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Two novel antimicrobial defensins from rice identified by gene coexpression network analyses

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ABSTRACT

Defensins form an antimicrobial peptides (AMP) family, and have been widely studied in various plants because of their considerable inhibitory functions. However, their roles in rice (*Oryza sativa* L.) have not been characterized, even though rice is one of the most important staple crops that is susceptible to damaging infections. Additionally, a previous study identified 598 rice genes encoding cysteine-rich peptides, suggesting there are several uncharacterized AMPs in rice. We performed *in silico* gene expression and coexpression network analyses of all genes encoding defensin and defensin-like peptides, and determined that *OsDEF7* and *OsDEF8* are coexpressed with pathogen-responsive genes. Recombinant *Os*DEF7 and *Os*DEF8 could form homodimers. They inhibited the growth of the bacteria *Xanthomonas oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *Erwinia carotovora* subsp. *atroseptica* with minimum inhibitory concentration (MIC) ranging from 0.6 to 63 μg/mL. However, these *Os*DEFs are weakly active against the phytopathogenic fungi *Helminthosporium oryzae* and *Fusarium oxysporum* f.sp. *cubense*. This study describes a useful method for identifying potential plant AMPs with biological activities.

1. Introduction

Plants have evolved natural resistance to abiotic and biotic stresses (e.g., adverse environmental conditions and infection by microbial and insect pathogens). The cell wall functions as a physical barrier, and is the first line of defense. Additionally, plants produce several specialized metabolites to protect against infections or physical damage [52]. There are also antimicrobial peptides (AMPs) that function in innate immune systems, and are essential for protecting plants from microbial invaders. In addition to plants, AMPs are present in many organisms, including bacteria, fungi, insects, and vertebrates. The existence of AMPs has been known for decades.

Most plants AMPs are small cysteine-rich peptides (CRPs) that have antimicrobial properties. They can be divided into several categories based on their common patterns of disulfide bond formation. These include defensins (DEFs), thionins (THIONs), lipid transfer proteins (LTP), hevein-type peptides, and knottin-type peptides [3,11]. Almost all of these AMPs possess four to eight cysteine residues forming two to four disulfide bonds that stabilize the overall folding of the polypeptide.

DEFs form a family of AMPs that is broadly dispersed among eukaryotes. There has been considerable interest in DEFs because of

Abbreviations: AMP, antimicrobial peptide; BSA, bovine serum albumin; CRP, cysteine rich peptide; DEF, defensin; DEFL, defensin-like; GST, glutathione *S*-transferase; MIC, minimum inhibitory concentration; MS, mass spectrometry; MM/PBSA, molecular mechanics/Poisson-Boltzmann surface area; MM/GBSA, molecular mechanics/generalized Born and surface area; LTP, lipid transfer protein; PCR, polymerase chain reaction; PDA, potato dextrose agar; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; THION, thionin; TSB, tryptic soy broth; *Xoc*, *Xanthomonas oryzae* pv. *oryzicola*; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*.

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their broad-spectrum antibacterial and antifungal activities. Additionally, they have several advantages over antibiotics, such as a greater diversity in their antimicrobial activities, an ability to specifically target the bacterial plasma membrane, and the fact they are unaffected by common mutations leading to antibiotic resistance [21].

Although plants possess genes encoding different classes of AMPs, they can still be susceptible to pathogens. This may be because AMPs are expressed at low levels in the infected tissues. Most plant AMPs are highly expressed in seeds and reproductive organs [51], but are rarely expressed in leaves, which are common targets for many pathogens. Rice (*Oryza sativa* L.) is an example of an important crop whose grain yields are lowered by bacterial and fungal infections of the leaves. There have been many attempts to overexpress AMPs from various organisms in rice to confer increased disease resistance. Transgenic rice overexpressing shrimp AMPs, specifically Np3 and Np5, were more resistant to bacterial blight than wild-type controls [61]. Crude leaf extracts from transgenic rice overexpressing *MjAMP2*, which is a DEF from *Mirabilis jalapa*, had fungicidal activities that inhibited the growth of the rice blast fungus, *Magnaporthe grisea* [3]. Furthermore, overexpression of an *Avena sativa* THION gene in rice resulted in abundant THION peptides in cell walls, and revealed the bacterial blight pathogen occurred only on the outer stomatal surface [26]. These results suggest AMPs can efficiently control bacterial infections. It is noteworthy that there has been little research on rice AMPs. To the best of our knowledge, only two rice AMPs, namely non-specific LTP and LTP110, have been investigated. These two peptides have been produced in *Escherichia coli*, and the purified products exhibited antifungal activities against the rice pathogen *Pyricularia oryzae* [13]. In 2007, 598 genes encoding CRPs were identified in the rice genome [51], and some of the genes were classified as AMPs from different families. However, the possible activities of most rice AMPs have not been determined.

We used bioinformatics tools, such as gene coexpression network analysis [35] and gene expression pattern analysis [27], to identify and characterize rice DEFs. Coexpression network analysis uses gene expression profiles from microarray data, and is based on the assumption that coexpressed genes participate in the same biosynthetic pathways/processes. Functional gene association networks have been studied in several organisms [34]. In plants, these networks have been used to functionally characterize biosynthetic genes in *Arabidopsis thaliana* [15,40,59] and rice [9,35]. Coexpression analyses have also been used to identify transcriptional regulators of metabolic pathways in rice [10]. In this study, we identified two rice DEFs that are coexpressed with pathogen-responsive genes. We then evaluated the antimicrobial activities of these two DEFs against different microbes, including rice pathogens. This study describes a viable method to identify novel AMPs with desirable activities from a large group of AMPs.

2. Materials and methods

Analytical grade and/or molecular biology grade chemicals and reagents were purchased from the following manufacturers: Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), and Ajax Finechem (Sydney, Australia). Commercial kits, restriction enzymes, and DNA markers were purchased from Qiagen (Hilden, Germany), New England Biolabs (Ipswich, MA, USA), and Bio-Rad (Hercules, CA, USA). Primers were synthesized and DNA sequencing was completed at Bio Basic Inc. (Toronto, Canada). Rice cDNA samples were obtained from the Rice Genome Resource Center (Ibaraki, Japan). The bacteria and fungi used in the antimicrobial assays were purchased from the DOAC Culture Collection Centre, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. These include *Xanthomonas oryzae* pv. *oryzae* (DOAC 4-1570 isolated from leaves of *O. sativa* L.), *Xanthomonas oryzae* pv. *oryzicola* (DOAC 4-1561 isolated from leaves of *O. sativa* L.), *Erwinia carotovora* pv. *atroseptica* (DOAC 4-0039 isolated from tubers of *Solanum tuberosum* L.), *Fusarium oxysporum* subsp. *cubense* (DOAC 0110 isolated from pseudostems of *Musa sapientum*), and *Helminthosporium oryzae* (DOAC 1570 isolated from leaves of *O. sativa* L.).

2.1. In silico *analyses*

We searched for *O. sativa* ssp. *japonica* DEF and DEF-like (DEFL) peptide genes using the Phytozome [17] and Gramene [62] databases. The coexpression of rice DEF genes with pathogen-responsive genes was analyzed using PlantArrayNet [35], with data collected from 183 microarrays involving rice leaves, roots, flowers, and calli at various developmental stages. The cut off percentage was set at 60% (i.e., $r_{\text{two-spots}}$ at 6.0). The expression levels in rice tissues were determined using the Rice eFP browser (microarray database) [27]. Expression profiles were generated for seedlings, roots, mature and young leaves, and shoot apical meristems, as well as for panicle (P1–P6) and seed (S1–S5) developmental stages. The stages were categorized according to panicle length and the number of days after pollination according to a previously described method [25]. Furthermore, the Genevestigator tool [24] was used to analyze the expression levels of candidate rice DEFs that were affected by seven pathogens, including plant-pathogenic bacteria (i.e., *Agrobacterium tumefaciens*, *Xanthomonas oryzae*, *Magnaporthe oryzae*, and *Magnaporthe grisea*), a plant-pathogenic fungus (*Mycosphaerella graminicola*), a nematode (*Meloidogyne incognita*), and an insect larva (*Orseolia oryzae*). Subcellular localizations and the presence of a secretory signal peptide were predicted using Wolfpsort [22] and SignalP [45], respectively.

2.2. Phylogenetic analysis

The *OsDEF7* (EMBL: BAF09407) and *OsDEF8* (EMBL: BAF10767) genes underwent neighbor-joining estimation analysis using the MEGA4 program, with bootstrap resampling set as the default [57]. Plant DEFs with known functions [60] were also used to construct the phylogenetic tree, and are listed in Supplemental Table S1.

2.3. Construction of recombinant plasmids for rice AMP production

The pUC18 plasmid containing either *OsDEF7* or *OsDEF8* was used as a template. The following primers were designed to exclude the *OsDEF* signal peptides: *OsDEF7* forward 5′-AAAGGATCCATGAGGCACTGCCTGTCGCAGAG-3′, *OsDEF7* reverse 5′-AAAGTCGACCTAGCAGACCTTCTTGCAGAAG-3′, *OsDEF8* forward 5′-AAAGGATCCATGGGGCCGGTGATGGTGGCGGA-3′, and *OsDEF8* reverse 5′-AAAGTCGACTCAGGGGCAGGGCTTGGT-3′. The underlined sequences represent either *Bam*HI or *Sal*I restriction enzyme sites. The polymerase chain reaction (PCR) was completed using a T100 Thermal Cycler (Bio-Rad). The PCR products were inserted into the glutathione S-transferase (GST)-containing expression vector, pGEX-6P-3, for the expression of a fusion protein consisting of *Os*DEF and GST (Supplemental Fig. S1). This prevented the host cells from being damaged by the encoded DEF genes. All constructs were sequenced to confirm the presence of the correct insertion.

2.4. Expression and purification of recombinant rice AMPs

The pGEX-6P-3-*OsDEF7* and -*OsDEF8* plasmids were transformed into Rosetta-gami *E. coli* (DE3) cells, which enable cytosolic disulfide bond formation. To produce GST-fused *Os*DEF7 and