**Gene expression analysis, subcellular localization, and in planta antimicrobial activity of rice (*Oryza sativa* L.) defensin 7 and 8**

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**Abstract**

Defensins are a group of plant antimicrobial peptides. In a previous study, it was reported that two recombinant rice (*Oryza sativa* L.) defensin (*OsDEF*) genes (*OsDEF7* and *OsDEF8*) produced heterologously by bacteria inhibited the growth of several phytopathogen. Here, we analyzed gene expression patterns in Thai jasmine rice (*O. sativa* L. ssp. *indica* ‘KDML 105’) using quantitative reverse transcription-polymerase chain reaction and compared them with those in Japanese rice (*O. sativa* L. ssp. *japonica* ‘Nipponbare’). Although the cultivars exhibited similar gene expression patterns at the developmental stages examined, the expression levels differed between organs. Upon *Xanthomonas oryzae* pv. *oryzae* infection in the leaves, both *OsDEF*s were highly upregulated at 8 days post-infection, suggesting that they play a role in pathogen defense. Moreover, in silico analyses revealed that *OsDEF* expression levels were affected by drought, cold, imbibition, anoxia, and dehydration stress. Using green fluorescent protein (GFP) fusions, we found that both OsDEFs were in the extracellular compartment, confirming their functions against pathogen infection. However, when recombinant OsDEFs (without GFP) were produced in tobacco BY-2 cells or *Nicotiana benthamiana* leaves, they could not be detected in either the culture medium or the cells. Yet, *N. benthamiana* leaves infiltrated with OsDEF7 or OsDEF8 constructs exhibited in planta inhibitory activity against the phytopathogen *Xanthomonas campestris* pv. *glycines*, suggesting that recombinant OsDEFs were present. Additionally, when targeting them to the ER compartment, recombinant OsDEFs could be detected. Lower inhibitory activity was observed when recombinant OsDEFs were targeted to the ER. These results suggest that OsDEFs play a role in controlling plant diseases.

*Keywords**:* Antimicrobial peptide; Defensin; Infection; *Nicotiana benthamiana*; Production; Rice; Subcellular localization

*Abbreviations:* AMP, antimicrobial peptide; DEF, defensin; GFP, green fluorescent protein

**1. Introduction**

Antimicrobial peptides (AMPs) are key components of the innate immune system in both plants and animals. Plant AMPs comprise many families, such as defensins (DEFs), thionins, and snakins, and are small, cysteine-rich peptides that generally contain fewer than 100 amino acids. They exhibit non-specific antimicrobial activity against bacteria and fungi by invading their cell membranes (Jenssen et al., 2006); consequently, they are more effective than other antibiotics, because pathogens find it difficult to develop resistance against them. Most of these peptides contain eight cysteine residues that form four disulfide bonds, which are responsible for stabilizing their three-dimensional structures and protein folding (de Oliveira Carvalho and Gomes, 2009). Although plants possess many genes that encode different classes of AMP, they are expressed at low levels in infected tissue (Tantong et al., 2016). Most plant AMPs are highly expressed in reproductive organs and seeds (Silverstein et al., 2007), but are rarely expressed in leaves, which are a common target of many pathogens. Subcellularly, plant AMPs are localized to different parts of the cell, depending on their signal peptides and possible functions. Plant DEFs have been reported to be localized either in extracellular spaces (Gao et al., 2000; Jha et al., 2009; Terras et al., 1995) or in the vacuole (Lay et al., 2003). Both the snakin-1 (SN1) protein from *Solanum tuberosum* and the DEF Rs-AFP2 protein from *Raphanus sativus* have been localized to the plasma membrane (Jha and Chattoo, 2010; Nahirnak et al., 2012) The TaGASR1 protein from wheat has been localized to either the cell membrane or cytosol (Zhang et al., 2016), and OsGASR1 and OsGASR2 proteins from rice (*Oryza sativa* L.) have been localized to the apoplasm or cell wall (Furukawa et al., 2006). It is probable that AMPs are in the apoplast, because it is where they can come into contact with pathogenic microorganisms during the early stages of infection.

DEFs have been widely studied in various plant species because of their inhibitory activity against pathogens (Lay and Anderson, 2005). The overexpression of heterologous DEFs results in resistance against the fungus *Cercospora beticola* in *Arabidopsis thaliana* (De Coninck et al., 2010), and against the fungi *Magnaporthe oryzae* and *Rhizoctonia solani* in rice (Jha and Chattoo, 2010). Although rice is globally one of the most important agricultural crops and pathogen invasion results in a reduction in grain yield, DEFs in rice have received little attention. Despite having many annotated DEFs in its genome, most rice DEFs have not been studied. Previously, based on in silico analyses, our group found that rice DEF7 (*OsDEF7*; LOC\_Os02g41904) and DEF8 (*OsDEF8*; LOC\_Os03g03810) from Japanese rice (*O. sativa* L. ssp. *japonica* ‘Nipponbare’) were co-expressed with pathogen-responsive genes. Recombinant OsDEF7 and OsDEF8 produced in *Escherichia coli* exhibit antimicrobial activity against phytopathogens such as *Xanthomonas oryzae* pv. *oryzae* and *Fusarium oxysporum* f.sp. *cubense*,suggesting that they could be used in disease control (Tantong et al., 2016). However, the in planta characterization of these two DEFs has not been conducted.

In this study, we compared the expression patterns of these two genes using quantitative real-time polymerase chain reaction (qRT-PCR) in Thai jasmine rice (*O. sativa* L. ssp. *indica* ‘KDML 105’) and Japanese rice. We also investigated *OsDEF* expression patterns upon pathogen infection, and under other conditions. We confirmed the subcellular localization of both DEFs, and produced recombinant DEFs in tobacco BY-2 cells and *Nicotiana benthamiana* leaves. In addition,in planta antimicrobial activity was measured. Our results suggest a potential use of both DEFs in pathogen resistance, and provide additional information concerning alternative hosts that produce AMPs for other biological applications.

**2. Materials and methods**

*2.1. Plant materials*

Thai jasmine rice (*Oryza sativa* L. ssp. *indica* ‘KDML 105’) seeds were germinated in deionized water at room temperature without light for 2–3 days until the coleoptiles appeared. Germinated seeds were separated into three replicates (eight plants per replicate) and grown in Yoshida’s nutrient solution (Yoshida et al., 1976). The plants were grown in a growth chamber under 60% relative humidity, 25 °C, a light intensity of 6000 lux, and a 16-h photoperiod. Plant materials were collected at 5 days (germinating seed), 2 weeks (seedling), and 1 month (mature). Flowers were also collected, and leaves and roots were collected separately. The samples were wrapped in aluminum foil and frozen by liquid nitrogen. The frozen tissues were then ground into a fine powder using a mixer mill (MM400, Retsch, Germany).

*Nicotiana benthamiana* seeds were germinated in small pots with peat moss and grown under controlled conditions of 25 °C, at light intensity of 4000 lux, and a 16-h photoperiod for 1.5 months.

Tobacco BY-2 cells were obtained from RIKEN BRC, Japan, and cultured on Murashige and Skoog (MS) salt mixture medium containing 3% sucrose, 0.2 mg/mL of KH2PO4, and 0.2 μg/mL of 2,4-dichlorophenoxyacetic acid in the dark at 25 °C. Plant cultures on agar plates were subcultured once a month, and the cultures in the liquid medium were subcultured every week with shaking at 120 rpm on an orbital shaker.

*2.2. Bacterial strains*

*Escherichia coli* DH5α and *Agrobacterium tumefaciens* strain GV3101 were used. *Xanthomonas* *oryzae* pv. *oryzae* (DOAC 4-1570, isolated from the leaves of *O. sativa* L.) was purchased from the DOAC Culture Collection Center, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. *Xanthomonas campestris* pv. *glycines* (TISTR 786) was obtained from the Institute of Scientific and Technological Research Culture Collection, Thailand.

*2.3.* OsDEF7 *and* OsDEF8 *expression patterns in Thai jasmine rice under normal growth conditions*

Total RNA was isolated from the ground, frozen tissue samples at different developmental stages and in different organs using a RNeasy® Plant Mini Kit (Qiagen, USA). Total RNAs were treated with RNase-Free DNase for 10 min at room temperature. The cDNAs were synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, USA) using 1 μg of RNA as the template. The qRT-PCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad). Each PCR contained 5 μL of EvaGreen® Supermix, 2 μL of the diluted cDNA (20 ng of RNA), 2 μL of nuclease free water, and 10 μM of each primer, in a total reaction volume of 10 μL. The primers used to amplify *OsDEF7* were def7RT-F (5′-GTGAGCAGCAACAACTGCG-3′) and def7RT-R (5′-CGACGAGCAATGCGACTG-3′), and those used to amplify *OsDEF8* were def8RT-F (5′-TGATCGATGAACCAGCAGCTA-3′) and def8RT-R (5′-GGATGGATGGTGGATGCAC-3′). The PCRs were performed under the following conditions: 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 10 s at 58 °C. Amplicon dissociation curves were analyzed after cycle 40 by heating from 65 to 95 °C with a ramp speed of 0.5 °C for 0.15 s. Relative gene expression levels were calculated by the comparative CT method (Schmittgen and Livak, 2008) using *Os*EF-1α as a reference gene. The primers used to amplify *OsEF*-1α were *OsEF*1α-F (5′-ATGGTTGTGGAGACCTTC-3′) and *OsEF*1α-R (5′-TCACCTTGGCACCGGTTG-3′). The gene expression patterns in Thai jasmine rice were compared with those in the GENEVESTIGATOR® microarray database of Japanese rice (Hruz, 2008).

*2.4.* OsDEF7 *and* OsDEF8 *expression patterns in infected Thai jasmine rice*

X. *oryzae* pv. *oryzae* was cultured in a nutrient broth for 48 h at 28 °C, with 250-rpm shaking. The cultures were diluted with nutrient broth to an appropriate optical density at 600 nm (OD600) of 0.2. The first three leaves of two-week-old plants were then cut about 1 inch from the tip and dipped in a suspension culture of *X*. *oryzae* pv. *oryzae* for 1 min. For the control treatment, the cut leaves were dipped into nutrient broth. The infected and control plants were left for 6 h, 4 days, and 8 days. The treated leaves were collected two inches from the cut edge. Subsequently, RNA was extracted from the samples, cDNAs were synthesized, and qRT-PCR was performed, as described in *2.3*.

*2.5. In silico* *analysis*

The GENEVESTIGATOR® database (Hruz, 2008) was used to analyze *OsDEF7* and *OsDEF8* expression levels in Japanese rice at different developmental stages and in different organs. Effects of the abiotic stressors drought, cold, imbibition, anoxia, and dehydration on *OsDEF7* and *OsDEF8* expression in the rice cultivars were also examined using the same database. WoLF PSORT software (Horton et al., 2007; http://wolfpsort.org) was used for subcellular localization, and membrane anchor-sequence predictions were obtained using AMPHIPASEEK (Sapay et al., 2006).

*2.6. Construction of plant expression vectors*

A pUC18 vector containing either *OsDEF7* or *OsDEF8* was used as a template, and *OsDEF7* and *OsDEF8* expression constructs were generated using Gateway® cloning technology. The following primers were designed to exclude the stop codon: DEF7-F (5′-ATGGCTCCGTCTCGTCGCATG-3′), DEF7-R (5′-GCAGACCTTCTTGCAGAAGCA-3′), DEF8-F (5′-ATGGAGGCTTCACGCAAGGTGTT-3′), and DEF8-R (5′-GGGGCAGGGCTTGGTGCA-3′). PCR amplifications were performed using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, USA) as follows: 30 s at 98 °C, 30 cycles of 15 s at 98 °C, 30 s at 60 °C, and 45 s at 72 °C, followed by 5 min at 72 °C. The PCR products were ligated into a pCR®8/GW/TOPO®TA Cloning Kit (Thermo Scientific, USA). Subsequently, the *OsDEF* coding region was recombined into either the C-terminal green fluorescent protein (GFP) fusions destination vector, pGWB5 (Nakagawa et al., 2007) to generate pGWB5-*OsDEF*s, or the C-terminal His-tag fusions destination vector, pEAQ-HT-DEST3 (pEAQ3) (Peyret and Lomonossoff, 2013) to generate pEAQ3-*OsDEF*s using LR Clonase® II (Thermo Scientific). For ER-targeting OsDEFs, *OsDEF*s were codon-optimized for *N. benthamiana* using a codon optimization tool (http://www.idtdna.com/CodonOpt) (Supplemental Fig. S1). Synthetic,codon-optimized *OsDEF7* (opt*OsDEF7*) and *OsDEF8* (opt*OsDEF8*) (gBlocks® Gene Fragments, Integrated DNA Technologies, Singapore) that included a His-tag and the SEKDEL ER-retention motif downstream were amplified as previously described, and finally recombined into the pEAQ-HT-DEST1 (pEAQ1) vector (Peyret and Lomonossoff, 2013) to generate pEAQ1-opt*OsDEF*-His-SEKDELs. All of the expression vectors constructed were transformed into *A. tumefaciens* GV3101 by electroporation.

*2.7. Subcellular localization*

2.7.1. Agroinfiltration into *N. benthamiana* leaves

A single colony of recombinant *A. tumefaciens* containing each of the pGWB5-*OsDEF*s was cultured in lysogeny broth containing 50 μg/mL of kanamycin, 50 μg/mL of hygromycin B, 50 μg/mL of rifampicin, and 50 μg/mL of gentamycin, and grown for two nights at 30 °C with shaking at 250 rpm. The cultures were harvested by centrifugation at 5000 × g for 15 min and the pellet was resuspended in 10 mM MMA buffer (MES-MgCl2-acetosyringone) to an OD600 of 1, and then incubated at room temperature for 2–3 h. The cells were used for co-infiltration at a 1:1 ratio with *A. tumefaciens* containing the p19 gene, a silencing suppressor (Lindbo, 2007), into the abaxial leaves of 6-week-old plants using a needleless 1-mL syringe. The infiltrated plants were left for 5 days. Pieces of the infiltrated leaves were sampled from the infected area and observed under a confocal laser scanning microscope (FluoView FV1000, Olympus, Japan). GFP was excited at 488 nm, and the emission filter wavelengths were 497 to 526 nm.

2.7.2. Transient transformation of BY-2 cells

The *Agrobacterium* culture containing each of the pGWB5-*OsDEF*s was harvested and resuspended in MS medium to an OD600 of 1. In total, 100 μL of each culture of *Agrobacterium* and 100 μL of *A. tumefaciens* containing p19 were co-incubated with 5 mL of a tobacco BY-2 cell suspension at an exponential phase of growth (3 days after subculture), before 5 µL of 20 mM acetosyringone was added. The co-incubated culture was kept at 25 °C in the dark. After 5 days, the cultures were harvested by centrifugation at 12,000 × g for 15 min, and the supernatant was observed for GFP fluorescence under ultraviolet (UV) light.

*2.8. Production of recombinant* OsDEF*s*

2.8.1. BY-2 cells

A 5-mL BY-2 cell suspension at an exponential phase of growth was transferred to a culture dish and incubated at 25 °C with 100 μL of *A. tumefaciens* that harbored each transgene expression construct pEAQ3-*OsDEF* and 5 μL of 20 mM acetosyringone at an OD600 of 1 in the dark. After 48 h of cocultivation, the cell suspension was washed with MS medium three times and plated on MS agar medium containing 50 μg/mL of kanamycin and 500 μg/mL of carbenicillin. After 2–3 weeks, the transformed BY-2 cells were transferred onto fresh solid MS medium containing 50 μg/mL of kanamycin. Positive clones were inoculated separately into a MS liquid medium containing 50 μg/mL of kanamycin for 1 week with 120-rpm shaking. The supernatant was collected after centrifugation at 5000 × g for 15 min. Then the supernatants were dialyzed overnight against phosphate buffer and lyophilized. Total proteins of 1 μg per lane were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in tricine buffer and transferred to a polyvinylidene difluoride membrane, and the His-tag was detected using a Pierce™ His-tag mouse monoclonal antibody (Thermo Scientific) diluted 1:2000. Binding was detected using alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody diluted 1:1000 (Thermo Scientific). 5-bromo-4-chloro-3-indolyl-phosphate was used in conjunction with nitro blue tetrazolium (Thermo Scientific) for the colorimetric detection of alkaline phosphatase activity.

2.8.2. *N. benthamiana* leaves

*A. tumefaciens* harboring each of the pEAQ3-*OsDEF*s or pEAQ1-opt*OsDEF*-His-SEKDELs was infiltrated into 6-week-old plants as described in 2.6.1., without co-infiltration with p19. After 5 days, the infiltrated leaves were collected and frozen with liquid nitrogen. Frozen leaves (0.1 mg fresh weight) were ground into a fine powder using a mixer mill and extracted in 1 mL of lysis buffer (10 mM Tris-HCl (pH 6.2), 50 mM KCl, 6 mM MgCl2, 0.4 M NaCl, 1% (v/v) Triton X-100, and 10 mM ethylenediaminetetraacetic acid) (Company et al., 2014), centrifuged at 15,000 × g for 15 min at 4 °C, and then purified using His SpinTrap™ (GE Healthcare, UK). The purified protein was separated and detected by western blot analysis, as previously described.

*2.9. In planta inhibitory activity*

*A. tumefaciens* harboring each of the pEAQ3-*OsDEF*s orpEAQ1-opt*OsDEF*-His-SEKDELs at an adjusted OD600 of 2 was co-infiltrated at a 1:1 ratio with *X. campestris*, which was cultured overnight in nutrient broth at 28 °C, shaken at 250 rpm, and adjusted to an OD600 of 0.4, into the abaxial leaves of 6-week-old plants as described in 2.7.1. The infiltrated plants were left for 9 days, monitored for disease symptoms, and compared with controls that were 1) co-infiltrated with *A. tumefaciens* harboring an empty pEAQ3 vector and *X. campestris*,or 2) infiltrated with *X. campestris* at different positions on the same leaf. All of the experiments included at least five biological replicates.

**3. Results and discussion**

*3.1. Gene expression patterns of* OsDEF7 *and* OsDEF8

The GENEVESTIGATOR® microarray database provided *OsDEF* expression levels in the Japanese rice cultivar ‘Nipponbare’ at different developmental stages and in different organs (Supplemental Fig. S2), and we compared them with those in Thai jasmine rice at similar stages, including germination, the seedling stage, and flowering. The unique amplicons obtained in the qRT-PCR revealed that *OsDEF7* was most highly expressed at the germination stage, whereas *OsDEF8* was most highly expressed in flowers (Fig. 1). These findings were similar to the patterns found in the database. Notably, in almost all of the tissues examined, *OsDEF7* was more highly expressed than *OsDEF8*. Although similar gene expression patterns at different developmental stages were found between the Thai and Japanese rice, *OsDEF7* expression in the roots and leaves differed between the cultivars. *OsDEF7* in Japanese rice was equally expressed in the roots and leaves, but in Thai rice, *OsDEF7* was much more highly expressed in the roots than the leaves, in both seedlings and mature plants. These findings suggest a difference in gene regulation between the two cultivars.

High levels of *OsDEF7* expression could protect vulnerable, germinating seeds from pathogens. Similar results have been obtained for RsAFP1 and RsAFP2 from radish seeds, which are preferentially released during germination after disruption of the seed coat. The amount of released protein is sufficient to create a microenvironment around the seed in which fungal growth is suppressed (Terras et al., 1995). It is also possible that high levels of *OsDEF8* expression could protect flowers against pathogens.

*3.2. Upregulation of* OsDEF7 *and* OsDEF8 *under pathogen infection and other conditions*

In silico analysis has demonstrated that *OsDEF7* and *OsDEF8* are upregulated in various cultivars when infected with *M. oryzae* or *X. oryzae* pv. *oryzae* (Tantong et al., 2016). In the present study, Thai jasmine rice leaves were infected with *X. oryzae* pv. *oryzae* and collected at 6 h post-infection, 4 days post-infection (dpi), and 8 dpi. *OsDEF7* and *OsDEF8* were highly upregulated at 8 dpi (Fig. 2), and *OsDEF7* was more highly expressed than *OsDEF8*. The induction of both genes upon *X. oryzae* pv. *oryzae* infection suggests their involvement in pathogen defense. Rice plants may be susceptible to *X. oryzae* pv. *oryzae* infection because of low levels of *OsDEF* expression in the leaves under normal conditions, and delayed upregulation upon infection. Moreover, the expression levels of several rice pathogenesis-related genes, such as *OsPR1*s, are highly upregulated at 8 dpi with *X. oryzae* pv. *oryzae* (Mitsuhara et al., 2008)*.* If *OsDEF*s were expressed more rapidly, it is possible that rice plants would be resistant to pathogens. The upregulation of several *DEF*s in pea, tobacco, *Arabidopsis*, and spruce upon pathogen infection has also been reported (Lay and Anderson, 2005).

In addition to pathogen infection, we also investigated the expression of these two genes under abiotic stress using the GENEVESTIGATOR® tool. *OsDEF7* and *OsDEF8* expression levels were affected by several stressful conditions (Supplemental Fig. S3); *OsDEF7* was upregulated under imbibition and anoxic conditions, whereas *OsDEF8* was upregulated under drought, cold, and dehydration. This result suggests that *OsDEF*s are involved in other processes, which confirms previous reports, e.g., *Tad1* isolated from the crown tissues of winter wheat (*Triticum aestivum*) is induced during cold acclimation (Koike et al., 2002), and *CADEF1* mRNA accumulates in pepper leaves in response to wounding, high salinity, and drought (Do et al., 2004).

In addition to responses to abiotic stress, the involvement of *DEF*s during flower development has been reported. *DEF2* is expressed during early flower development in tomato, and antisense suppression or the constitutive overexpression of *DEF2* reduces pollen viability and seed production (Stotz et al., 2009). In *Zea mays*, the *DEF* *ZmES4* is expressed in the synergid cells, and is involved in pollen tube bursting by activating the potassium channel, KZM1 (Amien et al., 2010). Furthermore, in silicoanalysis has revealed that *OsDEF7* and *OsDEF8* are co-expressed with gibberellin-regulated proteins (Tantong et al., 2016). The question is whether or not these hormones/small molecules play a role in AMP expression when plants defend themselves against disease. A previous study reported that plant hormones do indeed regulate AMP expression: tgas118, a tomato DEF, seems to be regulated by gibberellins throughout flower development (Van den Heuvel et al., 2001). However, the molecular mechanisms underlying DEF function during these processes have not been completely elucidated.

*3.3. Subcellular localization*

According to WoLF PSORT, OsDEF7and OsDEF8 are localized to the extracellular compartment. To confirm the localization of both OsDEFsin plants, GFP was fused to the peptides’ C-terminus in a binary vector under a 35S promoter. Subsequently, either OsDEF7::GFP or OsDEF8::GFP fusion proteins were transiently expressed in *N. benthamiana* and tobacco BY-2 cell leaves by *Agrobacterium*-mediated gene transfer. *N. benthamiana* leaf cells were analyzed using confocal laser scanning microscopy at 5 days post-infiltration. Most cells only exhibited GFP fluorescence in the cell periphery (Fig. 3), indicating extracellular or membrane localization of the fusion proteins, in contrast to the GFP control, which exhibited GFP fluorescence in the nucleus and cell periphery (Bartetzko et al., 2009; Salinas et al., 2006). In addition, we did not find any membrane-anchor sequences in both DEFs. To confirm whether the peptides were localized to the cell membrane/cell wall or secreted, transient expression in BY-2 cells was investigated. Both OsDEF7::GFP and OsDEF8::GFP co-incubated with p19 exhibited greater fluorescence in the culture medium under UV light than OsDEFs::GFP without p19, while the GFP control did not exhibit any fluorescence in the culture medium (Fig. 4). The subcellular localization of OsDEFs strongly supports their involvement in pathogen responses, because they are secreted into the extracellular compartment where they first interact with the pathogen. AMP expression in the apoplast probably occurs because most pathogenic microorganisms are in the extracellular space during the early stages of infection. OsDEFs localized to the apoplastic region of the tissues may interact with the microbial membrane, leading to membrane destabilization, a reduced proliferation of the pathogen in the tissues, and enhanced disease resistance to phytopathogens (Jha et al., 2009). Moreover, the results from the transient BY-2 cell suspensions indicate that p19 facilitates transient protein expression. Lakatos et al. (2014) have shown that p19 from *Cymbidium ringspot* virus inhibits RNA silencing via its small RNA-binding activity in vivo. These results strongly suggest that both OsDEFs are secreted into the extracellular compartment, and p19 promotes greater protein production.

*3.4. Production of recombinant rice AMPs in Nicotiana spp.*

We have explored alternative production systems for these DEFs, from bacteria (Tantong et al., 2016) to plants. The advantages of a plant-based system include high product yield, quality, and homogeneity. Proper folding, glycosylation, and disulfide bond formation can also be achieved (Ma et al., 2003; Ramessar et al., 2008). To produce recombinant DEFs in plants, we cloned *OsDEF7* and *OsDEF8* into a pEAQ3 expression vector containing p19, a gene-silencing suppressor. Firstly, we evaluated production in tobacco BY-2 cells. We expected to detect OsDEF proteins in the culture medium, based on the subcellular localization experiment. Unfortunately, DEFs could not be detected in the culture medium of transgenic BY-2 cells expressing each of the OsDEFs (data not shown), although their gene expressions were detected using semi-qRT-PCR.

To investigate the transient expression of recombinant DEFs, infiltration of *N. benthamiana* leaves was performed. *A. tumefaciens* harboringeither pEAQ3-*OsDEF7* or pEAQ3-*OsDEF8* was infiltrated into the leaves, and then crude proteins were extracted.The OsDEFs could not be detected by western blot analysis. Unlike OsDEF::GFP, which could be secreted, the much smaller OsDEFs could not. These observations are in contrast to previous results that show that OsDEF::GFPs are secreted. As GFP (~27 kDa) forms a large part of hybrid OsDEF::GFP proteins and OsDEFs are only ~6 kDa, any interactions between OsDEFs and cell wall components would be disrupted. Company et al. (2014) suggested that DEFs that have strong cationic charges could strongly interact with cell wall components, and that the expression of a synthetic AMP (BP100) fused with the SEKDEL ER-retention signal at the C-terminal can preserve its antimicrobial activity and make detection easier. Therefore, we designed other constructs targeting OsDEFs to the ER compartment (pEAQ1-opt*OsDEF7*-His-SEKDEL and pEAQ1-opt*OsDEF8*-His-SEKDEL). In these constructs, *OsDEF* codons were optimized for *N. benthamiana*. Eventually, we were able to detect the recombinant peptides, which showed the bands at the expected mass of ~9 kDa (excluding the predicted signal peptide sequences, but including the SEKDEL plus 6xHis sequence), using a His-tag antibody (Fig. 5). However, an unknown band of ~17 kDa was also detected in the crude protein extracts from leaves infiltrated with *Agrobacterium* harboring either *OsDEF*s or the control GFP, suggesting that a non-specific protein in *N. benthamiana* interacted with the His-tag antibody. It should be noted that we did not observe a clear band of these peptides by SDS-PAGE, suggesting that there was only a small amount of recombinant peptides. In addition, the protein concentration remained the same at 3, 5, and 7 dpi. In contrast to our previous study, in which the recombinant OsDEF7 and OsDEF8 produced in *E. coli* were detected as ~15 kDa homodimers after column cleavage of glutathione-S-transferase fused to OsDEFs (Tantong et al., 2016), in this study, the recombinant ER-localized OsDEFs were detected as monomers. However, it cannot be ruled out that secreted forms of OsDEF would also form monomers. A previous study reported that the dimerization of NaD1, a DEF from *Nicotiana alata* flowers, enhances its antifungal activity when compared to a monomeric protein (Lay et al., 2012). Therefore, DEFs either form monomers or dimers, possibly depending on their primary amino acid sequences and environments; their overall structures would certainly affect antimicrobial activity. In this study, OsDEF7 and OsDEF8 were successfully expressed in a plant-based system, but they were produced in low concentrations and were difficult to extract and purify when compared with their use in *E. coli* (Tantong et al., 2016). Although the vector used in this study promotes very high protein production, and previous studies have reported its efficacy (Peyret and Lomonossoff, 2013; Sainsbury et al., 2009), it is possible that plants have a mechanism that prevents the overproduction of OsDEFs to a level that could result in an abnormal pleiotropic phenotype. The overexpression of tomato DEF2 results in small leaves and short sepals and carpels (Stotz et al., 2009). Because the protein concentrations were too low for in vitro OsDEF activity testing, we performed in planta activity testing instead.

*3.5. In planta antimicrobial activity of recombinant OsDEF7 and OsDEF8*

Although the in vitro antimicrobial activity of recombinantOsDEF7 and OsDEF8 produced from *E. coli* was confirmed, we also investigated the in plantainhibitory activity of these peptides. *A. tumefaciens* harboring either pEAQ3*-OsDEF*s or ER-localized pEAQ1-opt*OsDEF*s-SEKDEL were co-infiltrated with *X. campestris* into *N. benthamiana* leaves. After 9 days, both secreted OsDEFs and ER-localized OsDEFs exhibited disease resistance in comparison with both controls, which were co-infiltrated with *X. campestris* and *A. tumefaciens* with either an empty vector or not transformed (Fig. 6). These results strongly support the antibacterial activity of both types of OsDEF, although the secreted recombinant proteins produced by the pEAQ3-*OsDEF*s could not be detected. When the expression of these two types of construct was compared in infected leaves (Fig. 6), we found that pEAQ3*-OsDEF*swere more efficient than ER-localized pEAQ1-opt*OsDEF*s-SEKDEL, highlighting the importance of OsDEF subcellular localization. The secreted OsDEFs interacted with *X. campestris* much more rapidly than the intracellular, ER-localized OsDEFs. These results demonstrate that transgenic plants overexpressing OsDEFs can develop resistance against pathogen infection.

**4. Conclusion**

We analyzed gene expression patterns in Thai jasmine rice and Japanese rice by qRT-PCR. Although similar gene expression patterns were found at different developmental stages between the two cultivars, *OsDEF7* expression in the roots and leaves differed between them, suggesting a difference in gene regulation between the subspecies. In addition, Thai jasmine rice infected with *X*. *oryzae* pv. *oryzae* exhibited *OsDEF7* and *OsDEF8* upregulation, suggesting their involvement in pathogen responses. Moreover, in silico gene expression analyses using GENEVESTIGATOR® confirmed that the expression levels of these two genes were affected by drought, cold, imbibition, anoxia, and dehydration. For recombinant peptide production, both OsDEFs were localized to the extracellular compartment using a GFP-fusion approach. Although OsDEFs could not be detected in transformed tobacco BY-2 cell cultures, the ER-retained OsDEFs in *N*. *benthamiana* leaves were successfully produced at low concentrations. Both the secreted OsDEFs and ER-retained OsDEFs exhibited disease resistance in planta when infected with *X*. *campestris*. These results suggest several possible functions of secreted AMPs, and provide information of an alternative host to produce them.

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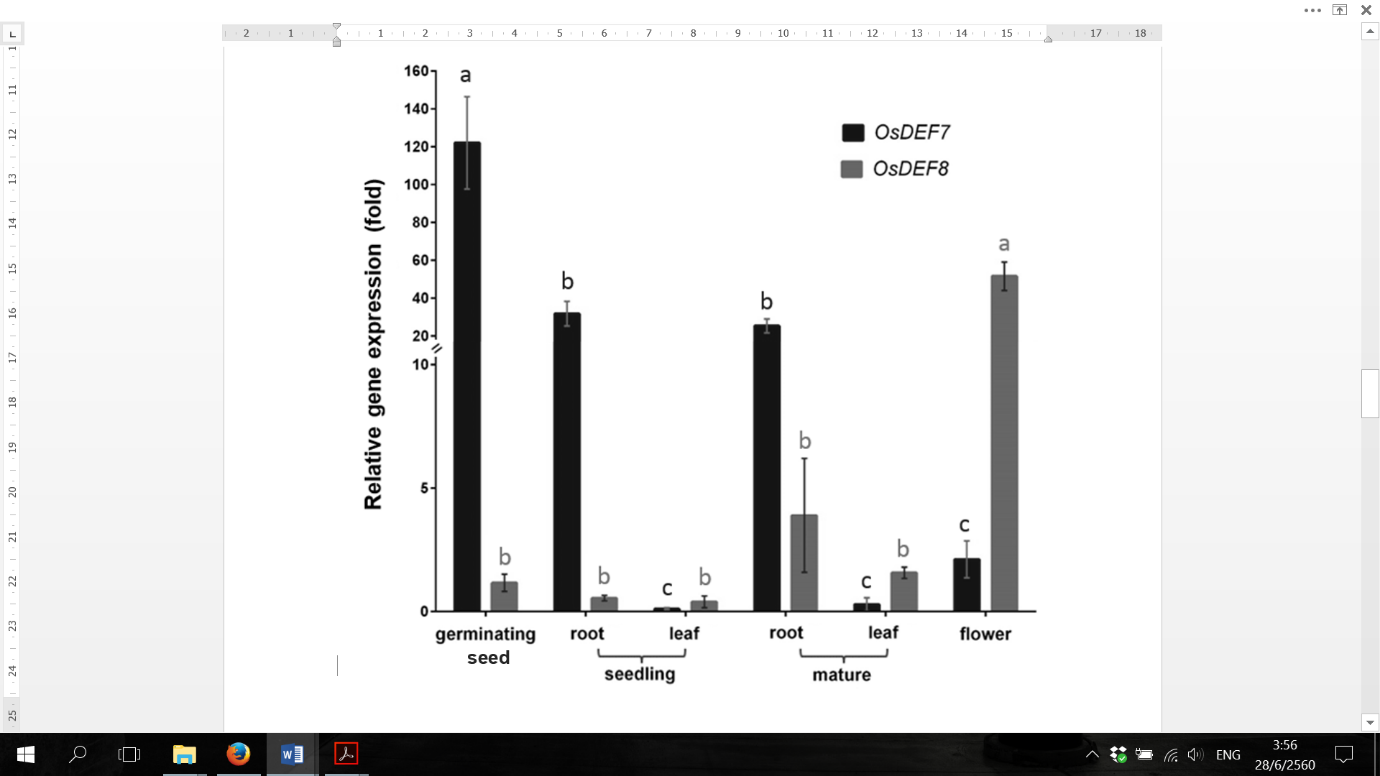
**Authors' contributions**

KW designed and performed the experiments and wrote the manuscript. SS designed the experiments, provided technical advice, and wrote the manuscript. GW, JM and WP provided technical advice and revised the manuscript. All of the authors have read and approved the final version of the manuscript.

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**Figures**



**Fig. 1.** Relative *OsDEF7* and *OsDEF8* expression levels in Thai rice. The levels of *OsDEF7* and *OsDEF8* were normalized to the standard (*EF1α*) and expressed as a fold relatively to the corresponding expression value of *OsDEF8* at germinating stage, which was given an arbitrary value of 1. Each value represents the mean ± SD (n = 3) with different letters indicate significant differences (P < 0.05) for each gene.

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**Fig. 2.** Relative *OsDEF7* and *OsDEF8* expression levels in Thai jasmine rice after infection with *Xanthomonas oryzae* pv. *oryzae*. Gene expression data were normalized to the standard (*EF1α*) and expressed as a fold relatively to the corresponding expression value of the controlsat each time point, which were given an arbitrary value of 1. Each value represents the mean ± SD (n = 3). Asterisks indicate a significant difference (P < 0.05) with the respective controls. hpi, hours post-infection; dpi, days post-infection.

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**Fig. 3.** Confocal images showing fluorescence signals from *Agrobacterium*-infiltrated leaf epidermal cells. *Nicotiana benthamiana* leaves were agroinfiltrated with (A) OsDEF7::GFP, (B) OsDEF8::GFP, or (C) a green fluorescent protein (GFP) control. Scale bars represent 50 μm.

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**Fig. 4**. Fluorescence observed in droplets of culture media of BY-2 cells transiently transformed with (A) 35S:GFP, (B) 35S:OsDEF7::GFP co-infected with 35S:p19, (C) 35S:OsDEF7, (D) 35S:OsDEF8::GFP co-infected with 35S:p19, or (E) 35S:OsDEF8.

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**Fig. 5.** Western blot analysis of crude and purified protein from *Nicotiana benthamiana* leaves after 5 days infiltration. Total soluble protein was extracted from 0.1 g/mL fresh weight of leaves in Tris-HCl buffer. For the purified protein, 2 mL of crude protein was purified using His SpinTrap™, and 20 μL of purified (lanes 4–7) or crude (lanes 1–3) protein was loaded per lane.

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**Fig. 6.** In planta antimicrobial activity of OsDEF7 and OsDEF8. *Agrobacterium tumefaciens* harboring (A) pEAQ3-*OsDEF7* and pEAQ3-*OsDEF8* or(B) pEAQ1-opt*OsDEF7-*SEKDEL and pEAQ1-opt*OsDEF8-*SEKDEL, with a blank pEAQ3 vector (control 1) or none (control 2), which was co-infiltrated at a ratio of 1:1 with *Xanthomonas campestris* into *Nicotiana benthamiana* leaves. This photograph was taken 9 days after infiltration.

C:\Users\DELL\Desktop\all manuscript\s1.tif**Supplementary Material**

**Supplemental Fig. S1.** Nucleotide sequence alignments of the original sequence and codon-optimized (A) *OsDEF7* and(B) *OsDEF8* in *Nicotiana benthamiana*, including 6xHis-tag and SEKDEL ER-retention at the C-terminal.

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**Supplemental Fig. S2.** *OsDEF7* and *OsDEF8* expression levels in *Oryza sativa* L. ssp. *japonica* (A) at different developmental stages (germinating seed, seedling (leaf and root), mature plant, and flowering) and in different organs, and (B) in seedling roots and leaves obtained from the GENEVESTIGATOR® microarray database.

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**Supplemental Fig. S3.** *OsDEF7* and *OsDEF8* expression levels under drought, cold, imbibition, anoxia, and dehydration stress obtained from the GENEVESTIGATOR® microarray database.