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Molecular cloning and characterization of three isoprenyl diphosphate synthase genes from alfalfa

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Abstract Isoprenoid is the precursor for the biosynthesis of saponins, abscisic acid, gibberellins, chlorophylls and many other products in plants. Saponins are an important group of bioactive plant natural products. The alfalfa (*Medicago sativa* L.) saponins are glycosides of different triterpene aglycones and possess many biological activities. We isolated three genes (*MsFPPS*, *MsGPPS* and *MsGGPPS*) encoding isoprenyl diphosphate synthases (IDS) from alfalfa via a homology-based PCR approach. The enzyme activity assay of purified recombined MsFPPS and MsGGPPS expressed in *Escherichia coli* indicated that they all had IDS activity. Expression analysis of the three genes in different alfalfa tissues using real time PCR displayed that they were expressed in all tissues although they had a different expression patterns. *MsFPPS* and *MsGPS* displayed a significant increase in transcript level in response to methyl jasmonate, but the transcript level of *MsGGPPS* decreased obviously. To elucidate the functions of the three IDSs, their overexpression driven by a constitutive cauliflower mosaic virus-35S promoter in tobacco plants was applied and analyzed. The T_0 transgenic plants of *MsFPPS* showed high levels of squalene content when compared with control. However, no differences were detected in T_0 transgenic plants of *MsGPPS* and *MsGGPPS*. In addition, the overexpression of *MsFPPS* induced senescence response in transgenic plant leaves. This result may indicate that *MsFPPS* performs a role not only in phytosterol and triterpene biosynthesis, but also in growth regulation.

Keywords Alfalfa · Isoprenyl diphosphate synthases · Methyl jasmonate · Recombinant proteins · Enzyme activity · Overexpression

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Introduction

In plants, many products are derived from isoprenoids for their growth and response to environmental changes [1]. Farnesyl diphosphate (FPP), geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) are three key isoprenoids to be converted into compounds necessary for plant, such as saponins, gibberellins, carotenoids, chlorophylls, isoprenoid quinones, and geranylgeranylated small G proteins [2]. Saponins, a group of natural products widespread throughout many plants, are glycosides of triterpenoid or steroidal aglycones [3, 4]. The foaming ability of saponins is caused by the combination of the hydrophobic sapogenin with the hydrophilic sugar substituent(s) [5]. Alfalfa (*Medicago sativa* L.) is of immense importance as livestock fodder as it contains a high amount of protein. The main antinutritional components present in this plant are saponins, and their unfavorable effects on animal performance have restricted the optimum use of this high-protein plant as an animal feed [6]. Alfalfa triterpene glycoside saponins are attracting increasing interest for their multiple biological activities. Some activities are positive and some others are negative. Thus, whereas some

saponins display allelopathic [7], antimicrobial [8, 9], and anti-insect activity, they can also be toxic to monogastric animals, act as antipalatability factors, or negatively impact forage digestibility in ruminants [10–12]. Some saponins have potentially useful pharmacological activities, including anticholesterolemic [10], anticancer [13, 14], adjuvant [15], and hemolytic activity [16]. Despite the obvious interest in facilitating or inhibiting production of triterpene saponins for crop improvement or development of pharmacological agents, most of the steps in their biosynthesis remained uncharacterized at the molecular level [17].

The biosynthesis of all terpenoids is initiated by the synthesis of isopentenyl diphosphate (IPP) via the mevalonic acid pathway or the methylerythritol phosphate pathway [18, 19]. GPP, FPP and GGPP are each believed to be formed by a specific isoprenyl diphosphate synthase (IDS) named for its product: geranyl diphosphate synthase (GPPS), FPP synthase (FPPS), and GGPP synthase (GGPPS) [19]. In subsequent steps, these linear diphosphates serve as substrates for terpene synthases, which form a complex variety of mono-, sesqui-, and diterpene carbon skeletons [20]. The process of terpenoid biosynthesis is partitioned among different cell compartments. The formation of GPP and GGPP and their subsequent conversion to mono- and diterpenes occur in the plastids, whereas the biosynthesis of FPP and its conversion to sesquiterpenes are localized in the cytosol [19]. Farnesyl diphosphate functions as a substrate in the synthesis of essential or secondary metabolites, including sesquiterpenoids, triterpenoids, and phytosterols. In plant several FPPS have been reported to detail the physiological functions or contributions of FPPS in secondary metabolite synthesis. FPPS was reported to play a regulatory role in sesquiterpene biosynthesis by the over-expression of a FPPS gene in transgenic plants [21]. Over-expression of *Arabidopsis thaliana* FPS1S reduced the level of endogenous zeatin-type cytokinins in the leaves [22]. Geranyl diphosphate is the universal precursor of the monoterpenes which produced by GPPS. GPPS have been isolated from several plant sources [23, 24] where it appears to participate primarily in the plastidial biosynthesis of monoterpenes [25, 26] by supplying the essential precursor of this family of natural products [27]. In mechanism and many reaction parameters, the GPPS resembles both FPPS and GGPPS [28]. Unlike farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase, GPPS are heterotetramers in which the large subunit shares functional motifs and a high level of amino acid sequence identity with geranylgeranyl diphosphate synthases (GGPPS) of plant origin. The small subunit, however, shares little sequence identity with other IDSs; yet it is absolutely required for GPPS catalysis [29]. Geranylgeranyl diphosphate is the precursor for the biosynthesis of gibberellins, carotenoids, chlorophylls, isoprenoid quinones, and geranylgeranylated proteins in plants [30, 31].

Many plant natural products, such as saponins, gibberellins, carotenoids, chlorophylls and so on play very important roles in organ development, abiotic and biotic stress regulation and many other conditions. For instance the important constitutive defenses of alfalfa plants include a complex mixture of about 30 saponin glycosides [32, 33]. In order to study the induced defense response to insect attack, methyl jasmonate (MJ) was often used to simulate insect attack [34, 35]. IDSs catalyze the branch-point reactions leading to GPP, FPP and GGPP, and so may channel flux among the different terpene classes present in saponins. So it's necessary to characterize the molecular and biochemical basis of IDSs. FPPS, GPPS and GGPPS activity have been investigated in *A. thaliana*, rubber tree (*Hevea brasiliensis*), banana (*Musa acuminata*), escobilla (*Scoparia dulcis*), spearmint (*Mentha spicata*) and maize (*Zea mays*) [22, 36–40]. There are two differentially expressed farnesyl-diphosphate synthase genes in *A. thaliana*. Northern blot analysis showed that At*FPS1* and At*FPS2* have different patterns of expression. AtFPS1 mRNA accumulates preferentially in roots and inflorescences, whereas *AtFPS2* mRNA is predominantly expressed in inflorescences [41]. There is a small gene family for GGPPs encoding five isozymes and one related protein in *Arabidopsis*. Subcellular localization of these GGPPs display that two were translocated into the chloroplast, two were localized in the endoplasmic reticulum and one localized in the mitochondria [41]. Naoumkina et al. [5] used comprehensive gene expression clustering analysis to identify candidate genes (including IDS genes) involved in the elaboration, hydroxylation, and glycosylation of the triterpene skeleton in the model legume *Medicago truncatula*.

In this study our aim is to clone and character the IDS genes in biosynthesis of saponins and other terpenoids in alfalfa. We hope the result may provide a basis for future studies to define genetically the roles of FPPS, GPPS and GGPPS in terpenoids biosynthesis and the multiple biological activities in alfalfa.

Materials and methods

Plant material

Cultivated variety Zhongmu-1 of alfalfa (*M. sativa* L.) was used, that had been cultivated by our institute. Seeds of Zhongmu-1 alfalfa were surface-sterilized in 0.01 % $HgCl_2$ for 10 min, then washed 15 min with sterile water and placed on Petri dishes with a single sheet of Whatman filter paper for germination. Seedlings were then transferred to

hydroponic culture of Hoagland's solution in a growth chamber at 25 °C under a 16 h light/8 h dark photoperiod and 75 % relative humidity. Seedlings used for experiments were grown for 30 days under these conditions, and induced by spraying with 0.5 mM methyl jasmonate in 0.5 % (v/v) Tween 20 as detergent. Control seedlings were sprayed only with Tween 20. Plant tissues (stems and leaves) were collected at different time points (0, 5, 10, 25, 40 and 60 h) and frozen in liquid nitrogen. Tobacco (*Nicotiana tabacum* cv. NC89) was used in the plant transformation experiment.

Amplification of *MsFPPS*, *MsGPPS* and *MsGGPPS* partial cDNAs

Using *FPPS*, *GPPS* (small subunit) and *GGPPS* sequences from *A. thaliana*, *Z. mays*, *G. max* and *M. truncatula*, degenerate primers were designed to different conserved regions by GeneFisher2 program (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/). For the amplification of *M. sativa* FPPS cDNA partial fragments, the upstream primer (5′-CTCCCATACACGTCVTGGTYAG-3′) and the downstream primer (5′-CCAAAGCAATCCAAATAHT CANCC-3′) were used. For the amplification of GPPS partial fragments, the primer combination (5′-CCACTTA CACCCACGAGMAT-3′) and (5′-CAGCACAATAATG TTAAGCVACA-3′) were used. For the amplification of GGPPS partial fragments, the primer combination (5′-CG AGGTAAACCTACAAACCNCAA-3′) and (5′-ACAAC CTGAAACAATWAACYGATA-3′) were used. Reverse transcription (RT) was carried out with total RNA and oligo-dT_{18} primers using Primescript reverse transcriptase (Takara, Japan), according to the manufacturer's instructions. PCR was performed with pre-denaturalization at 94 °C for 5 min, and then thermocycling was performed at 30 cycles of 94 °C for 30 s, annealing for 30 s, 72 °C for 60 s, and an additional polymerization step at 72 °C for 10 min in a thermocycler (Biorad, USA). PCR product was separated by electrophoresis on a 1 % agarose gel stained with ethidium bromide, and purified by using the DNA gel extraction kit (Takara, Japan). The products were cloned into the pMD-19T vector (Takara, Japan) and then transformed into *E. coli* DH5α. Recombinant plasmids were sequenced by the Beijing Genomics Institute (BGI, China).

RNA extraction and reverse transcription

Total RNA from 30 days-old plant stems and leaves was extracted by using Trizol (Invitrogen, USA). To remove contaminating DNA, the total RNA was treated with RNase-free DNase (Fermentas, USA). DNase-treated RNA samples were reverse-transcribed by using Primescript reverse transcriptase (Takara, Japan). The RT reaction was performed at 42 °C for 1 h using oligo-dT_{18} as a primer. The harvested cDNA was deposited at −20 °C until use for PCR amplification.

Amplification of full-length cDNAs by RACE-PCR

Rapid amplification of cDNA ends (RACE) was performed to amplify its unknown 3′ and 5′ ends. Total RNA was used to synthesize 5′-RACE-Ready-cDNA and 3′-RACE-Ready-cDNA according to the manufacturer's recommendation of BD SMARTTM RACE cDNA amplification kit (Clontech, USA). Based on the partial sequences already obtained, we designed gene-specific primers (5′-AGGAAACACTTCA AGGGAAAGCCGTA-3′), (5′-ATTCGTGTGGGGCTGT ATGTGGTG-3′) and (5′-CTGCGGCTTTGTTAGAAGGT GCTGTT-3′) to amplify the 5′-cDNA end of *MsFPPS*, *MsGPPS* and *MsGGPPS*. And designed primer (5′-CT CCATCATTCGCAGCAATCATTCCA-3′), (5′-GCATCT ATCACCCCTCCTCCTGAACC-3′) and (5′-CCAGCAAC GAGTCCTTCCGAGCCAA-3′) to amplify the 3′-cDNA ends of *MsFPPS*, *MsGPPS* and *MsGGPPS*. PCR reaction conditions were as follows: pre-denaturalization at 94 °C for 5 min, Thermocycling was performed at 30 cycles with 94 °C for 30 s, 68 °C for 2 min, and an additional polymerization step at 72 °C for 10 min. PCR product was separated by electrophoresis on a 1 % agarose gel stained with ethidium bromide, and then purified by using the DNA gel extraction kit (Transgen, China). The products were cloned into the pMD-19T vector (Takara, Japan) and then transformed into *E. coli* DH5α. And the recombinant plasmids were sequenced by the Beijing Genomics Institute (BGI, China). 5′-cDNA end and 3′-cDNA end sequences were assembled to the full-length cDNA sequence. *MsFPPS*, *MsGPPS* and *MsGGPPS* and its putative encoding proteins were subsequently analyzed for molecular characterization, such as the conserved motifs, sequence homology, phylogenetic analysis, transcript analysis.

Expression of MsFPPS, MsGPPS and MsGGPPS sequences in *E. coli* and isoprenyl diphosphate enzyme activity assay

The proteins encoded by *MsFPPS*, *MsGPPS* and *MsGGPPS* were predicted to have no signal peptide for plastid transport by signalIP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). The entire coding sequence of *MsFPPS*, *MsGPPS* and *MsGGPPS* were amplified with primers. (5′-TCggatccAT GGCAGATCTCAAGTCCACAT-3′) (*Bam*HI) and (5′-AC gagctcCTTCTGCCTCTTGTAAATTTTA-3′) (*Sac*I) were used to amplify *MsFPPS*, (5′-CTgatatcATGGCTACTA CAACTTCCCACCT-3′) (*Eco*RV) and (5′-ACgaattcAAT ATTCAAGAAAGTTTTAATTG-3′) (*Eco*RI) were used to

amplify *MsGPPS*, (5′-TCggatccATGAGTGCTGTGAATC TTAACAC-3′) (*Bam*HI) and (5′-GTgagctcGTTTTGCCT ATAAGCAATGTAAT-3′) (*Sac*I) were used to amplify *MsGGPPS*. The amplification was carried out with the PrimeSTAR High Fidelity PCR System (Takara, Japan), and the resulting cDNA fragments were cloned into the expression vector PET 30a (Novagen, Germany) which adds a tag containing six histidine residues to the C-terminus. Positive clones were first transferred into *E. coli* strain DH5α and then into strain BL21 (DE3) pLysS (Invitrogen, USA). Bacterial cultures were grown to an OD of 0.5 and transformants were induced with 0.5 mM IPTG at 25 °C. Bacterial pellets were resuspended in a buffer containing 20 mM Tris–HCl (pH8.0), 0.5 M/l NaCl, 1 mM EDTA, 1 mg/ml lysozyme. And sonified by using an ultrasonic instrument for 10 min, power 200 w. *MsFPPS*, *MsGPPS* and *MsGGPPS* proteins were individually purified over Ni–NTA agarose columns (Qiagen, Germany) according to the manufacturer's instructions. The recombinant proteins were eluted with 500 mM imidazole in the assay buffer.

Isoprenyl diphosphate enzyme activity assay of purified recombinant were performed as below. Recombinant IDSs were assayed in 50 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 % glycerol, 100 μM [1-^{14}C] IPP (55 mCi/mmol) and 200 μM DMAPP in a total volume of 100 μl. The reaction was initiated by addition of 100 μg/ml recombinant protein, and the assay mixture was overlaid with 1 ml hexane. The reactions were incubated for 40 min at 30 °C, 6 M HCl was added to stop the reaction, and incubation was continued for a further 10 min to solvolyze the acid-labile allylic diphosphates formed during the assay to their corresponding alcohols. Solvolysis products were extracted into the hexane phase by vigorous mixing for 15 s. An aliquot of the extracted hexane phase was then counted in a scintillation counter (Bioscan, USA).

Transcripts analysis by real-time fluorescent quantitative PCR

30 days old alfalfa (*M. sativa* L. cv. Zhongmu-1) seedlings were induced by spraying them with 0.5 mM methyl jasmonate. Total RNA from each time points and tissues was isolated by using Trizol (Invitrogen, USA). Using identical amounts of total RNA, template cDNA for subsequent PCR reactions was generated using Primescript reverse transcriptase (Takara, Japan). Real-time PCR was performed with SYBR® Premix Ex Taq™ (Takara) and a β-actin fragment of *M. sativa* to normalize transcripts of interest. The primers used were as follows: β-actin forward (5′-CACTCCCACATGCTATCCT-3′) and reverse (5′-GA CAATTTCCCGCTCAG-3′), *MsFPPS* forward (5′-CAC ACTGGAAGGAGAAAAAG-3′) and reverse (5′-AACA GGAAGGTAAAAAGAATAATA-3′), *MsGPPS* forward (5′-CTCAAGCCCACCACCGTCAGAA-3′) and reverse (5′-AGGTGGTGCATGGGCTCAAAAAC-3′), and *MsGGPPS* forward (5′-CCTTGTATGGATAACGACGAT-3′) and reverse (5′-CGAAAGCAAAAGCGAGAA-3′). Transcript abundance was quantified with ABI 7300 Real Time PCR Thermocycler (ABI, USA) using a program with a maximum of 45 cycles of 95 °C for 15 s, 60 °C for 30 s, followed by a melting curve analysis of transcripts. The transcript abundance of each gene was normalized to β-actin. The relative amount of transcript at the onset of treatment was used as calibrator. Each measurement was repeated with two independent biological replicates, each of which was represented by at least three technical replicates.

Transgenic vector construction and plant transformation

The complete coding sequences of *MsFPPS*, *MsGPPS* and *MsGGPPS* cDNA were PCR amplified by using primers. (5′-TCtctagaATGGCAGATCTCAAGTCCACAT-3′) (*Xba*I) and (5′-ACggatccCTTCTGCCTCTTGTAAATTT TA-3′) (*Bam*HI) were used to amplify *MsFPPS*. (5′-CTtct agaATGGCTACTACAACTTCCCACCT-3′) (*Xba*I) and (5′-ACggatccAATATTCAAGAAAGTTTTAATTG-3′) (*Bam*HI) were used to amplify *MsGPPS*. (5′-TCtctagaAT GAGTGCTGTGAATCTTAACAC-3′) (*Xba*I) and (5′-GT ggatccGTTTTGCCTATAAGCAATGTAAT-3′) (*Bam*HI) were used to amplify *MsGGPPS*.. The amplified fragment was cloned into vector *pBI121* (Clontech) to create *pBI-MsFPPS*, *pBI-MsGPPS* and *pBI-MsGGPPS* vectors. These vectors contains *GUS* (*uidA*) reporter under cauliflower mosaic virus-35S promoter. The *pBI121*also carries the *NPTII* (kanamycin) gene as a selectable marker. Tobacco (*N. tabacum*) leaf disks were transformed through a procedure [42] with *Agrobacterium tumefaciens* (LBA4404) containing *pBI-MsFPPS*, *pBI-MsGPPS* and *pBI-MsGGPPS*. Putative T_0 transgenic plants were regenerated from the callus in the presence of kanamycin and further screened by using PCR and β-glucuronidase (GUS) assay [43]. The GUS activity was visualized in the leaf tissue as described by Jefferson [43]. The transgenic plants were also analyzed by RT-PCR analysis. The RT was according to the method described previously. The transgenic vector construction primers of the three genes were used in the RT-PCR amplification.

Quantitative analysis of squalene

Freeze-dried T_0 transgenic tobacco leaves (1 g) were extracted twice with 20 ml of ethyl acetate at 100 rpm on a gyrator for 6 h at room temperature. The extracts were chromatographed on a silica gel cartridge column. The

eluent was extracted three times with 10 ml of aqueous 5 % KOH and then extracted twice more with 10 ml of aqueous 5 % HCl. The organic fraction was washed twice with 10 ml of water and water was eliminated with anhydrous sodium sulfate. The extract was evaporated and the residue was dissolved with 2 ml of hexane. Suspended particles were removed by 10 min of centrifugation at 6,000 rpm. An aliquot of the solution was analyzed via GC–MS. GC–MS analysis was conducted on a mass spectrometer (GCMS-QP2010 Plus, Japan) connected to a gas chromatograph. The analytical conditions were as follows: carrier gas He (4 ml/min), and column temperature 100–250 °C (20 °C/min). The squalene content was calculated from the ratio of the peak area of the respective compound to that of the standard.

Results

Isolation of three alfalfa genes encoding isoprenyl diphosphate synthases

To isolate alfalfa IDS genes, cDNA fragments were obtained by PCR using primers designed to conserved regions of known plant farnesyl diphosphate synthases (FPPS), GPPS and GGPPS with alfalfa (cultivated variety Zhongmu-1) RNA as a template. A 423-bp fragment of alfalfa FPPS, a 657-bp fragment of alfalfa GPPS, and a 377-bp fragment of alfalfa GGPPS were obtained by using these conserved region primers. These clones were sequenced and analyzed. These three fragments sequences had high homology with FPPS, GPPS and GGPPS genes of some other plants. Based on the partial sequences already obtained, rapid amplification of 5′ and 3′ cDNA ends (RACE) was adopted to amplify full-length cDNA clones of alfalfa IDS genes. The isolated clones were sequenced and designated *MsFPPS*, *MsGPPS* and *MsGGPPS*. The full-length cDNA of *MsFPPS* was 1,368 bp (GenBank ID: GU361537), which contained a 1,029 bp open reading frame (ORF) that encoded a protein of 342 amino acids. The molecular weight of the putative protein was about 38.98 kDa, and the *pI* is about 4.94. The full-length cDNA of *MsGPPS* was 1,293 bp (GenBank ID: JN241680), which contained a 906 bp ORF that encoded a protein of 301 amino acids. The molecular weight of the putative protein was about 32.84 kDa, and the *pI* is about 6.63. The full-length cDNA of *MsGGPPS* was 1,623 bp (GenBank ID: GU999998), which contained a 1,113 bp ORF that encoded a protein of 370 amino acids. The molecular weight of the putative protein was about 40.02 kDa, and the *pI* is about 6.33. The three putative proteins all contained a polyprenyl-synt PFAM domain. MsFPPS and MsGGPPS were predicted to contain polyprenyl synthases signature (Fig. 1). MsGPPS shared amino acid sequence identity with GPPS small subunit in some other plants (*H. brasiliensis*, *A. majus*, *M. piperita*), so MsGPPS may be the small subunit of GPPS in *M. sativa* and contained no polyprenyl synthases signature. Based on the amino acid sequence, a phylogenetic tree was constructed by using Neighbor Joining method. The result indicated that *MsFPPS*, *MsGPPS* and *MsGGPPS* were homologous with GPPS, GGPPS and FPPS of some other plants (Fig. 2).

Heterologous expression of alfalfa isoprenyl diphosphate synthase genes and enzyme activity assay

To confirm that MsFPPS, MsGPPS and MsGGPPS are functional IDS, we inserted the coding regions into PET30a vector with a C-terminal His tag. The recombinant vectors were transformated and expressed in *E. coli* (DE3).The bacterial extracts of the expressed proteins were analyzed with SDS-PAGE. The result revealed recombinant protein bands with an apparent molecular mass of approximately 39 kDa for MsFPPS, and 33 kDa for MsGPPS, and 40 kDa for MsGGPPS. The proteins were subsequently purified with Ni–NTA agarose columns (Fig. 3). In order to test whether the purified recombinant proteins had activity, extracts of cells expressing *pET30a-MsFPPS*, *pET30a-MsGPPS* and *pET30a-MsGGPPS* possessed much higher activity than extracts prepared from cells transformed with

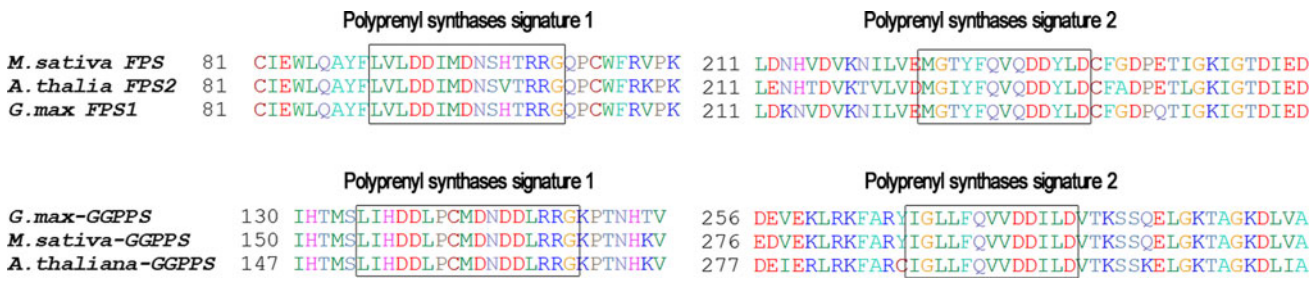

Fig. 1 Predicted polyprenyl synthases signature of MsFPPS and MsGGPPS. There was no predicted polyprenyl synthases signature in MsGPPS for it was predicted to be the small subunit of GPPS

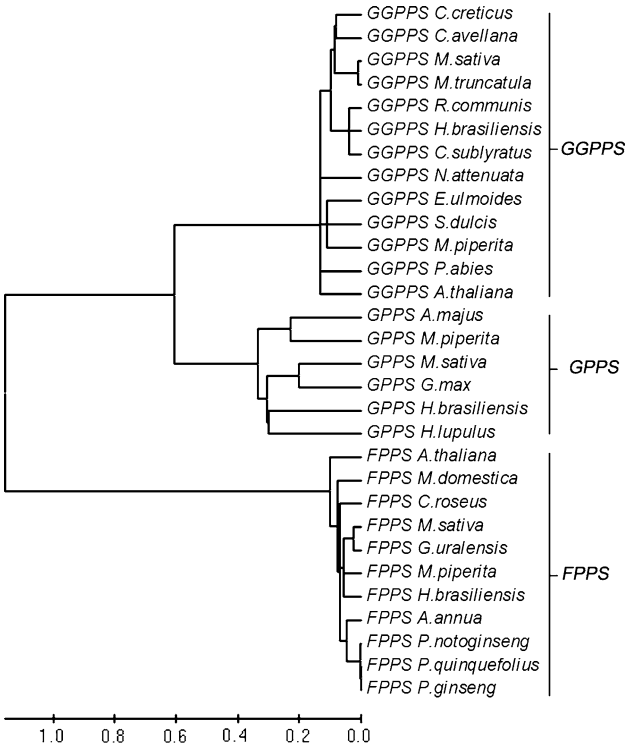

Fig. 2 Phylogenetic tree of the deduced amino acid sequences of *MsFPPS*, *MsGPPS*, *MsGGPPS* and other plants *GPPS*, *GGPPS* and *FPPS* sequences. Analysis was performed using Clustal X 2.0 and MEGA5.0. The accession numbers of the sequences are as follows: FPPS *M. sativa* ADC32809, FPPS *A. thaliana* NP_199588, FPPS *A. annua* AAC49452, FPPS *P. notoginseng* AAY53905, FPPS *M. piperita* AF384040, FPPS *P. quinquefolius* ADJ68004, FPPS *P. ginseng* AAY87903, FPPS *C. roseus* ADO95193, FPPS *M. domestica* AAM08927, FPPS *H. brasiliensis* AAM98379, FPPS *G. uralensis* ADE18770, GPPS *M. sativa* AEL29573, GPPS *G.max* ABY90133, GPPS *H. brasiliensis* BAF98300, GPPS *H. lupulus* ACQ90681, GPPS *A. majus* AAS82859, GPPS *M. piperita* AF182827, GGPPS *M. sativa* ADG01841, GGPPS *M. truncatula* ADG01842, GGPPS *C. sublyratus* BAA86284, GGPPS *C. avellana* ABW06960, GGPPS *H. brasiliensis* BAF98302, GGPPS *C. creticus* AF492022, GGPPS *R. communis* XP_002525096, GGPPS *A. thaliana* NP_195399, GGPPS *S. dulcis* BAA86285, GGPPS *N. attenuate* ABQ53935, GGPPS *P. abies* ACA21462, GGPPS *M. piperita* AF182828, GGPPS *E. ulmoides* BAB60820

the vector pET30a, as shown in Table 1. These data confirmed that the cDNAs of MsFPPS and MsGGPPS had IDS activity.

Expression analysis of *MsFPPS*, *MsGPPS* and *MsGGPPS*

A real-time PCR analysis was carried out to investigate the expression of *MsFPPS*, *MsGPPS* and *MsGGPPS* in different tissues and at different time points under MJ treatment. The alfalfa seedlings were induced by 0.5 mM MJ for 5, 10, 25, 40 and 60 h. And the seedlings grown on Hoagland's solution without treatment were used as control.

Transcripts of *MsFPPS* and *MsGGPPS* in different tissues were different. The transcripts of *MsFPPS* and *MsGGPPS* were higher in root and leaf than in stem, and the *MsGPPS* transcripts had no significant difference in different tissues. Transcripts of *MsFPPS* and *MsGPPS* genes increased continuously and reached a maximum at hour 5 after spraying MJ. In contrast, *MsGGPPS* transcript levels remained relatively constant after spraying (Fig. 4).

Overexpression of *MsFPPS* induces senescence-like response in tobacco leaves

During the early stages of growth, transgenic plants were morphologically indistinguishable from wild-type plants. However, by approximately 2 weeks of culture, the first produced rosette leaves of T_0 transgenic plants overexpressing *MsFPPS* started to show pale brown lesions with the appearance of regions in which premature cell death had occurred (Fig. 5). In addition, plants overexpressing *MsFPPS* grew much less vigorously than their non-transgenic counterparts. Consequently, the phenotypic alterations observed in leaves of plants overexpressing *MsFPPS* will be further referred to as a senescence-like phenotype. The phenotypes of T_0 transgenic plants overexpressing *MsGPPS* and *MsGGPPS* had no significant difference with wild type tobacco plant.

Squalene biosynthesis in transgenic plants

Squalene is the common precursor leading to both triterpene and phytosterol biosynthesis. To determine quantitatively, the amount of squalene in transgenic tobacco, GC analysis was conducted. The squalene content in the T_0 line of *MsFPPS* transgenic plant was increased, and about 2 times that of the control (Fig. 6). This indicated that the overexpression of *MsFPPS* in tobacco caused an increase in squalene content, which can be considered a positive effect on triterpene and phytosterol biosynthesis. The squalene contents in the lines of *MsGPPS* and *MsGGPPS* transgenic plants were not increased by comparing to the control.

Discussion

In this study we cloned and identified three cDNAs encoding *MsFPPS*, *MsGPPS* and *MsGGPPS* genes from alfalfa. The IDSs catalyze the branch-point reactions leading to the different classes of terpenes. A phylogenetic analysis of a range of known plant IDS genes, excluding heterodimeric forms, shows that *MsFPPS* sequences cluster separately from *MsGPPS* and *MsGGPPS* sequences. In order to verify the prediction about the putative functions

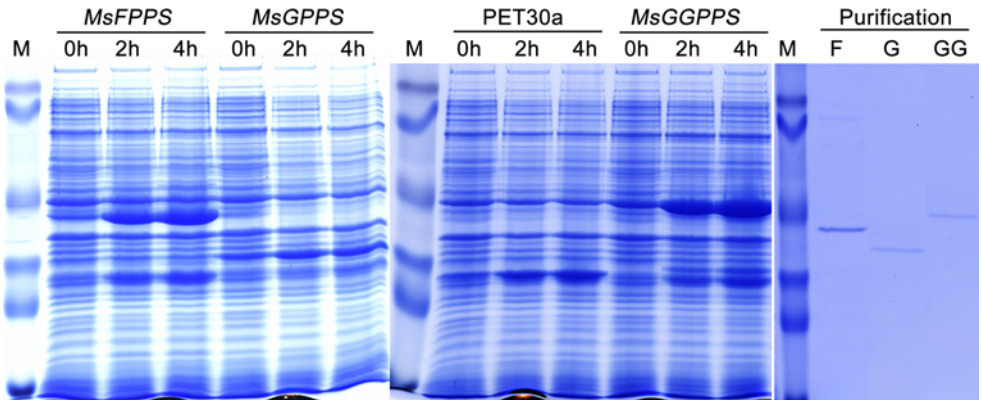

Fig. 3 SDS-PAGE analysis of recombinant MsFPPS, MsGPPS and MsGGPPS proteins expressed in *E. coli* and visualized by coomassie brilliant blue G250 staining. *Line 0*, *2* and *4 h* are bacterial extracts of recombinant expressing *PET30a-MsFPPS*, *PET30a-MsGPPS*, *PET30a-MsGGPPS* and *PET30a* empty vector induced for *0*, *2* and *4 h* with IPTG. *Line F*, *G* and *GG* are purified recombinant proteins of recombinant MsFPPS, MsGPPS and MsGGPPS after Ni–NTA agarose chromatography. *M* molecular mass markers (100, 62, 40, 30, 24, 14 kDa)

Table 1 Enzymatic activity of the three IDSs in alfalfa expressed in *E. coli* (DE3)

Recombinant IDS	Activity
MsFPPS	111.65 ± 1.85
MsGGPPS	129.52 ± 2.15
MsGPPS	8.23 ± 0.21
Empty vector	5.86 ± 0.11

The activity of extracts is expressed as nmol of IPP incorporated into IDS/(min mg) protein with standard deviations

of the three IDSs, the recombinant proteins of *MsFPPS*, *MsGPPS* and *MsGGPPS* were purified by heterologous expression in *E coli*. Then the enzyme activity assay confirmed that the cDNA encodes IDSs. To find the evidence for the regulatory role of IDS in triterpene saponin biosynthesis during insect attack, the induced defense response of alfalfa to insect attack was mimicked by spraying with MJ. At the transcriptional level, *MsFPPS*, *MsGPPS* and *MsGGPPS* were shown to be differently expressed in different tissues after treating with MJ. The

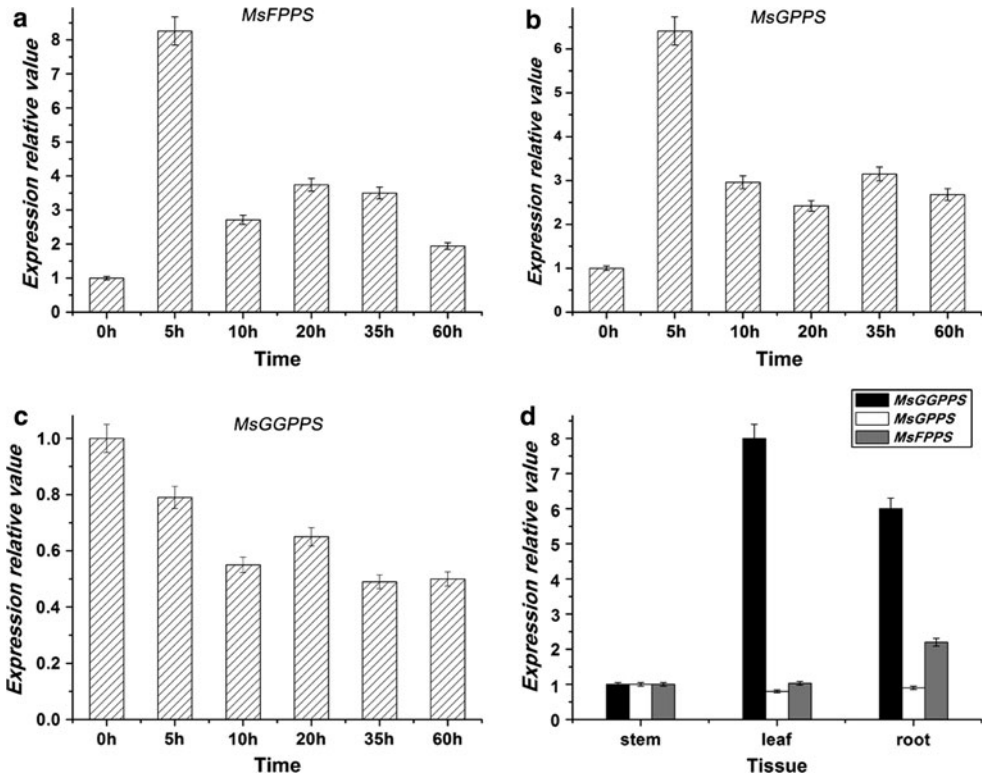

Fig. 4 The relative mRNA levels of *MsFPPS*, *MsGPPS* and *MsGGPPS* in alfalfa cultivar Zhongmu-1 in different tissues and at different time points under MJ treatment

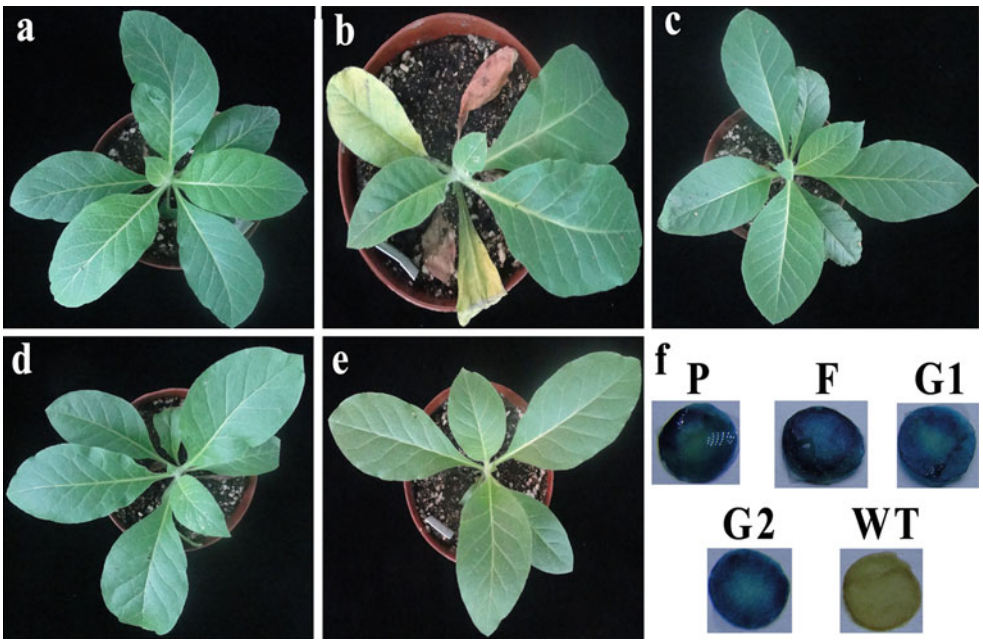

Fig. 5 The phenotype and growth of T_0 tobacco plants overexpressing *MsFPPS*, *MsGPPS* and *MsGGPPS*. **a–d** The transgenic lines of pBI121 empty vector, *MsFPPS*, *MsGPPS* and *MsGGPPS*. **e** wild type line. **f** GUS activity assay of transgenic T_0 lines and WT leaf disks (*P* pBI121 empty vector transgenic line, *F MsFPPS* transgenic line, *G1 MsGPPS* transgenic line, *G2 MsGGPPS* transgenic line, *WT* wild type line

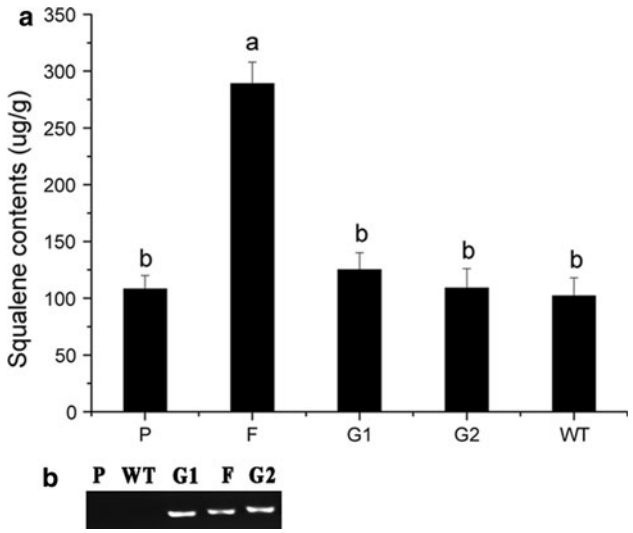

Fig. 6 The squalene content and RT-PCR analysis of T_0 tobacco plants of transgenic lines and WT. **a** Squalene content in the tobacco plant T_0 lines overexpressing pBI121 empty vector (*P*), *MsFPPS* (*F*), *MsGPPS* (*G1*), *MsGGPPS* (*G2*) and wild type line (*WT*). Vertical bars indicate the mean ± SE of three independent samples. Different letters indicate significant differences at $P \leq 0.05$. **b** RT-PCR analysis of transgenic plants and WT

transcripts of *MsFPPS* and *MsGPPS* increased continuously under MJ treatment. In contrast, the transcript of *MsGGPPS* decreased obviously. These increase of *MsFPPS* and *MsGPPS* in transcriptional level indicated that the synthesis of squalene path way in alfalfa may be enhanced when treated by MJ.

To elucidate the functions of the three IDSs, their overexpression driven by a constitutive cauliflower mosaic virus-35S promoter in tobacco plants was applied and analyzed. The T_0 transgenic plants of *MsFPPS* showed high levels of squalene content when compared with control. However, no differences were detected in T_0 transgenic plants of *MsGPPS* and *MsGGPPS* when compared with control. Chen et al. previously reported that A full-length cDNA encoding FPS was isolated from a *Gossypium arboreum* cDNA library and integrated into the *Artemisia annua* genome. As a result the artemisinin content of transgenic plants increased 2–3 fold [21]. Overexpressing FPSs in transgenic plants may permit the accumulation of triterpenoid saponins and related sesquiterpenoids. Our results raise the possibility that *MsFPPS*, may be key regulatory enzymes in triterpene saponin biosynthesis. It is interesting that overexpression of *MsFPPS* in tobacco triggered a phenotype of rapid cell death in intact leaves, whereas it induced premature senescence. This result indicates that *MsFPPS* performs a role not only in phytosterol and triterpene biosynthesis, but also in plant growth regulation.

Plant species in general defend themselves from herbivores by producing toxic phytochemicals. The important constitutive defenses of alfalfa plants include a complex mixture of about 30 saponin glycosides [33]. High levels of these substances are associated with antinutritional, even

toxic. The main antinutritional components present in alfalfa are saponins, and their unfavorable effects on animal performance have restricted the optimum use of this high-protein plant as an animal feed. According to our results, *MsFPPS* of alfalfa may play an active role in defense mechanism.

It is known from the literature that many IDS gene families have more than one member. So another direction of future studies is to clone and characterize more members of *MsFPPS*, *MsGPPS* and *MsGGPPS* in alfalfa. In some other plants, the IDS gene family members localize in different subcellular localizations, and there are some differences in function. For instance, there are two differentially expressed farnesyl-diphosphate synthase genes in *A. thaliana*, At*FPS1* and At*FPS2*, which have a different pattern of expression [41]. There are five GGPPS expressed in different organs are localized into three subcellular compartments in *Arabidopsis* [38]. Three GPPSs were also cloned and characterized in *Abies grandis*, their function are somewhat different [44]. So far, the genome sequence of *M. truncatula* has been released, which provides a way to find out new IDS genes in *M. truncatula*. It also provides evidence to clone IDS genes in alfalfa (*M. sativa*).

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