In Silico Analyses of Rice Thionin Genes and the Antimicrobial Activity of OsTHION15 Against Phytopathogens

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Accepted for publication 17 July 2018.

ABSTRACT

Thionins are a family of antimicrobial peptides. We performed in silico expression analyses of the 44 rice (*Oryza sativa*) thionins (OsTHIONs). Modulated expression levels of OsTHIONs under different treatments suggest their involvement in many processes, including biotic, abiotic, and nutritional stress responses, and in hormone signaling. OsTHION15 (LOC_Os06g32600) was selected for further characterization based on several in silico analyses. OsTHION15 in *O. sativa* subsp. *indica* ‘KDML 105’ was expressed in all of the tissues and organs examined, including germinating seed, leaves, and roots of seedlings and mature plants, and inflorescences. To investigate the antimicrobial activity of

OsTHION15, we produced a recombinant peptide in *Escherichia coli* Rosetta-gami (DE3). The recombinant OsTHION15 exhibited inhibitory activities toward rice-pathogenic bacteria such as *Xanthomonas oryzae* pv. *oryzae* and *Pectobacterium carotovorum* pv. *atroseptica*, with minimum inhibitory concentrations of 112.6 and 14.1 $\mu\text{g ml}^{-1}$, respectively. A significant hyphal growth inhibition was also observed toward *Fusarium oxysporum* f. sp. *cubense* and *Helminthosporium oryzae*. In addition, we demonstrated the in planta antibacterial activity of this peptide in *Nicotiana benthamiana* against *X. campestris* pv. *glycines*. These activities suggest the possible application of OsTHION15 in plant disease control.

Plant diseases are a threat to world agriculture and general food resources, and result in significant yield losses (Kenmore 1987; Teng and Gaunt 1980). On a global scale, the yield losses range from 20 to 40% of quantified crop production (Oerke 2006). Rice (*Oryza sativa* L.) is an important agricultural crop that is the staple food for several populations worldwide. In Asia, rice yield loss owing to pests and diseases was estimated at between 24 and 41% (Savary et al. 2000). The major diseases causing economic losses in rice-growing countries are bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Niño-Liu et al. 2006), bacterial leaf streak caused by *X. oryzae* pv. *oryzicola* (Niño-Liu et al. 2006), and seed-transmitted and bacterial sheath rot diseases caused by *Pectobacterium carotovorum* (Zhao et al. 2013). In addition, many serious rice diseases are caused by fungi such as root rot caused by *Fusarium oxysporum* (Saremi et al. 2011), brown leaf spot caused by *Helminthosporium oryzae* (Jatoi et al. 2016), and ufra disease caused by *Ditylenchus angustus* (Latif et al. 2011). To control diseases, various chemicals such as fungicides, bactericides (often called antibiotics), or nematicides have been extensively applied, depending on the targeted invasive organisms (Waard et al. 1993; Yamaguchi 2004). Nevertheless, chemical controls have several

disadvantages associated with bioaccumulation, and many bacterial and fungal pathogens develop resistance to chemical compounds.

Antimicrobial peptides (AMPs) are small, cysteine-rich peptides. They are naturally produced as host defense peptides against microbial invaders (Bolintineanu and Kaznessis 2011; De-Paula et al. 2008; Silverstein et al. 2007). AMPs have several advantages over antibiotics such as having more diverse antimicrobial activities, having the capability to specifically target the bacterial plasma membrane, and being unaffected by common mutations, which lead to antibiotic resistance (Hancock and Scott 2000). Thionins, a family of AMPs, are toxic to bacteria, fungi, yeast, and various mammalian cell types in vitro (Bohlmann et al. 1988; Carrasco et al. 1981; Fujimura et al. 2004). They can induce pathogen cell membrane leakage, resulting in antimicrobial and antifungal activities (Coulon et al. 2002; Thevissen et al. 1999). The overexpression of thionins can enhance plant resistance against a broad range of bacterial and fungal pathogens (Bohlmann and Broekaert 1994; Chan et al. 2005; Epple et al. 1997; Iwai et al. 2002; Muramoto et al. 2012; Shirasawa-Seo et al. 2002). Transgenic Carrizo plants (*Arundo donax*) expressing a modified thionin gene are less susceptible to *X. citri* infections (Hao et al. 2016). *Arabidopsis thaliana* thionin 2.4 (Thi2.4, At2g15010), which exhibits an antifungal activity, can be induced and accumulate in the cell wall after an *F. graminearum* inoculation. It is also found in the cell membranes of *F. graminearum* (Asano et al. 2013). Similarly, Iwai et al. (2002) reported that rice overexpressing *Avena sativa* thionin (Asthi1), which accumulates in the cell wall, revealed pathogenic bacterial staining only on the surface of stomata. Thus, thionins are effective in blocking bacterial attack at the target's surface (Asano et al. 2013; Iwai et al. 2002). Despite having potential applications, thionins have received less attention than other families of AMPs such as defensins. In rice, only one thionin has been functionally characterized. The transgenic *O. sativa* L. ‘Nipponbare’ overexpressing a thionin from rice, called OsTHI7 (LOC_Os06g32160), revealed a low susceptibility to the

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Funding: The research was supported, in part, by the Thailand Research Fund (Chulalongkorn University fund and IRG578008) and the Chulalongkorn Academic Advancement into Its 2nd Century Project. The 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund to K. Boonpa) is gratefully acknowledged.

K. Boonpa and S. Tantong contributed equally to this work.

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures and two supplementary tables are published online.

root pathogens *Meloidogyne graminicola* and *Pythium graminicola* (Ji et al. 2015), suggesting that the rice thionins (OsTHIONs) can efficiently control plant pathogens.

Because rice still possesses many uncharacterized thionins, in this study, we used gene expression data to identify all of the annotated OsTHIONs in the rice genome using in silico analyses. An uncharacterized thionin, OsTHION15, was selected to confirm its in vitro and in planta antimicrobial activities against phytopathogens. We suggest different biological functions for thionins and a potential use for OsTHION15 in disease control.

MATERIALS AND METHODS

Plant cultivation. Seed of Thai jasmine rice, *O. sativa* L. subsp. *indica* 'KDML 105', were sterilized with 70% ethanol for 2 min and 15% bleach for 30 min with shaking. They were washed with sterile distilled water and immersed for 4 days in the dark. The water was changed daily. On day 5, the coleoptiles were removed from the germinating seed for RNA extraction. The germinating seed continued growing in 0.5% Yoshida medium under the controlled conditions of 26°C with a relative humidity of 80% under a 6,000-lux light intensity with a cycle 16 h (day) and 8 h (night) for 2 weeks. The rice leaves and roots were collected from the seedlings for RNA extraction. After 1 month, some mature leaves and roots were collected for RNA extraction. The rest of the mature plants were then planted in clay pots and left outdoors until flowering. *Nicotiana benthamiana* seed were prepared for the in planta activity test. They were germinated in pots with peat moss and grown under controlled conditions of 25°C at a 4,000-lux light intensity with a 16-h photoperiod for 1.5 months. All of the plant materials were cultivated for three biological replicates for each experiment.

Microbial cultures. The bacterial and fungal indicators used in the antimicrobial assays were obtained from the DOAC Culture Collection Centre, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. They consisted of *X. oryzae* pv. *oryzae* (DOAC 4-1570, isolated from leaves of *O. sativa* L.), *Pectobacterium carotovorum* pv. *atroseptica* (DOAC 4-0039, isolated from tubers of *Solanum tuberosum* L.), *F. oxysporum* f. sp. *cubense* (DOAC 0110, isolated from pseudostems of *Musa sapientum*), and *H. oryzae* (DOAC 1570, isolated from leaves of *O. sativa* L.). For the in planta activity assay, *X. campestris* pv. *glycines* (TISTR 786) was obtained from the Thailand Institute of Scientific and Technological Research (TISTR) Culture Collection, Thailand.

In silico analyses. The genome of *O. sativa* subsp. *japonica* was searched for thionins using the Phytozome database (Goodstein et al. 2012) and the rice genome annotation project (Kawahara et al. 2013). The expression levels of OsTHIONs were determined in all rice tissues (seedlings, roots, mature and young leaves, and shoot apical meristem, as well as panicle [P2 to P6] and seed [S1 to S5] developmental stages) using the microarray database Rice eFP browser (Jain et al. 2007). The expression levels of OsTHIONs with greater than twofold changes ($P < 0.05$) under different treatment conditions were investigated using the Genevestigator tool (Hruz et al. 2008). The involvement of OsTHIONs with pathogen-responsive genes was analyzed using a coexpression network analysis available in the PlantArrayNet (Lee et al. 2009). The gene coexpression network was retrieved based on the correlation coefficient (r value) ≥ 0.6 . The subcellular localization and the presence of a secretory signal peptides were predicted using WoLF PSORT (Horton et al. 2007) and SignalP (Petersen et al. 2011), respectively.

Quantitative reverse-transcription polymerase chain reaction analysis. Total RNA was extracted from rice samples of four different developmental stages—germinating seed, seedling, mature, and flowering—using an RNeasy Plant Mini Kit (Qiagen Inc, Hilden, Germany) according to the manufacturer's instructions. The cDNA was prepared using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions using 1 µg of DNase-treated RNA as the

template. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using 5 µl of PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA), 2 µl of the diluted cDNA (20 ng of RNA), 2 µl of nuclease-free water, and 10 µM each primer in a total reaction volume of 10 µl. Thion15RT-F (5'-TGGCAACGAAGAAGGGAAC-3') and thion15RT-R (5'-ACTCCAGGGCAGCATATATAG-3') were used as the forward and reverse primers, respectively, for the *OsTHION15* amplification. The qRT-PCR conditions were as follows: 30 s at 95°C and 40 cycles of 5 s at 95°C and 10 s at 55°C. Amplicon dissociation curves were recorded 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 cycle threshold method (Schmittgen and Livak 2008) using *OsEF1α* as an internal control gene. Each value was derived from three biological replicates (P value < 0.05 , Tukey's honestly significant difference). The designed primers used to amplify *OsEF1α* were *OsEF1α*-F (5'-ATGGTTGTGGAGACCTTC-3') and *OsEF1α*-R (5'-TCACCTTGGCACCAGTTG-3'). The gene expression patterns in Thai jasmine rice were compared with those of Japanese rice in the Rice eFP browser (Jain et al. 2007) and Rice Expression Database (Xia et al. 2017).

Production of recombinant OsTHION in *Escherichia coli*. The cDNA of *OsTHION15* (Supplementary Fig. S1) was used as a template. The primers were designed to exclude the signal peptide and to contain either *Bam*HI or *Sal*I restriction sites for manipulation (underlined): forward 5'-AAGGATCCATGCAAGA GACGATCAAGGTGGG-3' and reverse 5'-AAAGTCGACTTAG GAAACGATAGTGACAATATCAGCT-3'. The expression vector was constructed by inserting amplified *OsTHION15* into the vector pGEX-6P-3 to express glutathione S-transferase (GST)-fused *OsTHION15*. The correctly inserted constructs were verified by sequencing. pGEX-6P-3-*OsTHION15* was transformed into competent *Escherichia coli* Rosetta-gami (DE3) that supports the formation of disulfide bonds in the peptides. A positive clone was inoculated into 5 ml of Luria-Bertani (LB) medium supplemented with kanamycin at 50 µg ml⁻¹, chloramphenicol at 34 µg ml⁻¹, tetracycline at 100 µg ml⁻¹, and ampicillin at 100 µg ml⁻¹. An overnight culture was inoculated and incubated at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.6. Then, the expression of GST-fused *OsTHION15* was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final 0.1 mM concentration and incubated at 37°C for 24 h. The GST-fused *OsTHION15* was purified from the supernatant using glutathione agarose bead affinity purification (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The PreScission protease (GE Healthcare, Chicago, IL, USA) was then applied to the spin column to cleave *OsTHION15* from the fusion protein. The recombinant *OsTHION15* was analyzed by 12% tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of purified recombinant *OsTHION15* was determined using a Bradford protein assay kit (Thermo Scientific) using bovine serum albumin as the standard. The protein sequence and the molecular mass of *OsTHION15* were predicted using the ExPASy Translate tool and the ExPASy Compute pI/Mw function, respectively (Gasteiger 2003).

Antimicrobial assay. The antibacterial activity of recombinant *OsTHION15* was determined in terms of minimum inhibitory concentrations (MICs). The peptide was serially diluted with fresh tryptic soy broth (HiMedia Laboratories, Mumbai, India) at concentrations that varied from 450 to 0.88 µg ml⁻¹ in a microplate. Bacteria causing plant diseases (*X. oryzae* pv. *oryzae* and *P. carotovorum* pv. *atroseptica*) were grown in tryptic soy broth medium at 37°C overnight. These cell suspensions were subsequently inoculated into the wells to produce a final cell concentration of 5×10^5 CFU ml⁻¹. The culture was incubated overnight at 30°C. The cell growth was determined from the OD₆₀₀ using a Model 550 microplate reader (Bio-Rad), with and without ampicillin as positive and negative controls, respectively.

The antifungal activities of the recombinant OsTHION15 were determined using the hyphal point technique (Bains and Bisht 1995). The fungal indicator strains *F. oxysporum* f. sp. *cubense* and *H. oryzae* were grown on potato dextrose agar (PDA) plates at 30°C for 7 days. Agar plugs of actively growing cultures on PDA plates were excised using a 10-mm sterile cork borer and placed on the center of new PDA plates supplemented with various concentrations of recombinant OsTHION15 (i.e., 7.0, 15.0, and 22.0 µg ml⁻¹). This similar range of concentration was used to demonstrate the antifungal activity of pepper CaThi (Taveira et al. 2017). The inoculated plates were incubated at 30°C for 7 days. The hyphal growth was recorded daily as diameter. PDA plates without and with benzoic acid (Shabana et al. 2008) were used as negative and positive controls, respectively. All experiments for antimicrobial activity assays were carried out with at least three replicates (*P* value < 0.05, *t* test).

In planta antibacterial activity. *OsTHION15* was inserted into a plant expression vector using Gateway cloning technology. The full-length *OsTHION15*, without a stop codon, was amplified from cDNA with the designed primers forward 5'-ATGAAAGGTGTGAAGAGTTTGATC-3' and reverse 5'-GGAAACGATAGTGA CAATATCA-3'. PCR amplification was performed with Phusion High-Fidelity DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA) in a T100 Thermal Cycler (Bio-Rad). *OsTHION15* was subsequently ligated using a pCR8/GW/TOPOTA Cloning kit (Invitrogen, Waltham, MA) before being integrated into the C-terminal His-tagged destination vector pEAQ-HT-DEST3 (pEAQ3) (Peyret and Lomonosoff 2013) using LR Clonase II (Invitrogen) to generate the expression cassette pEAQ3-*OsTHION15*. This expression vector was then transformed into *Agrobacterium tumefaciens* GV3101 by electroporation.

To determine the in planta activity, *A. tumefaciens* harboring pEAQ3-*OsTHION15* was cultured in LB medium containing a final concentration of kanamycin, rifampicin, and gentamycin at 50 µg ml⁻¹ each for 2 days at 30°C. The pellet of *A. tumefaciens* harboring pEAQ3-*OsTHION15* was collected and resuspended in the infiltration medium (10 mM morpholineethanesulfonic acid-MgCl₂-acetosyringone buffer) to an OD₆₀₀ of 2.0 and incubated at room temperature for 2 to 3 h. The overnight culture of *X. campestris* was prepared to an OD₆₀₀ of 0.4. The cultures of *A. tumefaciens* harboring pEAQ3-*OsTHION15* and *X. campestris* were mixed equally and coinfiltrated using a syringe onto leaves of 6-week-old *N. benthamiana*. The same leaf of *N. benthamiana* coinfiltrated with *A. tumefaciens* harboring the empty pEAQ3 vector and *X. campestris* was used as the positive control. The disease symptoms were observed for 8 days. The in planta activity assay was conducted with a minimum of three replicates of independent leaves.

RESULTS

In silico expression analyses of OsTHIONs. In the Phytozome database and the data from the rice genome annotation project, 44 *OsTHIONs* were annotated. All of the annotated genes and locus numbers are listed in Supplementary Table S1. We explored the expression levels of all 44 *OsTHIONs* in various rice tissues by in silico analysis using the Rice eFP browser (Jain et al. 2007). Of these *OsTHIONs*, the expression data of 34 genes could be retrieved. Seedling root, late stage inflorescence, and seed were the tissues in which relatively high expression levels were observed (Fig. 1). Most *OsTHIONs* showed organ-specific expression patterns, except *OsTHION30* and *OsTHION36*, which were expressed throughout all of the tissues (Fig. 1). We further investigated the expression levels of *OsTHIONs* under different treatment conditions using data from microarray experiments available in the Genevestigator tool, with a criteria of more than twofold changes and *P* < 0.05 (Fig. 2). Although microarray probes were not available for all of the *OsTHIONs*, 26 of the annotated *OsTHIONs* were covered in our Genevestigator analysis. *OsTHION15*,

OsTHION26, and *OsTHION30* showed significant upregulation when infected with the causal agent of rice bacterial blight, *X. oryzae* pv. *oryzae* (Fig. 2). *OsTHION15* showed an earlier upregulation than the other two thionins at 8 h postinfection in leaf. Similarly, *OsTHION34* was upregulated after being infected by *A. tumefaciens*. The downregulation could be observed for *OsTHION26*, *OsTHION29*, *OsTHION30*, *OsTHION32*, *OsTHION34*, and *OsTHION36* when infected with plant pathogens such as *A. tumefaciens*, *Magnaporthe grisea*, *M. graminicola*, *M. incognita*, *X. oryzae*, or an insect pest such as *Nilaparvata lugens*. Considering their antimicrobial activities, the genes upregulated during pathogen infection may be involved in defense mechanisms. Many *OsTHIONs* were also highly perturbed by abiotic stress. *OsTHION15* showed a high level of upregulation under drought- and salt-stress conditions. *OsTHION26* was upregulated under cold and heat treatments. Hormone treatments, chemical stresses, nutrient deprivation, gravistimulation, and imbibition also affect the expression levels of various *OsTHIONs*. In addition, *OsTHION15* was highly expressed during stamen development.

We performed a gene coexpression network analysis to evaluate the involvement of *OsTHIONs* in plant defense mechanisms. Among the 44 identified *OsTHIONs*, the coexpression data of 15 *OsTHIONs* (*OsTHION2*, *OsTHION7*, *OsTHION9*, *OsTHION15*, *OsTHION18*, *OsTHION24*, *OsTHION25*, *OsTHION26*, *OsTHION27*, *OsTHION29*, *OsTHION30*, *OsTHION32*, *OsTHION34*, *OsTHION36*, and *OsTHION39*) were retrieved from PlantArrayNet (Lee et al. 2009). The groups of coexpressed genes containing those *OsTHIONs* are summarized in Supplementary Table S2. We found 10 *OsTHIONs* (*OsTHION9*, *OsTHION15*, *OsTHION25*, *OsTHION26*, *OsTHION29*, *OsTHION30*, *OsTHION32*, *OsTHION34*, *OsTHION36*, and *OsTHION39*) that were coexpressed with plant-disease-resistant proteins, enzymes eliminating microbes (e.g., chitinase, glucanase, and amylase inhibitors), thaumatin-like proteins, and other AMP families. Some of the *OsTHIONs* were also coexpressed with genes involved in growth and development such as the rapid alkalization factor proteins (Pearce et al. 2001) and the gibberellin-stimulated family of proteins (Aubert et al. 1998).

Based on the in silico analyses, we focused on the characterization of *OsTHION15* because it was upregulated early upon *X. oryzae* pv. *oryzae* infection, coexpressed with genes known to participate in pathogen response processes such as genes encoding plant disease resistance proteins, pathogen-related proteins (X1), microbe-inhibiting enzymes (chitinase), and AMPs from other families (e.g., lipid transfer proteins, γ-purothionin, and glycine-rich proteins). In addition, it was coexpressed with genes involved in the phenylpropanoid pathway such as phenylalanine ammonia-lyase and 4-coumarate CoA ligase (Fig. 3). This pathway leads to cell wall biosynthesis and secondary metabolite production, which also play roles during pathogen infections (Miedes et al. 2014; Piasecka et al. 2015).

The expression of OsTHION15 in Thai jasmine rice (*O. sativa* subsp. *indica* 'KDML 105'). *OsTHION15* (408 bp) was predicted to encode 117 amino acids. We confirmed that the sequences of *OsTHION15* from Japanese rice and that from Thai jasmine rice were the same. *OsTHION15* was expressed in Thai rice in all of the tissues examined at four stages: germinating seed, seedling, maturity, and flowering. The highest expression level was detected in the flowering stage (Fig. 4). However, the expression pattern was different from those reported for *OsTHION15* in the database, which showed no expression in either the seedling roots or in the leaves at the young and mature stages (Fig. 1).

Heterologous production of OsTHION15 in *E. coli*. *OsTHION15* was predicted as an extracellular protein having a signal peptide cleavage site between Gln19 and Gln20. The signal peptide was excluded when the recombinant GST-fused *OsTHION15* was produced in *E. coli* Rosetta-gami (DE3). The fusion protein was produced because it could reduce the cellular toxicity that may be caused by *OsTHION15* and could be used for purification purposes.

The optimal conditions for the production were 37°C for 24 h with a 0.1 mM IPTG induction (Supplementary Fig. S2). The calculated molecular masses of the GST and OsTHION15 were 26 and 12.8 kDa, respectively. Thus, the expected molecular mass of GST-fused OsTHION15 was approximately 39 kDa, which was observed using an SDS-PAGE analysis. After the purification and on-column GST cleavage, a 13-kDa purified recombinant OsTHION15 at 0.84 mg liter⁻¹ was achieved (Supplementary Fig. S3).

In vitro and in planta antimicrobial activities of OsTHION15. The recombinant OsTHION15 peptide showed inhibitory activities against *X. oryzae* pv. *oryzae* and *P. carotovorum*, with MIC values of 112.6 and 14.1 µg ml⁻¹, respectively. The antifungal activity against *F. oxysporum* f. sp. *cubense* showed a negative correlation, in which higher concentrations of OsTHION15 could reduce the hyphal growth of *F. oxysporum* f. sp. *cubense* (Fig. 5A). The hyphal growth of *F. oxysporum* f. sp. *cubense* significantly decreased from 87 ± 0.7% at 7.0 µg ml⁻¹ to 71 ± 1.0% at 22.0 µg ml⁻¹. This indicates that OsTHION15 exhibited a dose-independent inhibition against *F. oxysporum* f. sp. *cubense*. In addition, the various concentrations of OsTHION15 had similar effects on the hyphal elongation of *H. oryzae* (Fig. 5B). Moreover, OsTHION15 showed in planta antibacterial activity against *X. campestris* pv. *glycines*, a common pathogen used to infect *Nicotiana benthamiana*. The *N. benthamiana*

leaves coinfiltrated with *A. tumefaciens* harboring the empty vector showed susceptibility to *X. campestris*. The leaves revealed gray necrosis, with a lesion diameter of 1.74 ± 0.22 cm spreading over the infiltrated area at day 8 (Fig. 6, right). The symptom was slightly visible on the leaves coinfiltrated with the pathogen and with *A. tumefaciens* harboring *OsTHION15*, with a lesion diameter of 0.35 ± 0.04 cm (Fig. 6, left). Although we did not detect the presence of OsTHION15 in the infiltrated *N. benthamiana*, we confirmed the expression of *OsTHION15* (Supplementary Fig. S4). This indicates that OsTHION15 exhibited an in planta antibacterial activity against the *X. campestris* infection.

DISCUSSION

Although rice possesses as many as 44 thionins, only 1 OsTHION, OsThi7, is known to exhibit an antimicrobial activity (Ji et al. 2015), and several OsTHIONs are regulated by phytohormones (Kitanaga et al. 2006) and root-knot nematode infections (Ji et al. 2015; Kyndt et al. 2012). Here, we further explored the annotated *OsTHION* expression data from different publicly available databases. Of the 39 *OsTHIONs*, 15 *OsTHIONs* were highly expressed in seedling root, inflorescence, and seed (Fig. 1). However, the expression patterns of *OsTHION15*, which was a

Fig. 1. Organ-specific expression profiles of selected rice thionin (*OsTHION*) genes. Expression profiles for seedling root, mature and young leaf, and shoot apical meristem (SAM), as well as for panicle (P2 to P6) and seed (S1 to S5) developmental stages, were retrieved from the rice eFP browser (Jain et al. 2007). The heat map is based on the values retrieved from the browser. Pixel intensities are proportional to the actual expression levels, which were calculated as log2 expression values. The bar indicates the magnitude of expression, from relatively low expression (gray) to high expression (black) levels.

focus of this study, in Thai jasmine rice (Fig. 4) are different from those of Japanese rice in the database (Fig. 1), suggesting subspecies-specific expression patterns between *indica* and *japonica* types. The type with the high *OsTHION* expression level may exhibit less susceptibility to pathogens by blocking pathogen penetration because many *OsTHIONs* were predicted to be localized at the cell wall. A few cell-wall-bound thionins interact with pathogen cell membranes (Asano et al. 2013; Iwai et al. 2002). Although *OsTHION15* expression could be detected in the mature leaves of Thai jasmine rice, this is still considered a cultivar susceptible to *X. oryzae* pv. *oryzae*. It is possible that (i) *OsTHION15* is not induced quickly enough to initiate a defense mechanism, (ii) its expression level is not high enough to overcome

the pathogen infection, or (iii) its inhibitory activity is not great enough against *X. oryzae* pv. *oryzae*. Upon infection, *OsTHION* expression levels were modulated. Previous studies demonstrated the downregulation of *OsTHIONs* upon root-knot nematode *M. graminicola* infection. Kyndt et al. (2012) showed the downregulation of *OsTHION5* and *OsTHION7* at 3 days after infection. Ji et al. (2015) observed the downregulation of *OsTHION1*, *OsTHION2*, and *OsTHION7+6* (the primers designed could bind to both *OsTHION6* and *OsTHION7*) using a quantitative real-time PCR analysis in *M. graminicola*-induced galls at 7 days postinoculation. They suggested that the downregulation of the *OsTHIONs* is the consequence of the pathogen-mediated suppression of plant defense mechanisms, which contributes to a successful

Fig. 2. Genevestigator heat map for rice thionin (*OsTHION*) expression under different treatments. The heat map presents the *OsTHION* expression levels under various conditions. The Genevestigator analysis (Hruz et al. 2008) was completed with a probe set for 26 *OsTHIONs*. The intensity for each gene expression profile was normalized, with the upregulated, unchanged, and downregulated expression levels represented in different shades. Data shown are for those with expression values having greater than twofold changes or $P < 0.05$. For *OsTHION11*, -18, -19, -20, -21, -22, -23, -24, -27, -38, and -41, the expression values are either less than twofold changes or $P < 0.05$. For *OsTHION1*, -2, -3, -4, -5, -6, -7, -8, -9, -13, -14, -16, -17, -39, -40, -42, -43, and -44, data are not presented due to probe unavailability.

infestation. They then demonstrated that the overexpression of *OsTHION7* in rice decreases its susceptibility to *M. graminicola* and the oomycete *Pythium graminicola* (Ji et al. 2015). Nevertheless, many pathogen-responsive genes are upregulated because of their important protective roles during infection (MacQueen and Bergelson 2016). Thus, in our study, we focused on the upregulated *OsTHIONs* (Fig. 2) that might be involved in pathogen responses because of their antimicrobial activities. A similar strategy was used to identify rice defensins with activities against plant pathogens (Tantong et al. 2016) and barley (*Hordeum vulgare*) thionins with activities against aphids (Escudero et al. 2017). In addition to fighting pathogen infection, roles for thionins in the defense against aphids have been recently established. The overexpression

of thionins, which show an upregulation in barley after interactions with the phloem-feeding insects *Myzus persicae* and *Rhopalosiphum padi*, also increased resistance in *N. benthamiana* to *M. persicae* (Escudero et al. 2017). However, in the in silico analyses, we only observed the downregulation of *OsTHIONs* in rice stems invaded by the aphid *Nilaparvata lugens* (Fig. 2). Because of the incomplete expression data for all of the *OsTHIONs*, their involvement in response to aphid infestations cannot be fully determined. The high similarity levels among *OsTHION* family members at the nucleotide level has made it difficult to specifically design probes for each of the *OsTHIONs*.

The regulation of many *OsTHIONs* is also linked to several abiotic factors (e.g., cold, drought, heat, and salt), plant hormones,

Fig. 3. Gene coexpression network analysis of rice thionin 15 (*OsTHION15*). Networks were analyzed using PlantArrayNet-Rice 300k. Coexpressed genes with the cut-off value for Pearson’s correlation coefficient of 0.6 are presented in separate groups based on their predicted functions. The locus number of each gene is provided in the boxes. The closeness of their relationships is not represented in the networks.

Fig. 4. Relative expression levels of rice thionin 15 (*OsTHION15*) in different stages and organs of Thai jasmine rice (*Oryza sativa* L. subsp. *indica* ‘KDML 105’). Levels of *OsTHION15* were normalized to the reference gene, *EF1α*, and expressed as fold relative to the expression value of *OsTHION15* in the mature leaf, which was given an arbitrary value of 1. Significant statistics are shown by different letters (P value < 0.05, Tukey’s honestly significant difference). Each value represents the mean $\pm$ standard deviation ($n = 3$).

and chemicals and to nutrient deprivation. To our knowledge, no information on the biological functions of thionins under many conditions is currently available. Nevertheless, an inverse correlation between gibberellic acid and several *OsTHION* expression levels has been documented (Kitanaga et al. 2006). In addition, indirect in planta evidence shows that some *OsTHIONs* are downregulated in the *Meloidogyne graminicola*-infected rice galls in which several gibberellin biosynthetic genes were activated (Kyndt et al. 2012). However, *OsTHION15*, which is the only *OsTHION* from the in silico analysis that is upregulated in the late stage of stamen development, might respond to gibberellin differently. Our coexpression analysis revealed the involvement of *OsTHION15*, which is coexpressed with gibberellin 20-oxidase, a biosynthetic enzyme involved in gibberellin biosynthesis, growth, and development (Fig. 3). These observations suggest a complex regulatory network involving the thionin family. Interestingly, Asano et al. (2013) reported that *Arabidopsis*' thionin (Thi2.4) exhibits not only an antifungal activity but also interacts with lectin in the fungal fruit body of *F. graminearum* to suppress its toxicity to plant cells. Thus, thionins appear to have extremely diverse functions in different plant processes. More studies are needed to provide a better understanding of the functions of the highly modulated thionins under various treatment conditions.

In this study, we functionally characterized *OsTHION15*, which is expressed throughout different developmental stages and in different organs, coexpressed with several pathogen-responsive genes, and upregulated upon *X. oryzae* pv. *oryzae* infection. Typically, a thionin contains an N-terminal signal peptide for targeting to the endoplasmic reticulum, a positively charged mature domain with conserved cysteine residues and a C-terminal acidic peptide with no consistent motif. Thionins are produced as preproteins that are usually processed to mature peptides (44 to 47 amino acids) of approximately 5 kDa (Stec 2006). Nevertheless, a full-length *OsTHION15* was predicted to contain a signal peptide of 19 amino acids and a mature peptide of 117 amino acids, with a molecular mass of 13 kDa on tricine SDS-PAGE. Although C-terminal processing can increase the stability and solubility of many thionins (Romero et al. 1997), not all thionins require this modification. For

Fig. 6. In planta inhibitory activity of rice thionin 15 (*OsTHION15*). Symptoms were observed on the same *Nicotiana benthamiana* leaves after coinfiltrating with either *Agrobacterium tumefaciens* harboring pEAQ3-*OsTHION15* (left) or the empty pEAQ3 vector (right), as a positive control, together with *Xanthomonas campestris*. At least three replicate tests were performed. The phenotype was photographed at 8 days after infiltration.

Fig. 5. Antifungal activities of rice thionin 15 (*OsTHION15*) against **A**, *Fusarium oxysporum* f. sp. *cubense* and **B**, *Helminthosporium oryzae*. Hyphal growth of the control experiment containing no *OsTHION15* was defined as 100%. Statistically significant differences, considered by comparison of the percent hyphal growth between treated and untreated control fungi, are shown by different letters (P value < 0.05, t test).

example, Thi2.4 has a molecular mass of 15 kDa, which is the calculated mass, in *Arabidopsis* cells, indicating that processing did not occur in the C-terminal region. This region may be important for its activity against the fungal pathogen *F. graminearum* (Asano et al. 2013). Although there is no in planta evidence for OsTHION15 processing, our study showed that unmodified OsTHION15 exhibits in vitro activities against several rice pathogens. The recombinant OsTHION15 had MIC values of 112.6 and 14.1 $\mu\text{g ml}^{-1}$ against *X. oryzae* pv. *oryzae* and *Pectobacterium carotovorum* pv. *atroseptia*, respectively, suggesting a relative high inhibitory activity against *P. carotovorum* pv. *atroseptia*. Additionally, at the lowest concentration used (7.0 $\mu\text{g ml}^{-1}$), recombinant OsTHION15 reduced the hyphal growth of *F. oxysporum* f. sp. *cubense* and *Helminthosporium oryzae* to 87 and 83% of the control, respectively (Fig. 5). The viability levels of *F. sporotrichioides* and *F. graminearum* were significantly reduced when Thi2.4 at 15 and 150 ng ml^{-1} , respectively, was tested (Asano et al. 2013). Thionins from the endosperms of wheat and barley were more active against bacterial species such as *Clavibacter michiganensis* and *Pseudomonas solanacearum* than fungal pathogens such as *Rosellinia necatrix*, *Colletotrichum lagenarium*, and *F. solani* (Molina et al. 1993). Thionins AX1 and AX2 from sugar beet (*Beta vulgaris*) at 2 to 4 $\mu\text{g ml}^{-1}$ were sufficient for the 50% growth inhibition of the fungi *Cercospora beticola* but they had limited effects against bacteria (Kragh et al. 1995). Thus, OsTHION15 could still be an antibacterial or antifungal peptide. Additionally, the overexpression of OsTHION15 exhibits an in planta inhibitory activity toward *X. campestris* in *Nicotiana benthamiana*. The expression vector used in our study, pEAQ-HT-DEST3, produces milligram levels of recombinant proteins in plants (Sainsbury et al. 2009), making the rapid screening for novel peptides or proteins involved in plant defense mechanisms possible. Therefore, in planta transient expression has received more attention recently (Holaskova et al. 2015; Niknejad et al. 2016). In addition, posttranslational modifications, if any, that can occur in plants might contribute to an increase in peptide or protein activity. Also, Stover et al. (2013) suggested that AMPs have higher in planta activities because of their ability to form smaller AMP aggregates, in contrast to larger aggregates formed in vitro that could interfere with their inhibitory functions. Thus, transgenic plants overexpressing OsTHION15 could develop resistance against pathogen infections.

In summary, our in silico analysis for AMPs suggested the involvement of OsTHIONs in several processes, in addition to pathogen responses, that have not been previously reported. We successfully produced recombinant OsTHION15 in bacteria and provided both in vitro and in planta evidence for its antimicrobial activities against several phytopathogens. This peptide could be of great practical interest for utilization as an agricultural microbicide against phytopathogens.

ACKNOWLEDGMENTS

We thank G. Lomonosoff (John Innes Centre, Norwich, UK) and Plant Bioscience Limited (Norwich, UK) for supplying the pEAQ vectors.

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