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Overexpression of *Withania somnifera SGTL1* gene resists the interaction of fungus *Alternaria brassicicola in Arabidopsis thaliana*

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ABSTRACT

Heterologus transformed *Arabidopsis* plants improved tolerance against abiotic stress by the modulation of glycosylation of sterols and sterol glycosides. It is due to enhanced activity of *Ws*SGTL1 enzyme in different stresses. Presently, the effect of *WsSGTL1* gene was investigated, focusing on interaction of *A. brassicicola* fungus on transgenic lines. Overexpressed lines showed restricted lesion, less spore counts and few electrolyte leakages. Biochemical study suggested that the transgenic lines had more glycosylated sterol/phenolic compounds. Relative expression of JA biosynthesis gene and phenylalanine ammonia lyase (*PAL)* showed enhanced expression. Photosynthesis and chlorophyll fluorescence imaging revealed that transgenic plants had minimum damage of photosynthetic apparatus because of minimum PSII destruction.

1. Introduction

Plants endure challenges in adverse environmental conditions, such as biotic stresses (fungi, bacteria, viruses and pests) and abiotic stresses (high salt, drought, water logging, heat, cold, injury, and others), these effects their normal growth and development. To overcome the adverse environmental conditions plants have evolved a systematic defensive mechanism that includes multiple independent and crossed signaling pathways for recovery [1]. Glycosylation is a crucial defense mechanism of plants to protect themselves against the huge range of toxic compounds released by their attackers [2]. Sterol glycosyltransferases enzymes play diverse roles in cellular metabolism by modifying the important regulatory mechanism in the activity of sterols and secondary metabolites in higher organisms ranging from molds and plants to insects and mammals [3,4]. Sterols and secondary metabolites rarely accumulate in their free form but are often conjugated to carbohydrate moieties, particularly glucose, through the action of glycosyltransferases [5,6]. Glucose conjugation is thought to regulate bioactivity of aglycones, to enhance their solubility, enhance the free radical scavenging activity, to protect their reactivity toward cellular oxidases, and to alter their transport properties throughout the whole plant [7,8]. A large group of glycosterols called saponins, comprising glycosylated triterpenoids, steroids and steroidal alkaloids, occurs in many plants. These steroidal glycoalkoloid contain sugar chain coupled to C-3 hydroxyl group and possess antifungal activity [9]. Several publications showed clear evidence that fungal toxins can be modified and inactivated in planta [10,11]. *Sinapis alba* for example is able to hydroxylate and glycosylate destruxin B, a toxin produced by *Alternaria brassicae*, and simultaneously activate the production of phytoalexins which makes the plant species resistant to the blackspot fungus [11]. Plant pathogenic fungi of the genus *Fusarium* produced trichothecene deoxynivalenol (DON), a harmful mycotoxin which causes disease of small grain cereals and maize. Overexpression of glycosyltransferase UGT73C5 in transgenic *Arabidopsis* can improve its resistance against fungal toxins DON [12]. Downregulation of a pathogen responsive tobacco UDP glucose:phenylpropanoid glucosyltransferase reduces scopoletin glucoside accumulation, enhances oxidative stress and weakens virus resistance in tobacco [13].

Abbreviations: A. brassicicola, *Alternaria brassicicola*; Dpi, Days post inoculation; hpi, Hours post inoculation; MeJA, Methyl jasmonate; NPQ, Non photochemical quenching; LOX2, Lipoxygenase 2; PAD3, Phytoalexin deficient 3; SA, Salycilic acid; SGT, Sterol glycosyltransferase; Y (II), Yield.

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Withania somnifera is a source of variety of secondary metabolite like glycosylated steroidal lactones called withanosides, present in roots [14] and leaves [15]. It exhibits broad sterol specificity by glycosylating a variety of sterols/phenolic acids/withanolides with β-OH group at C-3 position [9,16]. Previously, we had shown *WsSGTL1* gene (*Withania somnifera* sterol glycosyltransferse *L1* gene) expression in *W. somniferra* was increased with treatment of heat, cold, SA and MeJA (Biotic elicitor) during different time intervals [17]. Their overexpression in *Arabidopsis thaliana* and *Nicotiana tabacum* enhanced modulation of sterols and other small molecules, therefore overexpressed transgenic plants showed more adaptive response against different abiotic stress and resistance towards *Spodoptera litura* [18,19]. Due to high expression and enhanced activity of *WsSGTL1* gene in presence of biotic elicitors [16,17], we have investigated the interaction of necrotrophic fungus *Alternaria brassicicola* on *WsSGTL1* overexpressed transgenic lines. The infected *Arabidopsis* with *A. brassicicola* has been extensively used as a model for diseases caused by fungal necrotrophs [20]. *A. brassicicola* causes dark spot disease, one of the most common and destructive fungal diseases of *Brassicaceae* worldwide. The results suggested that overexpression of *WsSGTL1* gene in *Arabidopsis* affected the glycosylation pattern of membrane sterols and enhanced the steroidal glycoalkaloids and glycosylated phenolics compounds. These molecules prevent the accumulation of ROS and free radicals after fungal infection and enhanced the immunity to sustain their own survival.

2. Materials and methods

2.1. Plant materials and growth conditions

To know the interaction of fungus *A. brasccicola in WsSGTL1* overexpressed lines we have selected previously characterized overexpressed *WsSGTL1* Line1, Line 2 and Line 3. All these transgenic lines were derived from independent transformation events. Line 1 and Line 2 has a single T-DNA insert, whereas the Line 3 has more than one copy of T-DNA inserts. RT-PCR analysis and enzyme activity showed that all *WsSGTL1* overexpressed lines significantly expressed and enhanced enzyme activity [18]. All genotype of *Arabidopsis* (Col-0) plants were grown to flowering stage in a shaded greenhouse at 22 °C under long day (LD) conditions (16 h light and 8 h dark) with white light illumination (120 μmol/m^2 s^{-2}) provided by fluorescent tubes [21].

2.2. Fungal strains and inoculation procedures

Fungal pathogen *A. brassicicola* was cultured on potato dextrose agar (PDA) medium at 28 °C. Conidia of *A. brassicicola* were collected from 12-day-old cultures in PDA (24 g l^{-1}). Fungal spores were scrapped in 1% gelatin upto spore count of 4×10^5 spores ml^{-1}. Then 10 μl suspension of 4×10^5 spores ml^{-1} in 1% gelatin was inoculated on leaves of individual 4-weeks-old plants. For mock treatment, 10 μl droplets of 1% gelatin were placed onto the leaves. Inoculated plants were kept at 100% RH at 24 °C with white light illumination (120 μmol/m^2 s^{-2}) provided by fluorescent tubes on a 12-h-light/12-h-dark cycle. After five days, lesion-size of *A. brassicicola* on the leaves was measured. The symptoms of fungal blight of *Arabidopsis* leaves was normally leaf wilting, discoloration, leaf drop, a little bit stunting, finally wilting and death.

2.3. Spore count assay

All inoculated leaves of individual plants were put into 0.8% agar media in petriplate for establishment of the infection. After 3–5 days, when infection was established, each leaf was crushed in 250 μl of 0.1% Tween-20 containing 0.85% NaCl. A hemocytometer was used to count the spores.

2.4. Total Chlorophyll content analysis

Chlorophyll contents were determined according to [22]. Sample of 20–25 mg was collected by taking at least five leaves each of non infected and infected plants. Chlorophyll was extracted in 80% acetone expressed as mg/g of FW of leaf tissue and calculated as follows,

$$\text{Chlorophyll } a = ((0.0127 \times 663 \text{ nm}) - (0.00269 \times 645\text{m}) \times 1000$$

$$\text{Chlorophyll } b = ((0.0229 \times 645 \text{ nm}) - (0.00468 \times 663 \text{ nm}) \times 1000$$

2.5. Electrolyte leakage

Electrolyte leakage of healthy and fungal infected plant leaves were measured with conductivity meter before and after autoclaving (121 °C for 20 min). Healthy and infected leaves were chopped in small pieces and kept in 10 ml distill water for 5–6 h. After incubation conductivity of the solution was measured. Thereafter autoclaved the sample containing solution and conductivity was again measured. Finally the percentage of electrolyte leakage was calculated as the ratio of the conductivity before autoclaving to that after autoclaving. It was assumed that the conductivity after autoclaving represents complete (100%) electrolyte leakage [18].

2.6. Trypan blue staining

For visualization of the symptoms fungal spores were sprayed on 28 d old plant leaves. After 3–4 days infected leaves were immersed in 5 ml of alcoholic lactophenol, (1 vol of phenol: lacic acid: glycerol: water (1:1:1:1) and 2 volume of ethanol) with 0.02 g of trypan blue. The leaves were evacuated 15 min then placed at 65 °C and incubated until they were completely cleared of chlorophyll (15–30min). these leaves were transferred to fresh alcoholic lactphenol for an additional 2–24 h. Autofluroscence was observed in the cleared leaf samples mounted in 25% glycerol by epifluroscent illumination (365 nm excitatation filter) on a light microscope (Leica).

2.7. Analysis of phenolic compounds through HPLC

Phenolic compounds in all *WsSGTL1* overexpressed plants were qualitatively and quantitatively analyzed through HPLC. For preparation of extract, leaves (100 mg) of each overexpressed plants and WT plants were extracted with methanol (HPLC grade) overnight at room temperature with brief agitation. Extracts of all the samples were prepared in 100% methanol. Flavonoids and phenolic quantification was performed by using mobile phase of gradient which was prepared from 1% (v/v) acetic acid of HPLC-grade water (component A) and acetonitrile (component B). The gradient was from 20 to 35% of component B in 0–14 min and from 35 to 50% of component B in 14–40 min. Quantification was carried out at 254 nm room temperature and 42 min retention time with flow rate of 0.6 ml min^{-1}. Qualitative and quantitative analysis of flavonoids and phenolics was performed by HPLC-PDA with a Shimadzu (Japan) LC-10 system comprising an LC-10AT dual pump system, SPD-M20A PDA detector and rheodyne injection valve furnished with a 20 μl sample loop. Compounds were separated on a Merck Purospher star® RP-C18 column (250 × 4.6 mm, 5 μm pore size) protected by