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Transgenically expressed rice germin-like protein1 in tobacco causes hyper-accumulation of H_2O_2 and reinforcement of the cell wall components

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ABSTRACT

Our recent report documented that the rice germin-like protein1 (OsGLP1), being a cell wall-associated protein involves in disease resistance in rice and possesses superoxide dismutase (SOD) activity as recognized by heterologous expression in tobacco. In the present study, the transgenic tobacco plants were analyzed further to decipher the detailed physiological and biochemical functions of the OsGLP1 and its associated SOD activity. The transgenic tobacco lines expressing SOD-active OsGLP1 showed tolerance against biotic and abiotic stresses mitigated by hyper-accumulating H_2O_2 upon infection by fungal pathogen (*Fusarium solani*) and treatment to chemical oxidizing agent (ammonium persulfate), respectively. Histological staining revealed enhanced cross-linking of the cell wall components in the stem tissues of the transgenic plants. Fourier transform infrared spectroscopy (FTIR) analysis of the biopolymer from the stem tissues of the transgenic and untransformed plants revealed differential banding pattern of the spectra corresponding to various functional groups. Our findings demonstrate that the OsGLP1 with its inherent SOD activity is responsible for hyper-accumulation of H_2O_2 and reinforcement of the cell wall components.

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1. Introduction

Germin-like proteins (GLPs) are ubiquitous plant glycoproteins belonging to cupin super family and they possess diverse functional role in different plant species [1]. More recent studies have shown that the rice germin-like protein1 (OsGLP1) is a cell wall-associated protein and has the superoxide dismutase (SOD) enzyme activity [2]. Like other GLPs and germins, the OsGLP1 possesses protective role against fungal pathogen attack [1,2]. Structurally, the OsGLP1 was found to show a significant homology with barley oxalate oxidase, which is a germin protein [2] but the detailed physiological role and the biochemical function of OsGLP1 are yet to be established.

In plants, during flow of electron through electron transport chains and in different metabolic reactions small fractions of electrons escape and combine with molecular oxygen to yield a few reactive oxygen species (ROS) including superoxide. These ROS are highly toxic to cells and are not compatible with the normal metabolism and they must be eliminated through antioxidative defense system [3]. Under stress condition, the generation of toxic oxygen species is increased causing the susceptibility of plants to photo-inhibition and subsequent chlorosis [3]. The SOD-mediated reaction is one of the main antioxidative defense systems in plant and the SOD converts superoxide into hydrogen peroxide (H_2O_2) and molecular oxygen. Peroxidase enzymes convert the H_2O_2 into water and there are many peroxidases which are present in the apoplastic space of the plant cells and they are ionically or covalently bound to the cell wall polymers. ROS causes a number of modifications of cellular components [4], whereas in the presence of H_2O_2 and phenolic substrates peroxidases are involved in peroxidative cycle and are involved in synthesis of lignin and other phenolic polymers [5].

Oxalate oxidase and SOD activity of the proteins belonging to cupin family members are found to be responsible for their disease resistance property. An earlier study demonstrated that after infection with a fungus (*Sclerotinia sclerotiorum*), the accumulation of H_2O_2 increased in the oxalate oxidase over-expressing transgenic *Brassica napus* leaves compared to the untransformed one [6]. It was found that the GLPs from barley play a complex function in basal resistance against the fungal infection caused by *Blumeria graminis* [7]. In a few earlier reports, GLPs from wheat and barley have been proposed to have some structural role in relation to cross-linking of the cell wall after pathogen attack [8,9]. On the other hand, the lignin, being one of the most important biopolymer present in the plant cell wall has been well documented to be involved in various stress conditions. Lignin molecules cross-link with

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carbohydrates (cellulose and hemicellulose) and thereby play some crucial role of the cell walls; such as enhancing rigidity, improved stability to mechanical stress, conferring resistance to pathogen attack and enabling solute transport in the xylem [10].

Here we report the detailed analyses of the transgenic tobacco plants expressing the OsGLP1 for tolerance against biotic and abiotic stresses, and established that the OsGLP1-associated SOD activity mitigates the antioxidative defense response due to hyper-accumulation of H_2O_2 and cross-linking of the cell wall components including lignin.

2. Materials and methods

2.1. Plant materials

Tobacco (*Nicotiana tabacum*) leaf discs were transformed by *Agrobacterium tumefaciens* strain LBA4404 harboring the over-expression construct of OsGLP1 gene prepared in pCAMBIA 1300 vector as described earlier [2]. Different transgenic lines were generated and the T_2 generation plants were used for various analyses.

2.2. Southern blotting

Southern hybridization was carried out by electrophoresing 10 μg of *Bam*HI digested genomic DNA samples from the untransformed and different transgenic lines (line 1–4) onto 0.8% agarose gel along with lambda DNA digested with *Eco*RI and *Hin*dIII enzymes as molecular weight marker. Hybridization was performed using [α-^{32}P]dCTP labeled OsGLP1 (642 bp) probe following standard protocol [11].

2.3. Northern blotting

For northern blotting of leaf samples, 30 μg of RNA from untransformed and different transgenic tobacco lines (line 1, 2 and 4) were electrophoresed in 1.2% agarose-denaturing (formaldehyde) gel. After electrophoresis, deformylation and transfer of RNA; subsequent hybridization using [α-^{32}P]dCTP labeled OsGLP1 (642 bp) probe was performed following standard protocol [11].

2.4. Protein extraction, electrophoresis and western blotting

Isolation of total proteins from the leaf tissues of untransformed and transgenic tobacco plants and the electrophoresis under complete denaturing as well as semi-native conditions were performed following the earlier protocol [2]. After electrophoresis, the protein samples were transferred onto Hybond-C nitrocellulose membrane (Amersham life sciences) and western blotting was performed using anti-OsGLP1 polyclonal antibody as reported earlier [2].

2.5. Superoxide dismutase assay

The superoxide dismutase activity on semi-native polyacrylamide gel was performed as described before [2].

2.6. Bioassay of leaf discs against fungal pathogen

For bioassay of OsGLP1-expressing tobacco plants, the leaf discs from healthy transgenic and untransformed plants were infected with the saturated pure culture of a laboratory isolate of *Fusarium solani*, which is a fungal strain. Different amounts of inocula (20, 30, 40 and 50 μl) were spotted on the leaf discs along with the untreated one (where no fungal inoculum was added). After 24 h of inoculation, the disease infestations were recorded in different leaf discs of untransformed and transgenic plants.

2.7. Detection of hydrogen peroxide

The visual detection of H_2O_2 in *F. solani* infected leaves was performed following an earlier report [6]. The leaves of the untransformed and transgenic plants were inoculated with *F. solani* for 12 h and subsequently the excised leaves were incubated using 3,3-diaminobenzidine (DAB) as a substrate. After dechlorophylization and clearing with boiling ethanol; the whole leaves were digitally photographed and subsequently the transverse sections of the leaves were observed under the microscope (Leica, DB IL) and digitally photographed.

2.8. Treatment with chemical oxidizing agent

The leaves of the untransformed and different transgenic plants were analyzed for tolerance against ammonium persulfate (APS), which is a strong oxidizing chemical under *in planta* condition. Twenty microliters of APS (10%) solution was spotted onto the leaves of untransformed and different transgenic plants. The phenotype was recorded after 6 h of treatment and intensity of the burnt lesions (whitish color) were measured by arbitrary unit for knowing the effect of APS on leaves.

2.9. Histological staining

Phloroglucinol staining and Mäule staining were performed following reported protocol [10] by taking the fresh transverse thin sections from the apical portion (below the pods) of the tobacco plants at the mid-maturation stage. The sections were then observed under light microscope (Leica, DB IL) and digitally photographed.

2.10. Fourier transform infrared spectroscopy analysis

Fourier transform infrared (FTIR) study for the biopolymers of the stem tissues from untransformed and transgenic tobacco plants was performed using Nexus 870 Nicolet FTIR spectrophotometer following an earlier reported protocol [12]. In brief, stem tissues were crushed in liquid nitrogen into fine powder and dried in desiccators. Equal weights of powder samples from different plants were taken and mixed with potassium bromide to form pellet and subsequently subjected to FTIR spectrophotometer and analyzed using OMNIC software.

3. Results

3.1. Expression and activity of OsGLP1 in transgenic tobacco plants

Several independent transgenic tobacco lines were developed with the OsGLP1 transgene through *Agrobacterium*-mediated transformation using the OsGLP1 over-expression chimeric gene construct which has been described in our recent report [2]. Among different transformants, four independent transgenic lines (line 1–4) were confirmed by Southern blotting (Fig. 1A) in T_2 generation. The expression pattern of OsGLP1-specific transcript was analyzed in three different transgenic lines (line 1, 2 and 4) along with the untransformed control by northern blot using the OsGLP1 gene-specific probe. A prominent OsGLP1-specific transcript band was detected in all the transgenic lines tested (Fig. 1B), whereas no OsGLP1-specific transcript band was detected in the untransformed plant (Fig. 1B). The levels of OsGLP1 expression were found to differ in different transgenic lines and between them two better expressing lines were analyzed further.

In western blotting, neither in semi-native condition nor in complete denaturing condition was any OsGLP1-specific protein band

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