Journal of Plant Physiology 224–225 (2018) 95–102

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Heterologous expression and antimicrobial activity of OsGASR3 from rice (*Oryza sativa* L.)

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ARTICLE INFO

Keywords:
GASR
Snakin
Rice
Antimicrobial activity

ABSTRACT

According to an in silico analysis, OsGASR3 (LOC_Os03g55290) from rice (*Oryza sativa* L.) was predicted to be involved in plant defense mechanisms. A semi-quantitative reverse transcription polymerase chain reaction assay revealed that *OsGASR3* is highly expressed in the inflorescences of Thai jasmine rice (*O. sativa* L. subsp. *indica* 'KDML 105'). To characterize the biological activity of OsGASR3, we produced an OsGASR3–glutathione S-transferase fusion protein in *Escherichia coli* Rosetta-gami (DE3) cells for a final purified recombinant OsGASR3 yield of 0.65 mg/L. The purified OsGASR3 inhibited the hyphal growth of *Fusarium oxysporum* f.sp. *cubense* and *Helminthosporium oryzae* at a relatively low concentration (7.5 μg/mL). Furthermore, OsGASR3 exhibited in planta inhibitory activity against *Xanthomonas campestris*, suggesting its involvement in defense mechanisms, in addition to its previously reported functions affecting growth and development. These observations indicate that recombinant OsGASR3 may be useful for protecting agriculturally important crops against pathogenic microbes.

1. Introduction

Snakin/GASA peptides are classified as cysteine-rich antimicrobial peptides (AMPs), and were named based on the fact they include amino acid sequences that are similar to that of a snake venom peptide. They consist of 12 conserved cysteine residues that potentially form six disulfide bonds. The genes encoding snakin/GASA peptides are members of the gibberellic acid-stimulated transcript-related (GASR) gene family because they are involved in signaling pathways that regulate hormone levels, and in biotic stress responses (Porto and Franco, 2013). They also contribute to different plant developmental processes such as cell division, cell elongation, flowering, and defense. These peptides have been identified in diverse monocotyledonous and dicotyledonous plant species (Nahirñak et al., 2012b).

Many snakin/GASA peptides reportedly exhibit antimicrobial functions. Additionally, silencing the *Nicotiana benthamiana SN2* snakin gene increases the susceptibility of the resulting plants to *Clavibacter michiganensis* and alters the plant cell wall composition (Balaji et al., 2011). The potato (*Solanum tuberosum*) snakin/GASA peptides (i.e., SN1–SN3) are affected by bacterial and/or fungal infections (Nahirñak et al., 2016). Previous studies confirmed that SN1 is localized to the plant cell wall (Segura et al., 1999; Nahirñak et al., 2012a), implying that snakin peptides might act as the first line of defense. Furthermore, a potato snakin gene expressed in wheat and potato plants limits the invasiveness of fungal and bacterial diseases (Rong et al., 2013; Mohan et al., 2014). The overexpression of the snakin-encoding *SN2* gene in tomato (*Solanum lycopersicum*) enhances the tolerance of transgenic plants against bacterial wilt disease (Balaji and Smart, 2012). These studies suggest that snakin peptides have important antimicrobial functions.

Rice (*Oryza sativa* L.) is an important food crop that is consumed by more people worldwide than any other crop. However, attacks by insects and microbes decrease rice yields, with diseases caused by bacteria and fungi representing a major problem for rice production. These

Abbreviations: AMP, antimicrobial peptide; GASR, gibberellic acid, stimulated transcript-related; GST, glutathione S-transferase; MIC, minimum inhibitory concentration; IPTG, isopropyl β-D-1-thiogalactopyranoside; PCR, polymerase chain reaction; PDA, potato dextrose agar; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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https://doi.org/10.1016/j.jplph.2018.03.013
Received 6 June 2017; Received in revised form 15 March 2018; Accepted 24 March 2018
Available online 27 March 2018
0176-1617/

diseases can lead to considerable yield losses in severely infected rice fields, including those consisting of Thai jasmine rice (*O. sativa* L. subsp. *indica* 'KDML 105'). Although several AMPs are encoded in the rice genome (Silverstein et al., 2007), transgenic rice plants producing AMPs from different organisms, such as a thionin from oat (Iwai et al., 2002), a defensin from shrimp (Wei et al., 2011), and a knottin-type AMP from *Mirabilis jalapa* (Prasad et al., 2008), have been generated for improved disease management. This is because most rice AMPs have not been functionally characterized. Nevertheless, a few rice snakin/GASA (OsGASR) proteins have been described. Two *OsGASR* genes, namely *OsGASR1* and *OsGASR2* (hereafter referred to as *OsGASR3* and *OsGASR4*, respectively, according to the Phytozome database), are highly expressed in proliferating rice tissues, suggesting they are involved in cell division, and possibly panicle differentiation (Furukawa et al., 2006). The expression of *OsGASR3* is induced by salt stress and abscisic acid, and a characterization of a knockout mutant suggests this gene influences seedling growth and amylase production (Lee et al., 2017). Wang et al. (2009) reported that the protein encoded by *OsGSR1* (hereafter referred to as *OsGASR7* according to the Phytozome database) is involved in gibberellin and brassinosteroid pathways. However, the roles of OsGASRs in responses to biotic stress have not been determined. To identify OsGASRs associated with plant responses to pathogens, we employed a similar strategy that we previously used to identify antimicrobial rice defensin 7 and 8 (Tantong et al., 2016). We conducted an in silico analysis to screen for antimicrobial OsGASRs and subsequently characterized their potential utility for disease management.

2. Materials and methods

2.1. Plant materials

Oryza sativa L. subsp. *indica* 'KDML 105' seeds were surface-sterilized with 70% ethanol for 2 min and 15% sodium hypochlorite for 30 min. They were then immersed in deionized water for 4 days in darkness. The water was changed daily. The hull and coleoptiles were removed from germinated seeds that were collected on day 5 for a subsequent RNA extraction. The remaining germinated seeds were incubated for 2 weeks on 0.5% Yoshida medium at 25 °C and 80% relative humidity, with a 16-h photoperiod (light intensity of 6000 lx). The rice seedlings were then collected, and RNA was extracted from the separated leaves and roots. Mature leaves and roots were collected from hydroponically grown 1-month-old rice plants. The rest of the mature plants were transferred to pots containing clay soil, and grown outdoors until flowering. The analyses were completed with three biological replicates, with either 10 seeds or five plants per replicate. Additionally, *N. benthamiana* seeds were germinated in small pots containing peat moss, and incubated for 1.5 months at 25 °C with a 16-h photoperiod (light intensity of 4000 lx). All samples were represented by three biological replicates.

2.2. Microorganisms

The following bacteria and fungi used in the antimicrobial assays were obtained from the DOAC Culture Collection Centre in the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand: *Xanthomonas oryzae* pv. *oryzae* (DOAC 4-1570; isolated from *O. sativa* L. leaves), *Erwinia carotovora* pv. *atroseptica* (DOAC 4-0039; isolated from *S. tuberosum* L. tubers), *Fusarium oxysporum* f.sp. *cubense* (DOAC 0110; isolated from *Musa sapientum* pseudostems), and *Helminthosporium oryzae* (DOAC 1570; isolated from *O. sativa* L. leaves). *Xanthomonas campestris* pv. *glycines* (TISTR 786) was obtained from the Thailand Institute of Scientific and Technological Research Culture Collection.

2.3. In silico analyses

We searched the Phytozome database for *OsGASR* sequences (Goodstein et al., 2012). The expression levels of the detected sequences in rice tissues were determined using the Rice eFP browser (Jain et al., 2007). Gene expression profiles were generated for seedlings, roots, mature and young leaves, and shoot apical meristems, as well as for the panicle developmental stages (P2–P6). These stages were categorized according to the panicle length and number of days after pollination (Jain et al., 2007). The Genevestigator tool (Hruz et al., 2008) was used to analyze the pathogen-regulated expression levels of candidate *OsGASR* genes. Furthermore, the coexpression of *OsGASR* and pathogen-responsive genes was analyzed using PlantArrayNet (Lee et al., 2009). The cut-off percentage was set at 60% (i.e., $r_{\text{two-spots}}$ at 6.0). Subcellular localizations and the presence of a secretory signal peptide were predicted using WoLF PSORT (Horton et al., 2007) and SignalP (Petersen et al., 2011), respectively.

2.4. Semi-quantitative RT-PCR analysis of OsGASR3 expression in O. sativa subsp. indica 'KDML 105'

Total RNA was extracted from different rice tissues using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). The following *OsGASR3*-specific primers were designed: forward 5′-ATGGGATCAGA TTTCTGCGACGG-3′ and reverse 5′-TCATGGGCACTTGGGCCTC-3′. The extracted mRNA was reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). A reverse transcription polymerase chain reaction (RT-PCR) was conducted using the Phusion® High-Fidelity DNA polymerase (New England Biolabs, Ontario, Canada), with 10 ng cDNA as the template and EF1α as the internal standard. The PCR program was as follows: 98 °C for 30 s; 30 cycles of 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 45 s; 72 °C for 5 min. The PCR products were then analyzed on a 1% (w/v) agarose gel, and the band intensities were quantified using Quantity One software. The intensity ratio of each band to *Ef1α* was determined.

2.5. Construction of a recombinant plasmid for OsGASR3 expression in Escherichia coli

The following OsGASR3 primers were designed to exclude the signal peptide: forward 5′-AATGGATCCATGGGATCAGATTTCTGCGACGG-3′ and reverse 5′-AAAGTCGACTCATGGGCACTTGGGCCTC-3′. The underlined sequences represent either *Bam*HI or *Sal*I restriction enzyme sites. A pUC18 vector containing *OsGASR3* was used as a template. The PCR was completed using a T100 Thermal Cycler (Bio-Rad), and the resulting amplification products were inserted into the pGEX-6P-3 vector for the subsequent production of an OsGASR3–glutathione S-transferase (GST) fusion protein (Supplemental Fig. S1 B). This protein may protect host cells from damages due to the encoded OsGASR3. All constructs were sequenced to confirm the presence of the correct insertion.

2.6. Production and purification of recombinant OsGASR3

The pGEX-6P-3-*OsGASR3* plasmid was inserted into competent *E. coli* Rosetta-gami (DE3) cells. The bacteria were grown in Luria-Bertani (LB) broth containing 50 μg/mL kanamycin, 100 μg/mL tetracycline, 100 μg/mL ampicillin, and 34 μg/mL chloramphenicol. When the culture reached an optical density (600 nm) of 0.6, the cells were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and continuously grown at 37 °C for 6 h *E. coli* Rosetta-gami (DE3) cells were pelleted by centrifugation and then lysed by ultrasonication. After that, the soluble extract was fractionated by centrifugation at 12,000 rpm at 4 °C for 10 min. The proteins in soluble fractions were purified using Pierce glutathione spin columns (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The GST fusion partner was cleaved from the purified proteins by an on-column digestion with