Original Article

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Cloning and expression analysis of squalene synthase, a key enzyme involved in antifungal steroidal glycoalkaloids biosynthesis from *Solanum nigrum*

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ABSTRACT: Steroidal glycoalkaloids (SGAs) are a family of nitrogenous secondary metabolites produced in solanaceous plants. In our present study, γ-solamargine and its aglycone solasodine from *Solanum nigrum* were found to inhibit hyphae formation of *Fusarium oxysporum*. As phytoalexins, the formation of SGAs was significantly increased in the plants when infected with the spore of *F. oxysporum*. In order to understand this inducible defense mechanism, the rate-limiting enzyme squalene synthase in the biosynthesis process of SGAs was investigated well. A full-length cDNA encoding squalene synthase was isolated from *S. nigrum* (the squalene synthase in *S. nigrum* was designated as SnSS). The full-length cDNA of SnSS was 1,765 bp and contained a 1,236 bp open reading frame (ORF) encoding a polypeptide of 411 amino acids. Bioinformatic analysis revealed that the deduced SnSS protein had a high similarity with other plant squalene synthases. Real-time RT-PCR analysis showed that SnSS was expressed constitutively in all tested tissues, with the highest expression in stems. After treatment with the spore of *F. oxysporum*, the mRNA level of SnSS was significantly increased in the infected plants in accordance with the change of SGAs.

Keywords: *Fusarium oxysporum, Solanum nigrum,* squalene synthase, steroidal glycoalkaloids

1. Introduction

Plants encounter many different pathogens during their lifetime, and are armed and ready to defend themselves with a combination of pre-formed and inducible defense mechanisms, including phytoalexin synthesis (*1,2*). Steroidal glycoalkaloids (SGAs) are secondary metabolites which exhibit antifungal activities in a wide range of plants including the Solanaceae. These secondary metabolites provide built-in chemical protection against pest and pathogen attack and can also influence induced defense responses. Increasing foliar SGA content to enhance plant resistance against pests has resulted in elevated levels of SGAs in the tubers, whereas reduction or abolishment of plant SGAs for the sake of food safety may weaken pathogen resistance (*3*). SGAs accumulation varies in different plant organs during development (*4*). In addition to anticancer and some other pharmacological significance (*5*), our present tests found SGA and its aglycone from *Solanum nigrum* inhibits hyphae formation of *Fusarium oxysporum*, which is a root-infecting fungal pathogen and responsible for vascular wilt in many different plant species (*6*). The switch between the yeast and hyphae form of the dimorphic fungus *F. oxysporum* is very necessary in its pathogenesis and the hyphae formation is a prerequisite to infect plants. As phytoalexins, the production of SGAs was significantly increased in the *S. nigrum* plants when infected with the spore of *F. oxysporum*, which means that fungus infection stimulates biosynthesis of SGAs and results in self-defense.

The biosynthesis of SGA is *via* the mevalonate/isoprenoid pathway. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the first step in SGAs biosynthesis. Downstream, squalene synthase, which is a membrane bound enzyme (*7*) that condenses two farnesyl diphosphate molecules into squalene catalyzes the next important step leading to sterols and SGAs. SGAs accumulation appears to be regulated at different steps in the biosynthetic pathway in response to environmental stress and development. High transcript levels of potato HMGR and squalene synthase encoding genes were shown to be associated with high SGA levels (*8*), implying that the regulation of SGA biosynthesis involves the committed step of the mevalonate pathway as well as later steps of SGA formation. It indicated that along with HMGR,

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squalene synthase is a potential site for the regulation of SGA production. Squalene synthase genes have been characterized in *Nicotiana tabacum* (*9*), *Panax ginseng* (*10*), *Glycyrrhiza glabra* (*11*), and other plants (*12-18*).

In order to gain new insights into the role of squalene synthase in the SGAs formation in *S. nigrum*, in this work, we report the antifungal activity of SGAs and the molecular characterization and expression analysis of SnSS in *S. nigrum* while infected.

2. Materials and Methods

2.1. *Plant material and fungal inoculation*

Plants of *S. nigrum* were cultivated in green house pots at 25°C with a 12 h light/12 h dark cycle. Plant materials, including root, stem, leaf, flower, premature fruit, and mature fruit were sampled and frozen immediately in liquid nitrogen, and stored at –80°C for RNA isolation.

The seeds of *S. nigrum* were surface sterilized using 5% sodium hypochlorite and cultured on 1/2 B5 medium at 25°C with a 12 h light/12 h dark cycle for 3 weeks for inoculation. *F. oxysporum* was donated by Dr. Huiying Zhang of school of life sciences of Shandong University. The pathogen was grown on PDA medium at 28°C. Sixty 3-week-old seedlings were inoculated with infectious *F. oxysporum* suspension (OD600 = 1.0) using a root-dip inoculation method. After inoculation, the cultured seedling of *S. nigrum* was cultured for another week. Samples were collected and frozen immediately in liquid nitrogen, and stored at –80°C for RNA isolation and compounds analysis.

2.2. *Effect of SGAs on the hyphal formation of F. oxysporum*

γ-Solamargine and its aglycone solasodine (Figure 1) were isolated from *S. nigrum* and the structures were confirmed by NMR in our lab (*19*). *F. oxysporum* (1×10^5 cells/mL in RPMI1640 medium) was incubated with γ-solamargine or solasodine in 96-well flat-bottomed microtitration plates at 37°C without shaking. Hyphae induction for microscopic observation was carried out in 96-well flat bottom non-tissue culture-treated plastic plates. After the incubation, they were microscopically photographed. Each experimental condition was tested in triplicate on a given day and repeated on separate days.

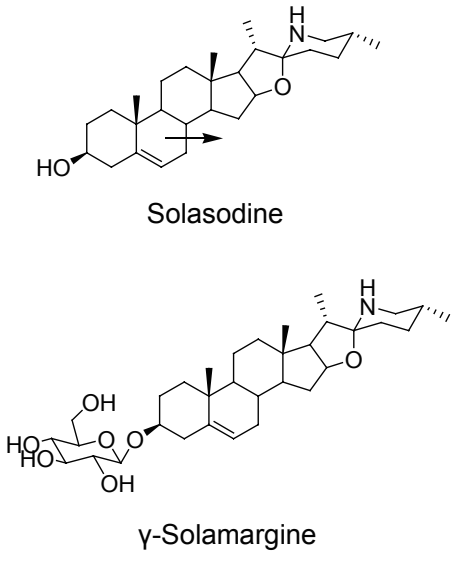

Figure 1. The structure of the two compounds.

2.3. *Liquid chromatography-mass spectrometry analysis of solasodine and γ-solamargine*

Lyophilized leaf and root tissue were immersed in a solution of ethanol-2 M HCl (4:1) for 24 h and ultrasonically extracted with 80% ethanol for 1 h. The extracts were centrifuged at 3,000 g for 60 min and the supernatant was removed and evaporated under vacuum. The dried residue was resuspended in 0.5 M HCl and extracted with $CHCl_3$ twice. The pH of the water phase was adjusted to 10.5 and then extracted with $CHCl_3$ three times and evaporated under vacuum. The dried residue was resuspended in 200 μL acetonitrile and analyzed using HPLC-DAD-MS.

HPLC-DAD-MS was performed using an Agilent 1100 series II LC system (Agilent Technologies) equipped with a photodiode array detector coupled to an API 4000 mass spectrometer (Applied Biosystems/MDS Sciex) equipped with an electrosprayionization source. A reverse-phase, Venusil XBP-C18 (5 μm, 2.1 × 50 mm) column (Agilent Technologies) was used for separations. The mobile phases consisted of solvent A (0.1% (v/v) CH_3COOH in water) and solvent B (acetonitrile), and separations were performed using a linear gradient of 25% to 65% B (v/v) over 70 min. The flow rate was 0.3 mL/min, and the temperature of the column was kept at 28°C. Positive-ion ESI was performed using an ion source voltage of 5.0 kV.

2.4. *Standard curve and related indicators*

The stock solution of the solasodine and γ-solamargine were prepared precisely (100 μg/mL) and diluted with methanol to 0.5, 1, 2.5, 10, 50, 200, 500, 1,000 ng/mL. A low, medium, and high concentration (1, 50, 800 ng/mL) was chosen to make the best quality control (QC). Concentration were tested for horizontal, analyte peak area for the vertical weighted ($W = 1/C^2$) least squares regression calculation.

The standard curves: $y = 6690x - 183$, $r = 0.9981$ (solasodine); $y = 3500x + 110$, $r = 0.9983$ (γ-solamargine) within the linear range of 0.5-1,000 ng/mL were obtained by a weight ($W = 1/C^2$) least squares regression calculation using compounds concentration as horizontal and peak area (MS, total ion) as vertical. The contents of solasodine and γ-solamargine were calculated according to the standard curves.

2.5. *RNA extraction and synthesis of the first-strand cDNA*

Samples of young leaves or other tissues were ground into fine powder and extracted using TRIzol reagent. The RNA pellets were washed with 70% ethanol. After a short drying at room temperature, the pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water. Total RNA obtained was used as template for cDNA synthesis. cDNA was synthesized using a reverse transcription kit (TOYOBO, Japan) according to the manufacturer's instructions.

2.6. *5' and 3' rapid amplification of cDNA ends (RACE) and amplification of full-length cDNAs*

On the basis of highly conserved amino acid sequences from squalene synthases of several plants, two degenerate primers were synthesized for the PCR amplification of the corresponding *S. nigrum* cDNA. The forward (SnSSP1) and reverse (SnSSP2) primers were 5'-CC ATCGACGACTACGACGAGTAYTGYCAYTA-3' and 5'-TGCCGATGGCCATGAYYTGNGGDAT-3', respectively. D, N, and Y represent mixtures of nucleotides A/G/T, A/C/G/T, and C/T, respectively. Using the above-mentioned *S. nigrum* cDNA as a template, degenerate PCR was carried out under the following conditions: 35 cycles of 94°C for 1 min, 45°C for 1.5 min, and 72°C for 2 min. A single DNA fragment was recovered, cloned into the PMD-19T-vector (TaKaRa, Japan), and subjected to nucleotide sequencing. The 5'- and 3'- ends of the SnSS cDNA were obtained by RACE, using the SMART RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. For the 5'-RACE, the gene specific primers that were used in the 1st PCR, the 1st nested PCR amplifications were: 5'-GAAAGATGGCAGGATCACGCAA-3' (SnSSGSP1) and 5'-GCCAGAACATACGACACTTAGG-3' (SnSSGSP2), respectively. Those for 3'-RACE were 5'-GACCAGTTCCATCACGTTTCGAC-3' (SnSSGSP3) and 5'-GCAAGGAGGTGGAAACAATCGA-3' (SnSSGSP4), respectively. The 5'- and 3'- RACE products were subjected to direct sequencing of both strands, and a consensus open reading frame (ORF) sequence was obtained. Based on sequencing results of the 3' and 5' RACE products, the SnSS ORF was amplified using single-stranded cDNA templates and two gene specific primers, SnSSFLF: 5'-TTGTTGAGAAGAATGGGGAC-3' and SnSSFLR: 5'-AGTTTGTGCTGTCTTCCCTG-3'.

2.7. *Expression patterns of SnSS*

PCR amplification was performed in an 8-tube strip format (Axygen, Union City, CA, USA) in triplicate. Each reaction contained 1 × SYBR Green PCR Master mix, 0.5 pmol/mL forward primer and reverse primer and 1 μL template cDNA in a final volume of 20 μL using a Mastercycler ep realplex apparatus (Eppendorf, Germany), with primers SnSSRT1 5'-GCCAGAACATACGACACT-3' (P1), SnSSRT2: 5'-AAGGAGGTGGAAACAAT-3' (P2). Elongation factor 1a (*EF-1a*) gene was also amplified as an internal control using EF1a-F (5'-ACCACTGGTGGTTTTGAAGC-3') and EF1a-R (5'-ACGACCAACAGGGACAGTTC-3') primers. The cycling profile included 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s. Data acquisition and the analysis of real-time PCR assays were performed using the Mastercycler ep realplex. Each fluorescent reporter signal was measured against the internal reference dye signal to normalize for non-PCR-related fluorescence fluctuations between wells. The threshold cycle represented the refraction cycle number at which a positive amplification reaction was measured and set at ten times the standard deviation of the mean baseline emission calculated for amplification cycles 3-15. All samples were taken in triplicate independent experiments.

3. Results

3.1. *Effect of steroidal alkaloids on growth form of F. oxysporum*

F. oxysporum was cultured in RPMI 1640 medium. After 12 h incubation, *F. oxysporum* cells were photographed (Figure 2). The results showed that *F. oxysporum*

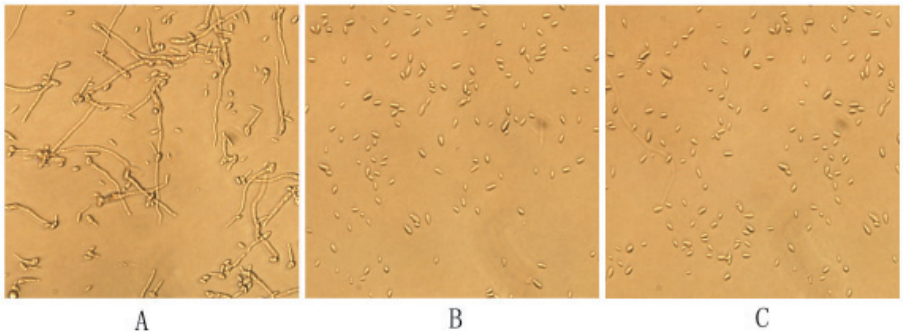

Figure 2. Microscopic observation of *F. oxysporum* treated with two compounds. *F. oxysporum* (1×10^5 cells/mL in RPMI 1640 medium) were incubated at 37°C for 6 h **(A)** and with 8 μg/mL solasodine **(B)**, 16 μg/mL γ-solamargine **(C)**.

suspended in RPMI 1640 medium grew in hyphal form. When solasodine and γ-solamargine were added to the medium, *F. oxysporum* grew in a yeast form. The ratio of hyphae formation was significantly inhibited by solasodine and γ-solamargine. These findings suggested that solasodine and γ-solamargine regulated the hyphae transformation of *F. oxysporum* cells.

3.2. *Isolation and sequence analysis of SnSS*

The full-length cDNA of SnSS was obtained through the RACE method. The SnSS cDNA contains an ORF encoding a protein of 411 amino acids. By aligning deduced sequences of SnSS with other squalene synthases from taxonomically diverse species, an SnSS amino acid sequence is 79.7, 95.8, 80.2, 79.7, 81.2, 74.6, 69.1% identical to those in *Glycyrrihiza glabra* (*11*), *Capsicum annuum* (*16*), *Panax ginseng* (*10*), S*olanum tuberosum* (*15*), *Euphorbia tirucalli* (*20*), *Arabidopsis thaliana* (*12,14,21*), *Oryza sativa* (*13*), respectively.

Six highly conserved peptide domains of 14-23 amino acids previously pointed out by Robinson (*22*) were also discernible within the SnSS amino acid sequence (Figure 3). Three domains (III, IV, and V) showed a highly conserved consensus sequence with other squalene synthase enzymes, while three domains (I, II, and VI) were much less conserved. Due to the high level of sequence identity shared with other squalene synthases reported so far, the SnSS cDNA might code for squalene synthase.

3.3. *F. oxysporum affects the biosynthesis of SGAs by regulating SnSS expression level*

The plants were kept in pots in a chamber at 25°C with light from fluorescent lights (12 h light/12 h dark). Plants (3-week-old) were inoculated and then the cultured seedling of *S. nigrum* was cultured for another week. The result indicated that the seedling inoculated with sterile water grew normally (Figures 4A and 4C) while the seedling inoculated with the spore suspension of *F. oxysporum* became small and yellow (Figures 4B and 4D). To investigate whether SnSS expression was positively correlated with the formation of SGAs, the concentration of solasodine and γ-solamargine and the expression level of SnSS were evaluated after inoculation of *F. oxysporum*. The concentration of solasodine and γ-solamargine increased in both leaves and roots after inoculation of *F. oxysporum* spores (Figure 4E). The solasodine concentration was 2.1- and 6-fold higher than the control in leaves and roots, respectively. The γ-solamargine concentration was 4.8- and 5.6-fold higher than the control in leaves and roots, respectively (Figure 4E). Real-time RT-PCR analysis was performed to determine transcription level of the *SnSS* gene in the solasodine and γ-solamargine biosynthetic pathway. The accumulation of SnSS transcripts increased, which was 2.3- and 3-fold higher than the control in leaves and roots after *F. oxysporum* spores inoculation (Figure 4F).

3.4. *Tissue-specific and developmental expression of SnSS*

The expression pattern of genes in different tissues reflects the distribution of secondary metabolites (*23*). To further characterize the tissue specific expression of SnSS, transcripts of the gene were examined using real-time PCR. In 3-month-old plants, the *SnSS* gene had the highest steady-state level of transcripts in mature leaves, and a lower level in flowers and fruits (Figure 5). This result indicated that the synthesis of SGAs is mainly located in leaves and may help to fight against vascular related diseases occurring on the leaves.

4. Discussion

The root-infecting fungal pathogen *F. oxysporum* is responsible for vascular wilt in over 100 different plant species, including banana, cotton, grain legumes, oil palm, vegetables, and ornamental plants (*6*). *F. oxysporum* is a dimorphic fungus that can switch between yeast and hyphae growth modes. The infection process for *F. oxysporum* includes the germination of spores in the soil, the fungal hyphae penetrating root tips through wounds or at the point of lateral root formation, and advancing through to the root cortex intercellularly, and ultimately reaching the xylem vessels as reported in potatoes (*24,25*). From this point, the pathogen travels through the vascular tissues, mostly upward towards the stem. So hyphae formation is a prerequisite for *F. oxysporum* infection in plants. Our results indicate that steroidal alkaloids isolated from *S. nigrum* exhibited an excellent inhibition effect on *F. oxysporum* hyphae formation and plays important roles in plant defense.

It has been reported that biosynthesis of steroidal alkaloids is *via* the mevalonate pathway in which squalene synthase is a key rate-limiting enzyme. Squalene synthase is a membrane bound enzyme (*7*) that condenses two farnesyl diphosphate molecules into squalene. Squalene synthase is generally described as a crucial branch-point enzyme for synthesizing sterol and is intriguing as a potential regulatory-point carbon flux into sterol. There is also a positive correlation between the expression level of squalene synthase and the amount of triterpenes and sterol produced. Our results suggest that squalene synthase in *S. nigrum* is also a key rate-limiting enzyme in steroidal alkaloid biosynthesis. The content of solasodine and γ-solamargine and the expression of SnSS were increased after *F. oxysporum* infection, which indicated that solasodine and solamargine play an important role in resisting fungi infection in *S. nigrum*.

In conclusion, in the present investigation, we demonstrate that the steroidal alkaloids solasodine and γ-solamargine isolated from *S. nigrum* exhibited an excellent inhibition effect on *F. oxysporum* hyphae formation, which may play an important role in defense from *F. oxysporum* infection. Squalene synthase, a key enzyme in the SGAs biosynthesis, was molecularly cloned and the expression level was enhanced after *F. oxysporum* infection in accordance with the content of solasodine and γ-solamargine.

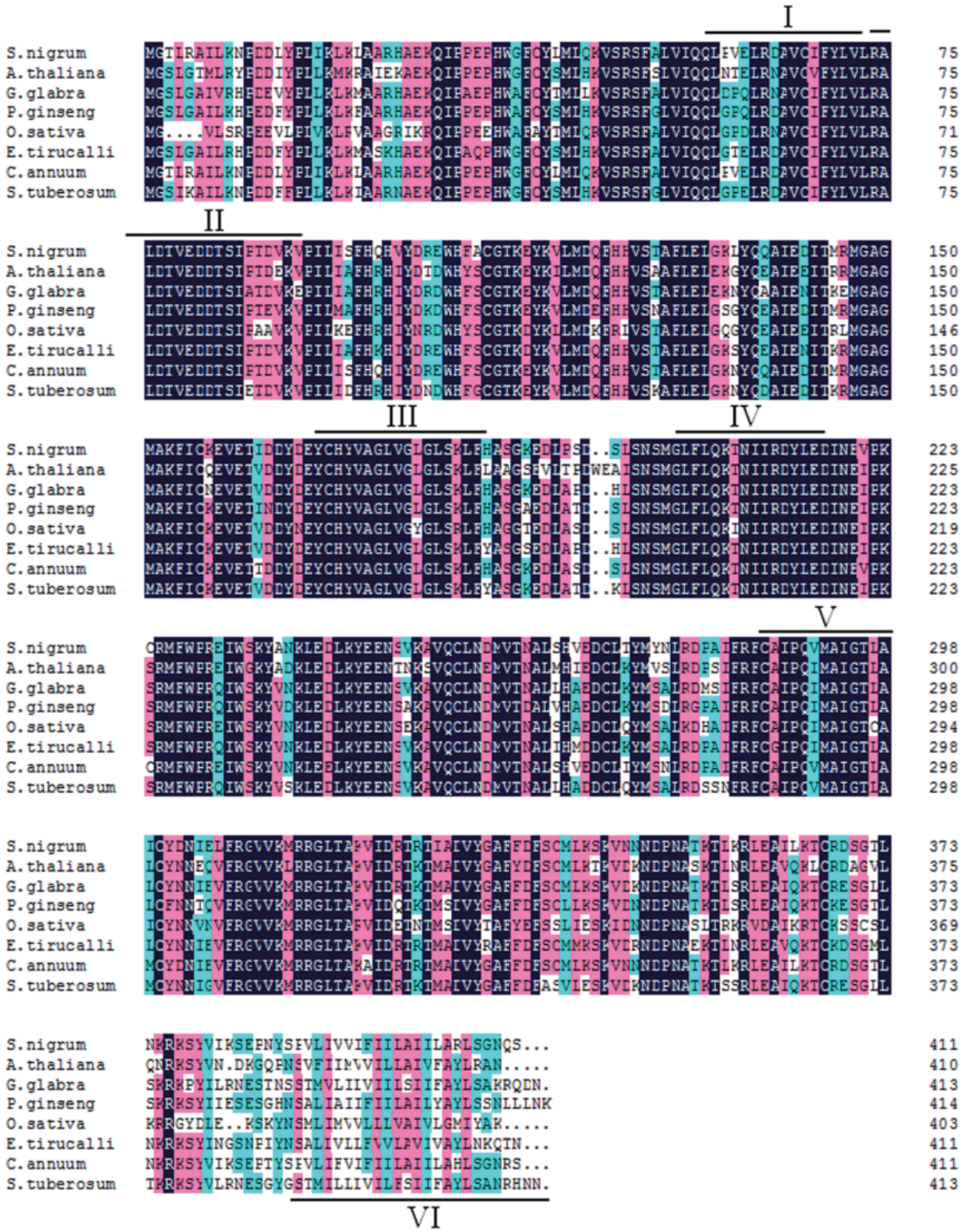

Figure 3. Multi-alignment of the deduced SnSS amino acid sequence with those of other plant squalene synthases. The DDBJ/GenBank/EMBL accession numbers of the retrieved sequences are AB433916 (*Euphorbia tirucalli*), D86409 (*Glycyrrhiza glabra*), AF124842 (*Capsicum annuum*), AB010148 (*Panax ginseng*), AB022599 (*Solanum tuberosum*), NM_119630 (*Arabidopsis thaliana*), and NM_001058160 (*Oryza sativa*).

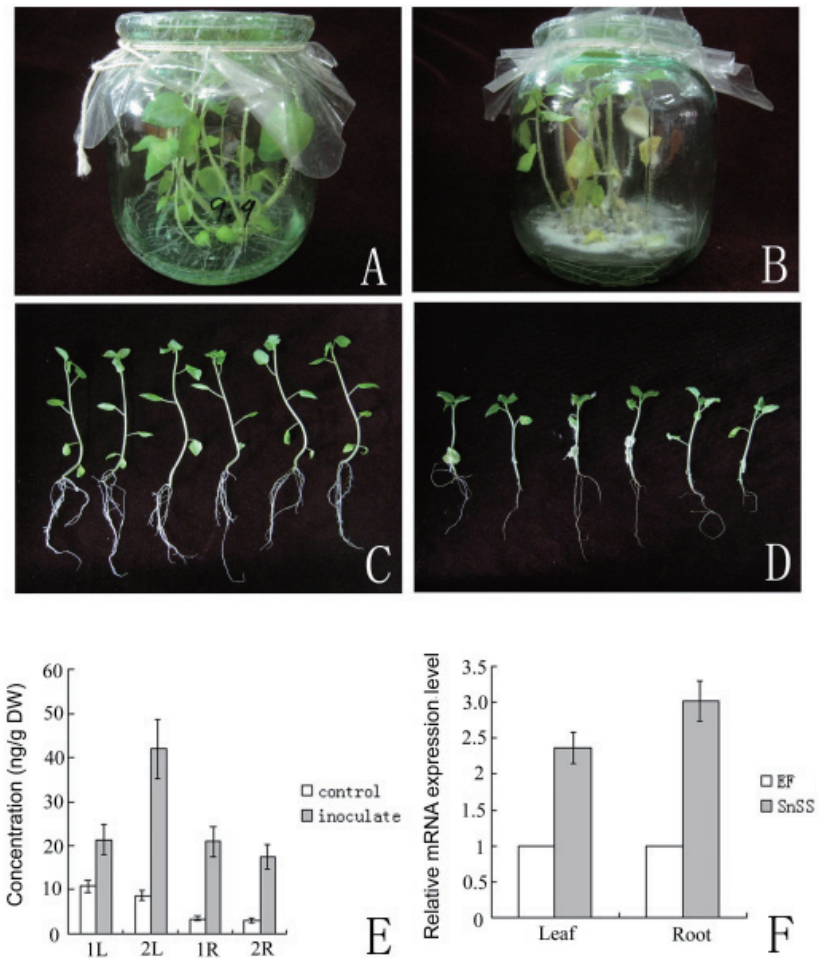

Figure 4. The plants were kept in pots in a chamber at 25°C with light from fluorescent lights (16 h light/8 h dark). 3-weeks old plants were inoculated with sterile water (**A** and **C**) or the spore suspension of *F. oxysporum* (**B** and **D**). Concentration of solasodine and γ-solamargine in leaves and roots of *S. nigrum* are shown in **E** and the changes of SnSS mRNA level after inoculation are shown in **F**. **1L**, **2L**, **1R**, **2R** means the content of compound 1 in leaf, compound 2 in leaf, compound 1 in root and compound 2 in root, respectively.

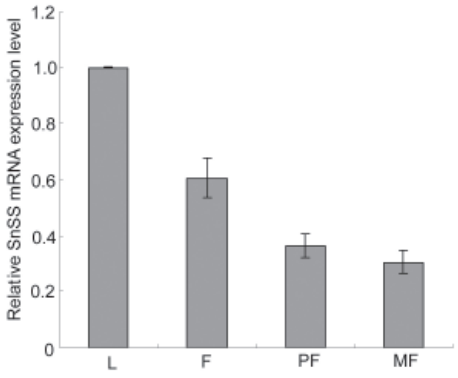

Figure 5. Real-time RT-PCR amplification of *SnSS*. PCR was performed using *S. nigrum SnSS*-specific primers and an equal amount of total RNA isolated from the leaves (**L**), the flower (**F**), premature fruits (**PF**) and mature fruits (**MF**). The elongation factor gene was amplified as a control.

Acknowledgements

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