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Overexpression of germin-like protein *GmGLP10* enhances resistance to *Sclerotinia sclerotiorum* in transgenic tobacco

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ABSTRACT

Germin-like proteins (GLPs) are ubiquitous water-soluble glycoproteins that are located in the extracellular matrix. These proteins have been reported to play vital roles in diverse biological processes. In the present study, a GLP in soybean (*Glycine* max L. Merr.), GmGLP10, was characterized. Sequence analysis revealed that the *GmGLP10* gene (GenBank Accession Number EU916258) encodes a 213-amino acid (aa) protein, which contains a N-terminal signal peptide at 1–22 aa and is highly homologous to the members of the GER2 subfamily. *GmGLP10* was highly expressed in the leaves, but very faint in the roots. The expression of *GmGLP10* was induced by methyl jasmonate (MeJA), ethylene (ET), salicylic acid (SA), oxalate acid (OA), and the infection of *Sclerotinia sclerotiorum*. Overexpression of *GmGLP10* in transgenic tobacco significantly enhanced tolerance to OA and *S. sclerotiorum* infection. Moreover, higher levels of H_2O_2 and the upregulated expression of a set of plant defense-related genes and HR (hypersensitive response)-associated genes were detected in the transgenic plants. These results suggest that *GmGLP10* functions as a positive regulator of resistance to *S. sclerotiorum.*

1. Introduction

Germins and germin-like proteins (GLPs) are ubiquitous water-soluble glycoproteins that are located in the apoplast or extracellular matrix [1,2]. Germin was first discovered in germinating wheat embryos [3], and since then, an increasing number of proteins with sequence similarity ranging from 30% to 70% to wheat germin have been found in diverse plant groups [4,5]. Due to their relatively lower sequence similarity, they are named as GLPs. Both germins and GLPs belong to the “cupin” subfamily that contains a conserved β-barrel core that is involved in manganese ion binding. Germins and GLPs show exceptionally high resistance to proteasome activity, heating, extreme pH, and detergents [6].

Sclerotinia sclerotiorum, is a non-host-specific and necrotrophic fungus causing a destructive disease in crop production. It infects several important commercial crops such as oilseed rape, sunflower, tobacco, potato, tomato, cotton, soybean, and alfalfa [7,8], thereby resulting in an extensive yield loss annually worldwide [9,10]. In recent years, quantitative trait locus (QTL) analysis [11,12] and genome-wide association (GWA) mapping methods [13,14] have made substantial progress in elucidating the mechanism underlying resistance to *S. sclerotiorum*. It is widely accepted that differences in resistance among varieties are regulated by multiple genes [15]. Therefore, improvements in breeding resistant strains have been hindered by the lack of immunotype germplasms.

Transgenic approaches have further proven that GLPs are associated with plant defense responses [16]. Overexpression of OsGLP2-1 in transgenic rice increases resistance to bacterial blight and fungal blast by influencing defense-related genes that are associated with the JA-dependent pathway, while elevating endogenous JA levels [17]. In addition, plants overexpressing HaGLP1 in *Arabidopsis* exhibit enhanced protection against fungal pathogens by promoting ROS (reactive oxygen species) accumulation [18]. Genome-wide analysis has revealed that soybean produces 21 types of GLPs [19]. Expression analysis has indicated that the majority of these genes are induced by abiotic stress [20]. Recent research studies have shown that *GmGLP7* is induced by various abiotic stresses. Transgenic *Arabidopsis* plants that overexpress *GmGLP7* exhibit improved salt tolerance to salt, drought,

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and oxidative stresses, and are hypersensitive to exogenous ABA treatment [20]. The overexpression of another member of the soybean GLP family, *GmGLP9*, significantly increases salt stress tolerance in tobacco [19]. These results indicate that soybean GLPs function as important regulators of stress responses. However, the functions of other *GmGLP* genes have not been established to date.

In the present study, the expression levels of *GmGLP10* in various tissues and treatments by salicylic acid (SA), ethylene (ET), methyl jasmonate (MeJA), oxalate acid (OA), and *S. sclerotiorum* were investigated. To elucidate the function of *GmGLP10* after *S. sclerotiorum* inoculation, *GmGLP10*-overexpressing transgenic tobacco was generated, and tolerance to OA and *S. sclerotiorum* in wild-type (WT) and transgenic plants was investigated. Furthermore, the content of endogenous H_2O_2 and transcript abundance of several known stress-related genes were analyzed. These results suggested that *GmGLP10* functions as a positive regulator of resistance to S. sclerotiorum.

2. Materials and methods

2.1. Plant growth conditions and treatments

Soybean cultivar “Maple Arrow” was used to analyze the expression patterns of *GmGLP10* under different treatments. Soybean seeds were sown in vermiculite and grown in a growth chamber under a 16-h light/8-h dark photoperiod. For treatments, leaves of 14-day-old seedlings were sprayed with 5 mM SA, 10 mM ET, 100 μM MeJA, and 5 mM OA, respectively. Leaves were harvested after 0 h, 1 h, 3 h, 5 h, 8 h, 12 h, and 24 h, respectively. For *S. sclerotiorum* challenge, leaves were inoculated with *S. sclerotiorum* discs punched in 5-mm diameter and covered to retain moisture. Leaves were harvested after 0 d, 1 d, 2 d, 3 d, 4 d, and 5 d. For tissue-specific analysis, the roots, stems, cotyledons, and leaves of 14-day-old seedlings were harvested. All samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. Bioinformatics analysis of GmGLP10

Domain analysis was conducted by SMART (http://smart.embl-heidelberg.de/). The signal peptide was predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). GlycoEP (http://www.imtech.res.in/raghava/glyc oep/) was used to assess potential glycosylation sites. Sequences showing highly similarity to *GmGLP10* were aligned using ClustalX.

2.3. qRT-PCR analysis

Total RNA was isolated using RNAiso Plus (Takara, Dalian, China). First-strand cDNA was synthesized and subsequently used for qRT-PCR analysis. The qRT-PCR was conducted on LightCycler 480 real-time PCR machine (Roche, Germany) using gene-specific primers. *GmACTIN4* (GenBank Accession Number AF049106) and NtEF1a (GenBank Accession Number D63396.1) was used as internal control. Relative expression levels were calculated using the $2^{-\triangle\triangle CT}$ method.

2.4. Generation of transgenic tobacco plants

The recombinant pCAMBIA3301 -*GmGLP10* plasmid was transformed into *Agrobacterium tumefaciens* strain EHA105 and subsequently used for transformation of tobacco cultivar “Havana 425” by *Agrobacterium*-mediated leaf disk transformation method [21]. Transgenic plants were identified by PCR and qRT-PCR methods using specific primers *GmGLP10I-F* and *GmGLP10I-R*.

2.5. OA tolerance assay

Assessment of OA tolerance was performed as described by Chen [22] with slight modifications. Briefly, seeds from WT and transgenic tobacco lines were sown on MS medium plates (calcium-free) with or without 1.2 mM OA, incubated at 4 °C for 3 days, and grown vertically at 22 °C, 16-h light/8-h dark for 10 days, followed by measurement of fresh weight and primary root length.

2.6. Analysis of S. sclerotiorum resistance

Resistance analysis was performed as described by S. Rietz [23] with minor modifications. Completely expanded leaves from six-week-old WT and transgenic lines were excised and placed on the moistened filter papers. Mycelium plugs 5-mm in diameter were separated from the margin of the mycelium-grown PDA plate and placed on the leaves, then sealed for moisture retention and incubated at 22 °C under a 16-h light/8-h dark photoperiod. Disease symptoms in the WT and transgenic lines leaves were monitored and photographed.

2.7. Detection of H_2O_2 levels and SOD enzyme activity

DAB staining was performed as previously described [24]. The H_2O_2 levels and SOD enzyme activity of the leaves of the transgenic and WT tobacco plants were measured using Hydrogen Peroxide Assay Kit (Colorimetric method) and Superoxide Dismutase (SOD) assay kit (WST-1 method) (Jiancheng, Nanjing, Jiangsu, China) following the instructions of the manufacturer.

2.8. Expression analyses of stress-related genes

To further understand the molecule mechanism underlying *GmGLP10*-mediated increase in *S. sclerotiorum* tolerance, the expression levels of HR- and defense-related marker genes, including *NtPR1* (GenBank Accession Number X06361), *NtPR2* (GenBank Acc. No. M60460), *NtPR4* (GenBank Acc. No. X60281), *NtPR5* (GenBank Acc. No. AF154636), *NtHSR201 (*GenBank Acc. No. X95343), and *NtHSR203* (GenBank Acc. No. X77136) were detected in WT and transgenic lines plants. *NtEF1α* (GenBank Acc. No. D63396) was used as an internal control. Leaves of six-week-old WT and transgenic lines plants were harvested and analyzed by qRT-PCR using gene-specific primers.

3. Results

3.1. GmGLP10 encodes a GLP in soybean

The full-length coding sequence of *GmGLP10* was 642 bp in length. The *GmGLP10* gene did not contain introns and was predicted to encode a 213-amino acid protein (Supplementary Fig. 1). The GmGLP10 protein was estimated to have a molecular mass of 22.23 kDa and with an isoelectric point (pI) of 6.03. The protein contained a cleavable N-terminal signal peptide of 22 amino acid residues and highly conserved motifs, namely germin boxes, which including BoxA, BoxB, and BoxC (Supplementary Fig. 1). Additionally, a putative N-glycosylation site and a KGD motif were identified within the sequence (Fig. 1A), glycosylation existed extensively in germin and germin-like proteins, previous reports shows that glycosylation can play vital roles in the folding, transportation and localiazation of protein, moreover, involved in many important biological processes such as receptor activation or signal transduction [25].

GmGLP10 is highly homologous to the members of the GER 2 subfamily, including PpABP19 (*Prunus persica*; AAD00295),

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