally and systemically some structural, physiological and molecular defense responses, including initiation of hypersensitive response (HR), accumulation of phenolic compounds and phytoalexins, production of reactive oxygen species (ROS), and upregulation of defense-related genes in host and nonhost plants33–45. SM1 and SM2 from Trichoderma virens, BcSpl1 from B. cinerea, and MoMSP1 (also known as MoMSP1) from M. oryzae could induce systemic disease resistance in rice, cotton, maize, tobacco, tomato and Arabidopsis against different pathogens43–37,38,40,42,45–47 and the activation of systemic resistance by CP from C. platani and BcSpl1 from B. cinerea was found to be regulated through stomatal perception, overexpression of salicylic acid (SA)- and ethylene-signaling genes and camalexin biosynthesis44,46. Further study revealed that the elicitor activity of the B. cinerea BcSpl1 resides in a two-peptide motif on the protein surface28. It was thus suggested that members of the CP family represent a novel class of proteins with potential PAMP activity as plant defense elicitors used in disease control22–24.

In our previous study, we demonstrated that MoSM1 from M. oryzae, when transiently expressed in leaves of Arabidopsis plants or stably expressed in transgenic Arabidopsis plants, conferred a broad-spectrum resistance against different pathogens such as Botrytis cinerea and Pseudomonas syringae pv. tomato DC3000, accompanied by accumulation of ROS and up-regulation of defense-related genes42. More recently, it was reported that recombinant MoMSP1 triggered cell death and elicits defense responses in rice, leading to potentiation of resistance to M. oryzae36. In the present study, we explored the potential of MoSM1 in improvement of rice disease resistance. Our results indicate that the MoSM1-overexpressing (MoSM1-OE) transgenic rice lines showed an improved resistance against blast disease, caused by M. oryzae, and bacterial blight disease, caused by Xanthomonas oryzae pv. oryzae (Xoo), probably through modulating SA/jasmonic acid (JA) signaling pathways, but did not affect resistance to sheath blight disease, caused by Rhizoctonia solani. Furthermore, the MoSM1-OE plants did not affect the drought and salt stress tolerance, caused by some of the important agronomic traits such as grain yield.

Results

Transient expression of MoSM1 in rice elicits HR and activates expression of defense-related genes. Before the investigation of function of MoSM1 in rice defense, we further examined the biochemical features of MoSM1 such as the role of signal peptide (SP) in activity and subcellular localization in planta. Because MoSM1 contains a 21 aa SP at its N-terminal42, we first examined whether this SP was necessary for its activity to induce HR. To do this, a SP-deletion vector, pINDEX3::MoSM1ΔSP, in which the SP region was deleted, was constructed. At 24 hr after agroinfiltration, transient expression of MoSM1 in leaves of N. benthamiana plants induced a typical HR cell death while transient expression of MoSM1ΔSP did not (Fig. 1a). We also examined the subcellular localization of MoSM1 in planta through generating an eGFP-MoSM1 fusion construct. When transiently expressed in leaves of N. benthamiana, significant accumulation of eGFP-MoSM1 fusion was detected by Western blotting, which was similar to the accumulation of eGFP alone, at 24 hr after agroinfiltration (Fig. 1b). Microscopic observation revealed that the eGFP-MoSM1 fusion targeted mainly to the plasma membrane, co-localized with a previously reported plasma membrane-targeted Arabidopsis aquaporin AtPIP1;4, whereas the eGFP alone distributed throughout the cells with distribution in nucleus, at 24 hr after agroinfiltration (Fig. 1c). These data suggest that SP in MoSM1 is required for its activity to induce HR and that MoSM1 targets to plasma membrane of cells in planta.

We next examined whether transient expression of MoSM1 in rice could induce defense responses such as HR and expression of defense-related genes. To this end, agrobacteria carrying pINDEX3::MoSM1 and empty pINDEX3::00 were infiltrated into leaves of 5-week-old rice plants and transient expression of MoSM1 was induced by exogenous application of 3μM DEX42. Transcript of MoSM1 in pINDEX3::MoSM1-infiltrated leaves was first detected at 12 hr after DEX treatment and the MoSM1 transcript levels gradually increased over a period of 12–48 hr (Fig. 1d). However, no MoSM1 transcript was detected in pINDEX3::00-infiltrated leaves (Fig. 1d). Water-soaked symptom initially occurred around the infiltration sites at 12 hr after DEX treatment in pINDEX3::MoSM1-infiltrated leaves and further enlarged and expanded into large area, leading to dried and colorless tissues at 48 hr (Fig. 1e). No obvious water-soaked symptom was observed in pINDEX3::00-infiltrated leaves.
Figure 1. Biochemical characteristics of MoSM1 and activation of rice defense response by transient expression of MoSM1. (a–c) Biochemical characteristics of MoSM1. (a) Requirement of SP for MoSM1 activity. Agrobacteria carrying pINDEX3::MoSM1, pINDEX3::MoSM1ΔSP or pINDEX3::00 were infiltrated into N. benthamiana leaves and HR symptom was examined 24 hr later. (b) and (c) Accumulation and subcellular localization of eGFP-MoSM1 fusion. Agrobacteria carrying pFGC-eGFP-MoSM1 or pFGC-eGFP were co-infiltrated with agrobacteria harboring pCAMBIA1300-AtPIP1;4-mCherry into N. benthamiana leaves and leaf samples were collected at 24 hr for Western blotting (b) or subcellular localization (c). (d–g) Activation of rice defense response by MoSM1. Fully expanded leaves of 5-week-old plants were infiltrated with agrobacteria carrying pINDEX3::MoSM1 or pINDEX3::00 construct and induced by spraying with 3 μM dexamethasone 24 hr after agroinfiltration. Leaf samples were collected at indicated time points (hr) and used for analysis of gene expression, measurement of electrolyte leakage and observation of hypersensitive response. (d) Expression of MoSM1 in pINDEX3::MoSM1- and pINDEX3::00-infiltrated leaves. (e) HR phenotype caused by transient expression of MoSM1 in pINDEX3::MoSM1-infiltrated leaves. (f) Increased electrolyte leakage caused by transient expression of MoSM1 in pINDEX3::MoSM1-infiltrated leaves. (g) Expression patterns of defense genes in pINDEX3::MoSM1- and pINDEX3::00-infiltrated leaves. Three independent experiments were performed with similar results in (a), (b), (c), (d), (e) and (g). Data presented in (f) are the means ± SD from three independent experiments and asterisks indicate statistically significant difference at p = 0.05 level between pINDEX3::MoSM1- and pINDEX3::00-infiltrated leaves.
(Fig. 1e). Similarly, electrolyte leakage in pINDEX3::MoSM1-infiltrated leaves increased significantly by 13%, 26% and 39% at 12, 24 and 48 hr after DEX treatment compared to the level at 0 hr, while the electrolyte leakage in pINDEX3::00-infiltrated leaves had no significant change (Fig. 1f). These results indicate that transient expression of MoSM1 in rice leaves can induce a typical HR.

We further examined whether transient expression of MoSM1 activated defense response by comparing the expression patterns of some selected defense-related genes between pINDEX3::MoSM1- and pINDEX3:00-infiltrated leaves after DEX treatment. As shown in Fig. 1g, induced expression of OsPR1b and OsPR10 was detected as early as 12 hr, and the expression levels increased further at 24 and 48 hr after DEX treatment in pINDEX3::MoSM1-infiltrated leaves, although relatively lower expression levels of OsPR1b and OsPR10 were also detected in pINDEX3::00-infiltrated leaves. These data indicate that transient expression of MoSM1 can also activate the expression of defense genes in rice and thus transiently expressed MoSM1 may function as an elicitor of defense response in rice.

**Generation and characterization of MoSM1-OE transgenic rice lines.** Transgenic rice lines with overexpression of MoSM1 (MoSM1-OE) were generated to precisely evaluate the function of MoSM1 in rice immunity. For this purpose, the MoSM1 full coding sequence including SP was constructed into a binary vector under the control of the maize ubiquitin promoter (Fig. 2a) and transformed into rice cv. Xiushui11. A total 36 independent transgenic lines were obtained through the Agrobacterium-mediated transformation method and two independent lines (SM1-70 and SM1-73) with single copy of the MoSM1 gene, as revealed by Southern blotting (Fig. 2b), were chosen for further studies. qRT-PCR analysis indicated that the expression level of the MoSM1 gene in plants of the T3 generations of the MoSM1-OE lines SM1-70 and SM1-73 was estimated to be 9 and 22 folds of the level of a rice Actin gene, respectively (Fig. 2c). However, no transcript of MoSM1 was detected in nontransgenic wild type (WT) plants (Fig. 2c). During our study, we did not observe significant changes in morphological and developmental phenotypes in the MoSM1-OE lines SM1-70 and SM1-73 at seedling and adult stages as compared to the WT plants (Fig. 2d and e). Furthermore, we also compared some important agronomic traits such as seed setting rate, panicle weight and grains per panicle of the MoSM1-OE plants with the nontransgenic WT plants grown under greenhouse condition. Overall, the panicles of the MoSM1-OE plants were comparable to those of the nontransgenic WT plants (Fig. 2f). No significant difference in some of key agronomic traits such as seed setting rate, panicle weight and grains per panicle was detected between the MoSM1-OE and nontransgenic WT plants (Fig. 2g–i). Taken together, these data indicate that the MoSM1-OE plants do not have abnormal growth, development and grain production.

**MoSM1-OE plants display an improved resistance against blast disease.** To explore the function of MoSM1 in rice disease resistance, we first evaluated the resistance level of the MoSM1-OE lines at seedling stage against blast disease, which is the most serious constraint on rice caused by *M. oryzae*. Two *M. oryzae* strains, 85-14 and 97-220, representing races ZB1 and ZE3, respectively, were used to inoculate the two-week-old transgenic and nontransgenic WT seedlings by foliar spraying of spore suspension and disease levels were evaluated by assessing disease scores and measuring in planta fungal growth. As shown in Fig. 3a,b and c, the overall blast disease phenotype on the MoSM1-OE lines SM1-70 and SM1-73 was less severe than those in the nontransgenic WT plants after inoculation with strains 85-14 and 97-220. Typical blast lesions were seen on leaves of nontransgenic WT plants, while almost no typical blast lesion was observed on leaves of the MoSM1-OE lines SM1-70 and SM1-73 (Fig. 3a,b and c). Similarly, significant cell death, as revealed by Trypan blue staining, in inoculated leaves of the nontransgenic WT plants was detected, while no remarkable cell death was seen in inoculated leaves of the MoSM1-OE lines SM1-70 and SM1-73 (Fig. 3a,b and c). At 7 days post inoculation (dpi) with strains 85-14 and 97-220, the averages of the blast disease scores on the nontransgenic WT plants were 5.8 and 4.7 grades, respectively, while the averages of the blast disease scores on the MoSM1-OE lines SM1-70 and SM1-73 were 0.2–0.3 grades (Fig. 3e), resulting in 4–5 grades lower than those on the WT plants. Further measurement of in planta fungal growth, as revealed by analyzing genomic DNA level of the 28S rDNA gene of *M. oryzae*, indicated that the MoSM1-OE lines SM1-70 and SM1-73 supported less growth of *M. oryzae* in inoculated leaves as compared with those in the nontransgenic WT plants (Fig. 3f), leading to a reduction of >110 folds in relative fungal growth. Taken together, these results and observations suggest that the MoSM1-OE lines showed an improved resistance to *M. oryzae*.

**MoSM1-OE plants exhibit an improved resistance against bacterial blight disease.** We next evaluated the resistance level of the MoSM1-OE lines against bacterial leaf blight disease, which is the most devastating bacterial disease in rice caused by *X. oryzae pv oryzae* (Xoo). The MoSM1-OE and nontransgenic WT plants at booting stage were inoculated using the leaf-clipping method with 2 different *Xoo* strains PXO96 and Zhe817 and the disease levels were evaluated by measuring lesion length at 15 dpi and the dynamics of bacterial growth in inoculated leaves. As shown in Fig. 4a and b, the overall bacterial blight disease severity in the MoSM1-OE lines SM1-70 and SM1-73 was lower than that in the nontransgenic WT plants after inoculation with strains PXO96 and Zhe817. At 15 dpi, the average of the lesion length on leaves of nontransgenic WT plants was 10.5 cm, while the averages of the lesion length on leaves of the MoSM1-OE lines SM1-70 and SM1-73 were 3.7 cm and 3.1 cm, respectively (Fig. 4c), leading to 65–70% of reduction in lesion length compared to the nontransgenic WT plants. Similarly, measurement of in planta bacterial growth showed that, the growth rates of *Xoo* strains PXO96 in inoculated leaves of the MoSM1-OE lines SM1-70 and SM1-73 were significantly lower than that in the nontransgenic WT plants, resulting in reduction of 10–100 folds at 6, 10 and 14 dpi (Fig. 4d). These results indicate that the MoSM1-OE lines also displayed an improved resistance to *Xoo*. 

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MoSM1-OE plants do not alter the resistance against sheath blight disease. We also evaluated the resistance level of the MoSM1-OE lines against sheath blight disease, another most important fungal disease on rice caused by *R. solani*, which is completely different in infection style from *M. oryzae*. Both *in vitro* detached leaf inoculation assay and *in vivo* sheath inoculation assay were used to evaluate the resistance level of the MoSM1-OE lines to *R. solani*. In the *in vitro* detached leaf inoculation assays, no significant difference in
lesion formed on the *R. solani*-inoculated leaves between the MoSM1-OE and nontransgenic WT plants was detected (Fig. 5a). Measurement of *in planta* fungal growth, as revealed by analyzing genomic DNA level of the G-protein \( \beta \)-subunit gene of *R. solani* with a rice Actin gene, also indicated no significant difference in fungal growth between the MoSM1-OE and nontransgenic WT plants at 3 dpi (Fig. 5b). In the *in vivo* sheath inoculation assays, lesions on the *R. solani*-inoculated sheath of the MoSM1-OE lines SM1-70 and SM1-73 were comparable in size with those on the sheath of nontransgenic WT plants at 15 dpi (Fig. 5c and d). Collectively, these results suggest that the MoSM1-OE lines did not alter the resistance to *R. solani*.

**MoSM1-OE plants contain elevated SA and JA levels.** SA and JA are most important plant hormones that play vital roles in regulating immune responses in rice\(^a\). To explore the molecular mechanism responsible for the improved resistance in MoSM1-OE lines, we examined whether the MoSM1-OE plants had altered levels of endogenous SA and JA under normal condition. The SA and JA levels in the MoSM1-OE lines SM1-70 and SM1-73 were significantly higher than those in the nontransgenic WT plants, leading to increases of 70–85% for SA and 90–141% for JA (Fig. 6a and b), respectively. These data indicate that the MoSM1-OE plants grown under normal growth condition accumulated elevated SA and JA levels.

**MoSM1-OE plants constitutively activate the SA and JA signaling pathways.** The improved resistance and the elevated SA and JA levels let us to examine whether the MoSM1-OE plants constitutively upregulated the expression of SA and JA signaling-related genes. Among them, 4 well-characterized SA pathway
genes were selected to compare their expression in the MoSM1-OE and nontransgenic WT plants under normal conditions. As shown in Fig. 6c, the expression levels of OsICS1, OsEDS1, OsNPR1 and OsPR1a, which are involved in the SA biosynthesis, signaling pathway and defense response, were markedly upregulated in MoSM1-OE plants, showing increases of 2.5, 30, 2.1 and 50 folds, respectively, as compared to the levels in nontransgenic WT plants. Similarly, the expression levels of OsAOS1, OsLOX1, OsACO7 and OsJAmyb, which are involved in JA biosynthesis and signaling pathway, were also upregulated markedly in MoSM1-OE plants, leading to increases of 2.1, 2.8, 7.8 and 3.1 folds, respectively, as compared to the levels in nontransgenic WT plants (Fig. 6d). These results indicate that the MoSM1-OE plants activated constitutively the SA and JA signaling pathways.

**MoSM1-OE plants do not have penalty on drought and salt stress tolerance.** To examine whether an improved disease resistance in MoSM1-OE lines leads to penalty on other biological processes such as abiotic stress tolerance, we compared the salt and drought tolerance of the MoSM1-OE and nontransgenic WT plants at vegetative stage. In salt stress tolerance assays, no significant difference in seed germination and seedling growth,
as revealed by the shoot and root length, was observed between the MoSM1-OE and nontransgenic WT seedlings on 1/2 MS medium with or without supplement of 150 mM NaCl, although the growth of the MoSM1-OE and nontransgenic WT seedlings was markedly inhibited on 1/2 MS medium containing 150 mM NaCl (Fig. 7a,b and c). In drought stress assays, the MoSM1-OE and nontransgenic WT plants showed similar growth status before

Figure 5. The MoSM1-OE plants show unaltered resistance against R. solani. Eight-week-old MoSM1-OE and nontransgenic WT plants were inoculated with R. solani strain GD-118 by placing mycelial discs onto detached leaves or attaching mycelial discs on sheath. (a) Disease symptom on the inoculated leaves at 3 dpi. (b) Quantification of fungal growth in inoculated leaves. Amounts of R. solani G protein β-subunit genomic DNA and rice Actin genomic DNA were estimated by qRT-PCR and relative fungal growth were shown as ratios of RsGβ/OsActin. (c) and (d) Disease symptom on the inoculated sheathes at 15 dpi. At least 20 plants in each experiment were used for assessment of the disease phenotype. Three independent experiments were performed with similar results in (a), (c) and (d). Data presented in (b) are the means ± SD from three independent experiments and no statistically significant difference at $p = 0.05$ level was detected between transgenic and nontransgenic lines.
drought treatment (Fig. 7d). Comparable drought symptom such as leaf rolling and wilting was observed between the MoSM1-OE and nontransgenic WT plants after drought stress treatment by withholding water for 12 days (Fig. 7e) and, no significant difference in growth phenotype (Fig. 7f) and survival rates (Fig. 7g) were observed between the MoSM1-OE and nontransgenic WT plants after re-watering regularly for 12 days. Collectively, these data demonstrate that the MoSM1-OE plants did not have adverse effect on drought and salt stress tolerance.

MoSM1 levels in transiently expressed leaves and stably expressed transgenic rice. To explain the phenomenon that transient expression of MoSM1 in rice leaves caused significant cell death but stable expression of MoSM1 in transgenic rice did not cause visible cell death, we detected the accumulation of MoSM1 in transiently expressed and stably expressed rice leaves. As shown in Fig. 8, a MoSM1 polyclonal antibody, prepared by immunizing rabbits with a peptide CNDLTNGQAGSLGRI, detected the accumulation of MoSM1 in both transiently expressed and stably expressed rice leaves whereas no signal was observed in transiently expressed eGFP leaves or in nontransgenic WT plants. Comparatively, the level of MoSM1 accumulation in transiently expressed rice leaves was relatively higher than those in the MoSM1-OE plants and this was much evident when the MoSM1 accumulation was normalized with the anti-β-tubulin antibody hybridized β-tubulin bands (Fig. 8). Thus, it is likely that the absence of visible cell death in leaves of the MoSM1-OE plants is largely due to a relatively low level of MoSM1, as compared with the amount of MoSM1 and the significant cell death in transiently expressed leaves.

Discussion
Many members that belong to the cerato-platanin family have been characterized from filamentous fungi including *M. oryzae* and *B. cinerea* and some of them have shown to trigger immunity in a number of plant species. We and others have shown that, transient or stable expression of *MoSM1* in Arabidopsis or application of...
Figure 7. The MoSM1-OE plants show unaltered salt and drought stress tolerance. (a–c) Growth performance (a), root length (b) and shoot length (c) of 10-day-old MoSM1-OE and nontransgenic WT seedlings grown on 1/2 MS medium with or without 150 mM NaCl. (d–f) Phenotype of 2-week-old MoSM1-OE and nontransgenic WT plants at different stages during the drought stress experiment. (g) Survival rates of the MoSM1-OE and nontransgenic WT plants at 12 days after re-watering. Three independent experiments were performed with similar results in (a), (d), (e) and (f). Data presented in (b), (c) and (g) are the means ± SD from three independent experiments and no statistically significant difference at $p = 0.05$ level was detected between transgenic and nontransgenic lines.
accumulation of MoMSP1 in rice apoplast is required for activation of defense response.\(^30,32\)

in cellular localization assays revealed that MoSM1 was targeted to the plasma membrane when transiently expressed into apoplast is critical for its PAMP activity in triggering immune response. Furthermore, our subcellular localization assays confirmed that transiently or stably expressed rice plants. We previously found that, when induced with low concentrations of DEX, MoSM1 did not produce toxic effect in MoSM1-OE Arabidopsis plants.\(^42\) Our Western blot assays confirmed that the level of MoSM1 accumulation in transiently expressed rice leaves was relatively higher than those in the MoSM1-OE plants (Fig. 8). This is probably due to the fact of only a single copy of the transgene MoSM1 in the MoSM1-OE plants vs a quite large number of copies of the MoSM1 gene in the transiently expressed rice leaves. Thus, unlike the necrosis produced by MoSM1 in transiently expressed rice leaves (Fig. 1e), the amount of MoSM1 in the tissues of the MoSM1-OE plants is not enough to cause observable necrosis in the MoSM1-OE plants. The lack of necrosis in the MoSM1-OE plants features an advantage for the use of these stable transgenic lines in development of novel rice varieties with improved disease resistance as significant necrosis may not be acceptable for practical use.

Most of CPs including MoSM1/MoMSP1 from M. oryzae have been shown to induce defense responses related to HR. Several HR characteristic features were observed for some CPs, e.g. the induction of ROS and autofluorescence, appearance of cytoplasm shrinkage and activation of defense gene expression.\(^35,41,37,44\) The present study further shows that expression of MoSM1 in transgenic rice confers an improved resistance against blast and bacterial blight diseases, probably through modulating SA/JA signaling pathways, demonstrating that MoSM1 is a promising elicitor that can be used in improvement of rice disease resistance.

No enzymatic activity has been reported to CPs including SM1 of T. virens.\(^40\) Although not all reported CPs can induce necrosis in plants,\(^33,37\) some CPs including a CP from C. platani, MoSM1/MoMSP1 from M. oryzae and BcSpl1 from B. cinerea have been shown to cause phytotoxic effects on different plants. For instance, when applied at 80μM\(^25\), purified C. platani CP induced significant necrosis in tobacco leaves and caused several structural and physiological changes, i.e. plasmolysis and accumulation of phenolic compounds.\(^34,35\) The B. cinerea BcSpl1 was found to produce a faster and stronger necrosis in tobacco leaves than the C. platani CP at a lower concentration.\(^31\) Similar to our previous observations that transiently or stably expressed MoSM1 caused typical necrosis in Arabidopsis, tobacco and tomato leaves,\(^42\) we also observed that transiently expressed MoSM1 in rice leaves did cause necrosis symptom and increased leakage of electrolyte (Fig. 1e and f). The difference in phytotoxic effects of the CPs may attribute to the intrinsic differences in CPs, the different plant species used in the assays and/or the methods used for application of the CPs to tested plants.\(^31\) Surprisingly, stably expressed MoSM1 in transgenic rice did not produce any morphological and growth change as compared to the WT plants (Fig. 2d). The difference in appearance of HR-like necrosis in transient expression of MoSM1 in rice leaves and in stable expression of MoSM1 in transgenic rice may be caused by the amounts of MoSM1 accumulated in transiently or stably expressed rice plants. We previously found that, when induced with low concentrations of DEX, MoSM1 did not produce toxic effect in MoSM1-OE Arabidopsis plants.\(^42\) Our Western blot assays confirmed that the level of MoSM1 accumulation in transiently expressed rice leaves was relatively higher than those in the MoSM1-OE plants (Fig. 8). This is probably due to the fact of only a single copy of the transgene MoSM1 in the MoSM1-OE plants vs a quite large number of copies of the MoSM1 gene in the transiently expressed rice leaves. Thus, unlike the necrosis produced by MoSM1 in transiently expressed rice leaves (Fig. 1e), the amount of MoSM1 in the tissues of the MoSM1-OE plants is not enough to cause observable necrosis in the MoSM1-OE plants. The lack of necrosis in the MoSM1-OE plants features an advantage for the use of these stable transgenic lines in development of novel rice varieties with improved disease resistance as significant necrosis may not be acceptable for practical use.\(^32\)

Most of CPs including MoSM1/MoMSP1 from M. oryzae have been shown to induce defense responses related to HR. Several HR characteristic features were observed for some CPs, e.g. the induction of ROS and autofluorescence, appearance of cytoplasm shrinkage and activation of defense gene expression.\(^35,41,37,44\) The present and our previous studies showed that MoSM1 can induce visible HR in both Arabidopsis and rice leaves, as verified by the appearance of rapid cell death and the induction of electrolyte leakage, and expression of defense genes (Fig. 1e,f and g). Similar results were also obtained with recombinant MoMSP1, which triggered cell death and induced H\(_2\)O\(_2\) production in both suspension-cultured cells and rice leaves.\(^32\) On the other hand, it was shown that MoMSP1 is secreted into culture medium in vitro and into apoplast in planta during infection and that a high level of accumulation of MoMSP1 in rice apoplast is required for activation of defense response.\(^30,32\) We observed that transient expression of MoMSP1,\(^35\), which lacked the SP, in N. benthamiana leaves abolished HR (Fig. 1a), which is similar to the previous observation that deletion of SP led to loss of the activity of MoMSP1 to up-regulate expression of defense genes in rice leaves.\(^32\) These data support the hypothesis that MoSM1/MoMSP1, along with other CPs studied so far, indeed function as typical PAMPs that can trigger PTI and that the secretion of MoSM1 into apoplast is critical for its PAMP activity in triggering immune response. Furthermore, our subcellular localization assays revealed that MoSM1 was targeted to the plasma membrane when transiently expressed in N. benthamiana leaves (Fig. 1c), which is similar to the observation that B. cinerea BcSpl1 associates with
the plasma membrane of cells in tomato and tobacco. It was found recently that Arabidopsis BAK1, a critical component of the PAMP signaling complex, plays a role in the induction of necrosis by BcSpl1. Similar to the common knowledge that some well-known PAMPs such as FLS2 for flg22 were found to be recognized by the extracellular regions of the plasma membrane surface-localized pattern recognition receptors, a yet unknown receptor that recognizes MoSM1/MoMSP1 may be localized on the plasma membrane of plant cells. Therefore, it can be speculated that, during infection or when expressed in rice, MoSM1/MoSP1 is secreted through a SP-guided process into apoplast, and is then recognized by a plasma membrane-localized receptor(s), followed by the initiation of HR and PTI. This speculation explains, at least partially, the importance of MoSM1/MoSP1 secretion into apoplast in triggering the immunity, and the feature of the MoSM1/MoSP1 plasma membrane localization (Fig. 1c). Further identification of MoSM1/MoMSP1 receptor or receptor complex and its associated components will broaden our understanding of the molecular mechanism of MoSM1/MoMSP1, especially the clarification of the earliest events and downstream signaling pathway involved in MoSM1/MoMSP1-induced HR and immunity.

Several PAMPs such as flg22 and lipopolysaccharides have been shown to induce a systemic defense response with the characteristics of SAR. Indeed, a number of CPs from different filamentous fungi, e.g. SM1, SM2 and Ep1 from the biocontrol agents T. virens and T. atroviride, as well as BcSpl1 from B. cinerea and a CP from C. platani, were shown to induce both local and systemic disease resistance in host and nonhost plants against different life style pathogens. In the present study, we found that overexpression of MoSM1 in transgenic rice plants confers an improved resistance against two strains representing different races of O. oryzae and Xoo, as revealed by reduced disease severity and suppressed in planta pathogen growth (Figs 3 and 4). Similarly, application of recombinant MoMSP1 at a sub-lethal concentration was also found to potentiate resistance against M. oryzae, with no significant cell death on rice leaves. However, the MoSM1-OE plants seem to be more effective to blast disease than to bacterial blight disease, as the blast disease severity and the in planta growth of O. oryzae were almost completely suppressed (Fig. 3) while the bacterial blight disease severity were suppressed by ~70% (Fig. 4). In addition, MoSM1 was previously found to induce resistance in Arabidopsis in planta Xoo growth revealed by reduced disease severity and suppressed in planta pathogen growth (Figs 3 and 4). These data indicate that MoSM1, like other CPs from both beneficial and pathogenic fungi, confers a broad-spectrum resistance against pathogens with different life styles in host and non-host plants, providing a great potential for application of MoSM1 in genetic improvement of disease resistance in other crop plants. The MoSM1-OE plants did not show a decreased resistance against sheath blight disease, although they did not gain an increased resistance (Fig. 5). This may be due to that the molecular mechanism responsible for defense response against R. solani differs from those for defense response against M. oryzae and Xoo. Similar results were also observed in OsWAK25-OE plants, which show resistance to both M. oryzae and Xoo but display increased susceptibility to R. solani.

SA and JA signalings are effective against both biotrophic and necrotrophic pathogens in rice. Given that MoSM1 functions as a PAMP, MoSM1 is hypothesized to be recognized by an unknown receptor, resulting in initiation of signal transduction pathway to activate defense response in rice. We found that the MoSM1-OE plants contained elevated levels of both SA and JA, two critical defense signaling hormones, and upregulated expression of SA- and JA-related biosynthesis and signaling genes (Fig. 6), indicating that the MoSM1 plants constitutively activate the SA- and JA-mediated defense signaling pathways. This is similar to the function of MoSM1 in transgenic Arabidopsis plants. Generally, rice plants contain high levels of endogenous SA that are weakly responsive to pathogen infection; however, SA can act in both of OsNPR1- and OsWRKY45-dependent pathways in rice defense response. The improved resistance in MoSM1-OE plants may be due to the upregulated expression of SA signaling regulatory genes such as OsNPR1, whose overexpression in rice leads to constitutive activation of defense response, rather than the elevated level of SA. The involvement of SA signaling in defense response of the MoSM1-OE plants is partially supported by the upregulated expression of OsPR1s and OsPR10 (Figs 1g and 6c), defense genes that are activated by the SA signaling pathway, by transiently or stably expressed MoSM1 in rice plants. This is in agreement with the observations that a CP from C. platani in Arabidopsis and BcSpl1 from B. cinerea in tobacco were shown to function through SA signaling but differs from the action of MoMSP1 in rice, in which SA suppressed MoMSP1-induced H2O2 production and cell death in suspension cultured rice cells. Whereas exogenous SA applied at a high concentration (e.g. 5 mM) suppressed MoMSP1-induced cell death and H2O2 production in suspension cultured cells, MoSM1 in transgenic rice plants promoted endogenous SA level (Fig. 6a). Alternatively, SA is differentially involved in MoSM1/MoMSP1-induced cell death and defense response in rice. On the other hand, manipulation of the JA biosynthesis by overexpression of OsAOS2, encoding allene oxide synthase that is a key enzyme involved in JA biosynthetic pathway, increased the endogenous JA level, which lead to activation of defense gene expression and enhancement of resistance to M. oryzae42. MoSM1 activates JA signaling in the MoSM1-OE plants by upregulating the expression of JA biosynthesis and signaling genes (Fig. 6b and d), similar to the observation that JA enhanced MoMSP1-induced cell death in rice. It is therefore likely that JA signaling is involved in MoSM1/MoSP1-regulated defense response. This is inconsistent with a recent finding that function of the CP from C. platani triggers SA- and ethylene (ET)-signaling pathways, but not the JA-signaling pathway, as revealed by the expression pattern of marker genes. Overall, the CP-triggered defense signaling pathways may be complicated and depend on specific CPs and/or the tested plant species.

The effect of CPs on plant growth and development as well as on the abiotic stress tolerance is not known yet. In the present study, we were unable to observe any morphological and developmental changes at seedling and adult stages and grain yield in the MoSM1-OE plants in comparison to the WT plants (Fig. 2), indicating that MoSM1 does not have effect on plant growth and development. On the other hand, we also did not observe distinguishable difference in salt and drought tolerance between the MoSM1-OE and WT plants (Fig. 7), indicating that overexpression of MoSM1 in transgenic rice did not disturb the abiotic stress tolerance.
Conclusion

We demonstrate in the present study that MoSM1, a member of cerato-platanin family from *M. oryzae*, can activate defense response in rice and that the MoSM1-OE plants gain an improved broad-spectrum resistance against fungal blast and bacterial blight, two most devastating diseases of rice worldwide. We also show that the enhanced resistance in the MoSM1-OE plants is regulated through a combination of SA- and JA-mediated signaling pathways. Furthermore, the MoSM1-OE plants do not have any deleterious effect on drought and salt tolerance as well as on important agronomic traits. Taken together, our data demonstrate that overexpression of MoSM1 in rice confers an improved broad-spectrum resistance against blast and bacterial blight diseases without any penalty on abiotic stress tolerance and grain yield, providing a promising potential for application of MoSM1 in improvement of disease resistance in other crop plants. Further identification of putative MoSM1 receptor(s), characterization of the MoSM1-triggered signaling pathway and genome-wide expression profiling of MoSM1-regulated genes will greatly help to elucidate the molecular mechanism by which MoSM1 regulates plant immunity.

Methods

**Plant materials and growth.** Rice (*Oryza sativa* L.) subsp. *indica* cv. Yuanfengzao was used in the transient expression experiments while *subsp. japonica* cv. Xiushui11 was used in transgenic study. Rice and *N. benthamiana* plants were grown in a growth room set at 28°C, 14 hr light (>3,000 lux)/2°C, 10 hr dark.

**Transient expression assays.** Infiltration suspension of agrobacteria carrying pINDEX3::MoSM1 or empty pINDEX3::00 construct was prepared as described previously. To construct the SP-deletion vector, the fragment of *MoSM1* without SP was amplified using primers *MoSM1-ΔSP-F* and *MoSM1-ΔSP-R* (Supplementary Table S1), digested with EcoRI/Sall, and then inserted into pINDEX3 vector, yielding pINDEX3::MoSM1ΔSP. Agrobacteria carrying pINDEX3::MoSM1, pINDEX3::MoSM1ΔSP or empty pINDEX3::00 were infiltrated into leaves of 8-week-old *N. benthamiana* or 5-week-old rice plants using a 1-µL syringe without a needle. The agroinfiltrated plants were foliar sprayed with 1 µM DEX solution 24 hr later to induce the expression of MoSM1. Leaf samples were collected at indicated time points after DEX treatment and used for analyses of electrolyte leakage and defense-related gene expression. For measurement of electrolyte leakage, 200 mg leaf samples were put into 5 ml distilled water for 3 hr at room temperature and the initial conductivity was measured using a DDS-IIAT digital conductivity meter. The samples were then boiled for 15 min to disrupt plant tissue, and the total conductivity was measured using the same meter. Percentage of electrolyte leakage was calculated as (initial conductivity before boiling)/(total conductivity after boiling) × 100%. Experiments were repeated three times using at least 6 leaves from 6 individual plants per treatment in each experiment.

**Subcellular localization assays.** The MoSM1 coding region was amplified using primers MoSM1-SL-F and MoSM1-SL-R (Supplementary Table S1), digested with BamHI/XbaI, and cloned into pFGC-eGFP, yielding pFGC-eGFP-MoSM1. Similarly, the coding sequence of *AtPIP1;4*, encoding for a previously reported membrane-localized aquaporins, was cloned into pCAMBIA1300-mCherry, yielding pCAMBIA1300-AtPIP1;4-mCherry. The recombinant plasmids pFGC-eGFP-MoSM1, pCAMBIA1300-AtPIP1;4-mCherry and the empty vector pGFP-EGFP were introduced into *N. benthamiana* plants using 1-µL needleless syringes. The agroinfiltrated plants were allowed to grow in a growth room at 25°C for 24 hr and agroinfiltrated leaves were collected. GFP/mCherry fluorescence were imaged using a Zeiss LSM710 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

**Western blotting assays.** Samples from transiently expressed leaves of *N. benthamiana* and rice plants or from leaves of the stable transgenic rice plants were ground in 200 µL extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 1 × protease inhibitor cocktail, and 1 mM PMSF) and then added 100 µL loading buffer. After boiling for 5 min, the extracts were centrifuged at 10000 g for 10 min at 4°C. The supernatant was separated on a 15% SDS-PAGE gel, followed by transferring onto PVDF membrane via wet electroblotting. Detection of GFP in transiently expressed *N. benthamiana* leaves was performed using a mouse monoclonal GFP antibody (Huaan Company, Hangzhou, CA, USA). Agrobacteria harboring pCAMBIA1300-AtPIP1;4-mCherry into leaves of 8-week-old *N. benthamiana* plants using 1-µL needleless syringes. The agroinfiltrated plants were allowed to grow in a growth room at 25°C for 24 hr and agroinfiltrated leaves were collected. GFP/mCherry fluorescence were imaged using a Zeiss LSM710 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

**Generation and characterization of MoSM1-OE lines.** The full coding sequence of MoSM1 including SP was amplified using primers MoSM1-OE-F and MoSM1-OE-R (Supplementary Table S1), digested with BamHI/Smal, and inserted into the binary vector pCoUm under the control of a maize *ubiquitin* promoter, yielding plasmid pCoUm-Ubi::MoSM1. The resulting construct was introduced into *A. tumefaciens* strain EHA105 and transformed into rice calli of cv. Xiushui11 through the Agrobacterium-mediated transformation protocol. The obtained MoSM1-overexpressing transgenic lines were screened by planting seeds on 1/2 MS medium containing 50 µg/L hygromycin (Hgr) and lines showing 3:1 (Hgr-resistant: Hgr-susceptible) segregation were selected as candidates of single-copy transgenic lines. Individual lines of T3 generations showing 100% Hgr resistance on selective medium were selected as homozygous lines. Further characterization of the MoSM1-OE plants do not have any deleterious effect on drought and salt tolerance as well as on important agronomic traits. The obtained MoSM1-overexpressing transgenic lines were screened by planting seeds on 1/2 MS medium containing 50 µg/L hygromycin (Hgr) and lines showing 3:1 (Hgr-resistant: Hgr-susceptible) segregation were selected as candidates of single-copy transgenic lines. Individual lines of T3 generations showing 100% Hgr resistance on selective medium were selected as homozygous lines. Further characterization of the MoSM1-OE plants.
A 589 bp fragment of the HptII gene was amplified using a pair of primers HptII-Probe-F and HptII-Probe-R (Supplementary Table S1) and labelled with DIG by the random priming method using a DIG High Prime DNA Labeling and Detection kit I (Roche Diagnostics, Shanghai, China). Prehybridization, hybridization and detection were performed according to the manufacturer’s recommendations. Homozygous lines with single-copy of transgene were used for all experiments.

Disease assays. M. oryzae strains 85-14 (race ZB1) and 97-220 (race E3) were grown on CM medium at 25°C for 10 days and spores were collected to prepare inoculum. Two-week-old seedlings at three leaf stages were inoculated by spraying with 5 × 10^6 spores/mL spore suspension containing 0.02% Tween-20. The inoculated plants were kept in the dark for 24 hr at 25°C with 100% relative humidity and then moved to the growth room with same condition. Disease severity was evaluated using a standard international 0–9 scale (0 = resistant and 9 = susceptible) from at least 30 plants for each line at 7 dpi. Fungal growth in inoculated leaves was estimated using qRT-PCR by analyzing the genomic DNA level of a R. solani mycelial agar plugs inoculated on the detached leaves were removed and genomic DNA was extracted from the phenotypic tissues. For measurement of fungal growth in inoculated leaves, the mycelium was collected by centrifugation. Eight-week-old plants were inoculated by attaching mycelial agar plugs onto detached leaves. Disease phenotype was examined at 15 days after inoculation. For detached leaf inoculation assays, R. solani strain GD-118 was grown in potato dextrose agar medium and inoculation was performed by placing mycelial agar plugs onto detached leaves. Disease phenotype was examined at 3 days after inoculation. For measurement of fungal growth in inoculated leaves, the mycelial agar plugs inoculated on the detached leaves were removed and genomic DNA was extracted from the leaf tissues. The fungal amount was determined using qRT-PCR by analyzing the genomic DNA level of a R. solani cDNA was synthesized from 1 μg of total RNA using AMV reverse transcriptase (TaKaRa, Dalian, China). First-strand cDNA was synthesized from 1 μg of total RNA using AMV reverse transcriptase (TaKaRa, Dalian, China) according to the manufacturer’s recommendations. The qRT-PCR was performed using a CFX96 real-time PCR system (BioRad, Hercules, CA, USA) using Fast Essential DNA Green Master kit (Roche Diagnostics, Shanghai, China). The rice Actin gene was used as an internal control to normalize the data and relative expression levels of genes of interest were calculated using the 2^−ΔΔCt method. Gene-specific primers used in qRT-PCR are listed in Supplementary Table S1. Three independent biological samples were performed.

Quantification of SA and JA content. Quantification of SA and JA was carried out according to the method described previously. Briefly, 300 mg leaf tissues were ground in liquid nitrogen with mortar and pestle, extracted with 2 mL 80% methanol and kept overnight at −20°C. After centrifugation at 4°C for 10 min at 12,000 × g, the supernatant was collected, dried under nitrogen gas and then dissolved in 0.5 mL 2% ammonia solution. The collected extracts were loaded on preconditioned Oasis MAX SPE columns (Waters Corp., Milford, MA) and sequentially washed with ammonia solution (2%) and 2 mL methanol. SA and JA in the column were eluted with 4 mL methanol containing 1% formic acid. The eluent was dried under nitrogen gas and finally dissolved in 0.5 mL 80% methanol. Quantification of SA and JA was performed by a HPLC-Triple quadrupole liquid chromatography-mass spectrometry system (Model 1290/6460, Aglient Technologies, Santa Clara, CA) with stable isotope-labeled SA and JA as standards. The experiments were repeated three times and data were averaged.

Drought and salt tolerance assays. For drought tolerance assays, 4-week-old seedlings were grown in the same pot and were subjected to drought stress treatment by withholding watering for 12 days, followed by recovery with normal water supply for another 7 days. Plants with green and healthy young leaves at 12 days after re-watering were regarded as survivors and survival rate was calculated as percentage of the survived plants in total treated plants. At least 10 plants for each line were used in each experiment. For salt tolerance assays, 100 seeds were germinated on 1/2 MS medium supplemented with or without 150 mM NaCl and allowed the seedling growth on the same medium. At 6 days after germination under 28°C/25°C (day/night) with a 12 hr photoperiod, shoot height and fresh weight of seedlings of each line were measured. The experiments were repeated three times and data were averaged.
Evaluation of grain yield. Rice plants were grown in a restricted greenhouse with natural sunlight and agronomic traits were evaluated. Harvested grains were air-dried and stored at room temperature for 1 month. Twenty random panicles from each line were chosen for measuring the panicle weight and total grains per panicle according to the conventional methods. Seed setting rate was calculated as percentage of fully filled seeds in total seeds of a single panicle. Experiments were repeated three times and data were averaged.

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Author Contributions
FS. conceived the study. Y.H. and F.S. designed the experiments. Y.H., Y.Z.H., L.H. and D.L. performed the experiments. Y.H. and F.S. analyzed the data. F.S. drafted the manuscript, and all authors read and approved the final manuscript.

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