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BRIEF COMMUNICATION

Overexpression of a stilbene synthase gene from *Fallopia multiflora* causes *trans*-piceid accumulation in transgenic *Arabidopsis*

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Abstract

Fallopia multiflora, also known as *Polygonum multiflorum*, is a traditional Chinese medicine with stilbenes as the main characteristic components. We previously isolated the presumed stilbene synthase gene *FmSTS1* from *F. multiflora* and inserted the *FmSTS1* gene into the genome of *Arabidopsis thaliana* under the control of cauliflower mosaic virus 35S promoter to evaluate its biological functions. Southern blot and Northern blot analyses were performed to confirm the integration and expression of *FmSTS1* in *Arabidopsis*. A new stilbene compound, which was identified as *trans*-piceid, was found to accumulate in the transgenic *Arabidopsis*. This study firstly reports*FmSTS1* from *F. multiflora* that was heterologously expressed in transgenic *Arabidopsis* and resulted in the accumulation of *trans*-piceid, which was originally not synthesized in *Arabidopsis* species.

Additional key words: CaMV promoter, HPLC detection, Northern blot, Southern blot.

Fallopia multiflora (Thunb.) Haraldson is one of the most important traditional Chinese medicinal plants (Committee 2010, Saier *et al.* 2018). Pharmacokinetic studies confirmed that stilbenes are the main characteristic components of *F. multiflora*. Twenty-one stilbene compounds have been separated from *F. multiflora*, including *trans*-resveratrol and a variety of 2, 3, 5, 4′-tetrahydroxystilbene glucosides (Lin *et al*. 2015, Xia *et al*. 2017). Therein, 2, 3, 5, 4′-tetrahydroxystilbene-*2*-*O*-*β*-D-glucopyranoside (THSG, the structure is shown in Fig. 1) has a similar structure with *trans*-resveratrol and is considered as the most important active component in *F. multiflora* (Xia *et al*. 2017). In Chinese medicine, THSG is often used as a chemical marker for the quality control of *F. multiflor*a (Zhao *et al*. 2014).

Plant stilbenes are derived from phenylalanine, and the last step of synthesis is catalysed by stilbene synthase (STS) (Chong *et al*. 2009) which belongs to type III polyketide synthases (Delaunois *et al.* 2007, Vannozzi

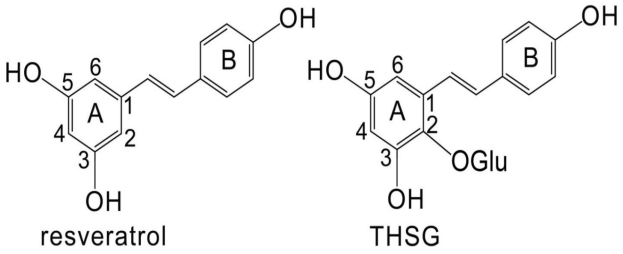

Fig. 1. The stucture of resveratrol and 2,3,5,4′-tetrahydroxy stilbene-2-*O*-β-D-glucoside (THSG).

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Abbreviations: CaMV - cauliflower mosaic virus; *FmSTS1 - Fallopia multiflora stilbene synthase 1*; HPLC - high performance liquid chromatography; STS - stilbene synthase.
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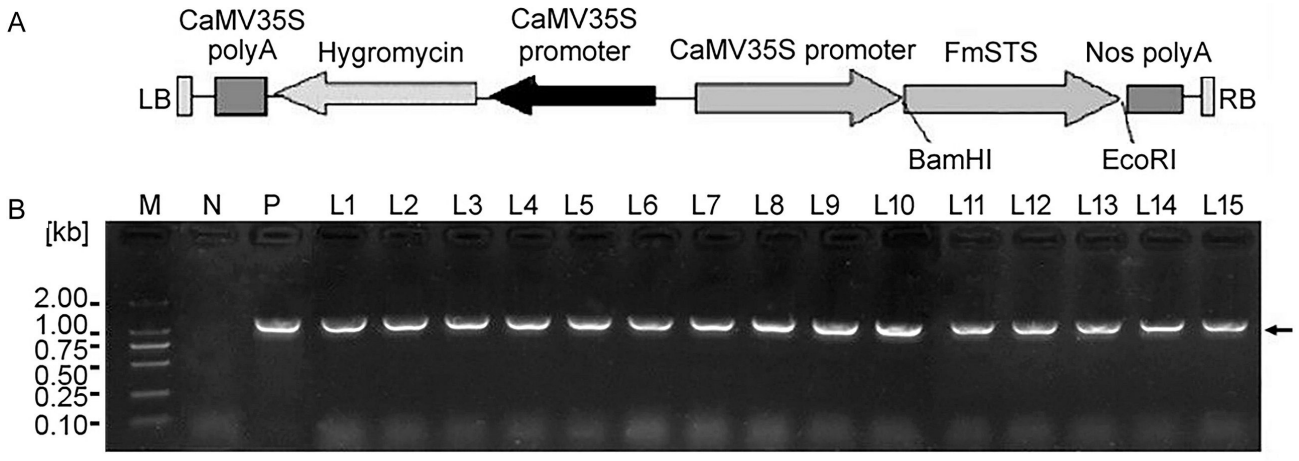

Fig. 2. Identification of transgenic *Arabidopsis* overexpressing *Fallopia multiflora stilbene synthase 1* (*FmSTS1*). *A* - A schematic representation of the construct used to overexpress *FmSTS1* in *Arabidopsis*. *B* - PCR analysis to confirm the presence of the transgene. N - wild type was used as a negative control; P - plasmid was used as a positive control; L1 to L15 - transgenic *Arabidopsis* lines of T1 progenies.

et al. 2012). The *STS* genes have been isolated from many plant species, such as peanut, grapevine, sorghum, and pine (Lu *et al*. 2016). *F. multiflora* is a rich source of stilbenes and have several *STS* genes. Our team has performed a series of studies to elucidate the biosynthesis pathway of THSG in *F. multiflora* and identified several presumed *STS* genes through digital gene expression profiling analyses (Zhao *et al*. 2014).

In our previous study, a presumed *STS* gene *FmSTS1* was isolated from *F. multiflora* (Sheng *et al*. 2010). To further confirm its function on the stilbene biosynthesis in *F. multiflora*, we inserted this gene into *Arabidopsis thaliana* L. genome for heterologous expression. As a result, a new stilbene compound, which was identified as *trans*-piceid, accumulated in the transgenic *Arabidopsis*.

The cDNA of *FmSTS1* (*NCBI* accession No. GQ411431) was PCR amplified with a gene-specific forward primer 5′-AAAGGATCCATGGCAGCTTCAGC-3′ and a reverse primer 5′-AAAGAATTCTTAAAAGATGGGCACACTTC-3′. The PCR-amplified *FmSTS1* was inserted into a pCAM-1 vector under the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 2). The recombinant *35S:FmSTS1* plasmid was transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation and then transformed into *Arabidopsis thaliana* (ecotype Columbia) by the floral dip method (Clough and Bent 1998). For the selection of transformants, sterilized seeds were germinated on agar plates containing hygromycin (25 μg cm^{-3}). After 2 weeks of selection, hygromycin-resistant plantlets were transplanted to soil. Transformed plants were identified by PCR and Southern blot analysis. Northern blot analysis was performed to investigate *FmSTS1* expression.

Approximately 10 μg of genomic DNAs were extracted from the leaves of wild-type and transgenic *Arabidopsis*. After complete digestion with *Eco*RI overnight, the samples were isolated by electrophoresis and transferred onto *Hybond-N*$^+$ nylon membrane (*Amersham Pharmacia*, Buckinghamshire, UK) (Zhou *et al*. 2010). The *FmSTS1* cDNA was labeled and used for filter hybridization with a *DIG-High Prime* kit (*Roche*, Mannheim, Germany).

For Northern blot, total RNA was separated from the leaves of wild-type and transgenic *Arabidopsis* plants. Approximately 20 μg of RNA was size fractioned by electrophoresis and transferred to a *Hybond-N*$^+$ nylon membrane. Probe preparation and detection were performed as described above for Southern blot analysis.

Transgenic lines with strong *FmSTS1* expression were screened until T3 progenies and analyzed for the presence of stilbene-related metabolites. Analysis was performed using the method previously carried out by our team (Liu *et al*. 2011). Triplicate samples (0.5 g each) were collected from the leaves of 4-week-old T3 plants and ground to fine powder in liquid nitrogen. Ground tissues were extracted with 5 cm^3 of 80 % (v/v) methanol, and supernatants were collected after centrifugation. The methanol fraction was evaporated to dryness under nitrogen. The residue was immediately redissolved in 0.5 cm^3 of 80 % methanol. After being filtered through a 0.22 μm film, 0.01 cm^3 of filtrate was analyzed on an *HP 1100* series high performance liquid chromatography (HPLC) system (*Agilent Technologies*, Palo Alto, CA, USA) by using a *Nucleosil C18* column (particle size 5 μm, 4.6 × 250 mm, *Macherey-Nagel*, Düren, Germany) and water-acetonitrile as eluent (acetonitrile/water, 25/75, v/v) at flow rate 0.2 cm^3 min^{-1}. A 306 nm detection wavelength was used.

Liquid chromatography effluent was then introduced into a turbo ion-spray source on a *Q/STAR-XL* quadrupole/time-of-flight (TOF) hybrid mass spectrometer (*Applied Biosystems*, Foster City, CA, USA). Negative ESI mass spectra were acquired over the range of 100 - 400 m/z. The electrospray voltage was set at -4.5 kV, and the source temperature was maintained at 350 °C.

The cDNA of *FmSTS1* was expressed in *Arabidopsis* under the control of a CaMV 35S promoter (Fig. 2*A*). Fifteen independent hygromycin-resistant transgenic *Arabidopsis* lines named L1 - L15 were regenerated, and the presence of *FmSTS1* was confirmed by PCR (Fig. 2*B*). Putative transgenic and control plants were selected for

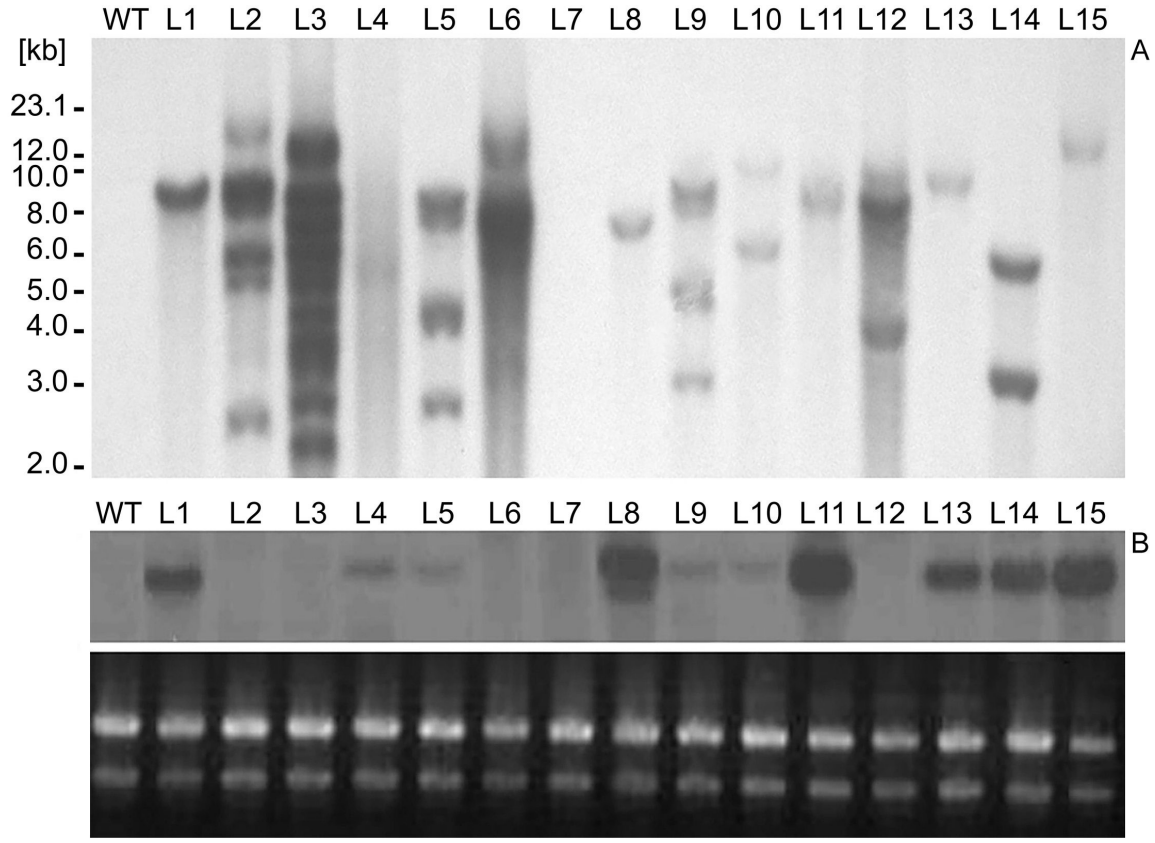

Fig. 3. Southern and Northern blot analyses. *A* - Southern blot analysis of genomic DNAs digested with *Eco*RI and hybridized with *Fallopia multiflora stilbene synthase 1* (*FmSTS1*) cDNA. L1, L4, L8, L10, L11, L13, L14, and L15 represent single or low copy integration in the transgenic *Arabidopsis* lines of T1 progenies. *B* - Northern blot analysis of transgenic *Arabidopsis* using PCR amplified *FmSTS1* as a probe. L1, L8, L11, L13, L14, and L15 represent relatively high expression of *FmSTS1*. rRNA was used as a loading control. WT - wild-type.

further studies.

Genomic DNAs from representative transformants and control plants were digested with *Eco*RI. Southern blot results indicated that besides L7, *FmSTS1* was inserted in all other transgenic lines. L2, L3, L5, L6, L9, and L12 contained multiple copies of the transgene. L1, L4, L8, L11, L13, and L15 contained a single copy (Fig. 3*A*). Northern blot analysis demonstrated no hybridization signal in the untransformed control (Fig. 3*B*). The single-copy- and low-copy-carrying transgenic lines (L1, L8, L11, L13, L14, and L15) showed a relatively high expression of *FmSTS1*. However, the high-copy transgenic lines (L2, L3, L5, L6, L9, L10, and L12) showed relatively low or absent expression. A comparison of data from Southern blot analysis and those obtained by Northern blot analysis indicates that *FmSTS1* expression negatively correlated with transgene copy number. A high copy number of the transgene together with the reduced expression of gene product suggests the occurrence of homology-dependent gene silencing in these lines (Tang *et al*. 2007, Liu *et al*. 2011).

The six transgenic lines (L1, L8, L11, L13, L14, and L15) with high expression of *FmSTS1* were selected for chemical analysis. HPLC and HPLC-ESI-MS were performed to analyze the stilbene metabolites in the leaves of T3 transgenic lines. Compared with the extracts of the wild type plants, transgenic plants contained an additional peak (Fig. 1 Suppl.). The peak was speculated as *trans*-piceid according to the retention time of 7.5 min, which was the same as the *trans*-piceid standard (Fig. 1 Suppl.). The peak was then analyzed by HPLC-ESI-MS (Fig. 2 Suppl.). The spectrum of the [M-H]- ion (m/z 389) with a prominent [M-H-$C_6H_{10}O_5$]- product ion at m/z 227 confirmed the new synthesized compound in transgenic plants as *trans*-piceid (Liu *et al*. 2011).

In this study, the products of *FmSTS1* found in the leaf tissue of T3 progenies of transgenic *Arabidopsis* were identified as *trans*-resveratrol glucosides (*trans*-piceid) by HPLC and LC-ESI-MS analyses. This result is consistent with the reports on purple sweet potato (Pan *et al*. 2012), *Nicotiana benthamiana* (Condori *et al*. 2009), white poplar (Giorcelli *et al*. 2004), kiwi (Kobayashi *et al*. 2000), and transgenic apple fruit (Rühmann *et al*. 2006). Resveratrol glycosylation is commonly found in plants and has been detected in many *STS* transgenic lines. This modification is an effective way for resveratrol storage as it can protect resveratrol from oxidation and degradation (Giovinazzo *et al*. 2012; Lu *et al*. 2016).

For the six selected transgenic lines, *trans*-piceid content estimated by HPLC ranged from 46.2 to 65.3

Table 1. *Trans*-piceid content [μg g-1(f.m.)] estimated by HPLC analysis from leaves of T3 plants. The values represent means ± SDs. Each experiment was repeated 3 times and included 15 individual plants.

Line	*Trans*-piceid content
Wild-type	0.00
L1	62.24 ± 1.74
L8	53.47 ± 2.06
L11	65.32± 2.18
L13	46.24± 1.46
L14	58.64± 1.87
L15	56.38± 1.83

μg g^{-1}(f.m.) (Table 1). Compared with previously published data, this study obtained stilbene content that was lower than that in transgenic *Arabidopsis* overexpressing a sorghum *STS* gene (up to 580 μg g^{-1}(f.m.) (Hipskind and Paiva 2000). The value is also lower than that in transgenic white poplar with a content of 309 - 615 μg g^{-1}(f.m.) (Giorcelli *et al*. 2004). However, in some other reports, the anticipated resveratrol or its derivatives were not detected. For example, in transgenic wheat (Serazetdinova *et al*. 2005), the expression of *STS* caused the accumulation of unknown stilbene derivatives. In transgenic strawberry (Hanhineva *et al.* 2009), *STS* gene transfer caused alterations in the phenylpropanoid metabolism. In *F. multiflora*, stilbene content is very different because of distinct growth areas. In Chinese pharmacopoeia, THSG content should not be less than 1 % in the dried root of *F. multiflora*. We determined the THSG content in our experimental sample of *F. multiflora* as high as 14.62 mg g^{-1}(f.m.) (Zhu *et al*. 2012). The content of THSG in *F. multiflora* is far higher than that in all of the *STS* transgenic plants. However, *STS* transgenic studies developed an effective way to improve nutritional quality or enhance the pathogenic resistance of plants.

Stilbene production in transgenic plants may be related to the promoters selected to drive the transgene, *STS* genes from different origins, and transgenic plant species. To date, genome sequencing has revealed a large array of *STS* genes present in plants of different origins. However, only a few are used for the metabolic engineering of plants. Therein, *Stsy* and *Vst1* from grape are the most commonly used genes (Delaunois *et al*. 2009). Other *STS*-encoding genes, such as *AhRS* gene from peanut (Hipskind *et al*. 2000), an *STS* gene from sorghum (Yu *et al*. 2006), an *STS* gene from *Polygonum cuspidatum* (Liu *et al*. 2011), and an *STS* gene from *Parthenocissus henryana* (Jeandet *et al*. 2018), have also been used. This study demonstrated that in transgenic *Arabidopsis*, the overexpression of the *FmSTS1* gene from *F. multiflora* leads to the accumulation of *trans*-piceid. Thus, *FmSTS1* provides a new resource for *STS* metabolic engineering.

THSG is the main component distributed in *F. multiflora*. Our team has performed considerable research aimed at characterizing the THSG biosynthesis mechanism (Xia *et al*. 2017). We previously showed that the expression pattern of *FmSTS1* analyzed by Northern blot corresponded well with the THSG distribution in different tissues of *F. multiflora*, implying that *FmSTS1* may play a crucial role in THSG biosynthesis (Sheng *et al*. 2010). In the current study, the anticipated THSG was not detected in the transgenic *Arabidopsis*. However, *trans*-piceid, 2-hydroxyl glycoside less than THSG, was detected, which may be attributed to the lack of specific hydroxylase in *Arabidopsis*. Given the structural similarity of piceid and THSG (Fig. 1), THSG may be synthesized by the hydroxylation of *trans*-resveratrol to form the corresponding tetrahydroxystilbene. This hypothesis was recently confirmed by our intro studies using stable isotope labeling and biocatalytic methods (Xia *et al*. 2017). Considering the results of previous and present study, we presumed that if *FmSTS1* was co-transformed with a hydroxylase from *F. multiflora*, THSG may probably be produced in transgenic *Arabidopsis. FmSTS* will be transformed into *F. multiflora* cell line or hair root in future studies to confirm whether it can improve THSG production.

In conclusion, this study shows that overexpression the *STS* gene *FmSTS1* from *F. multiflora* in transgenic *Arabidopsis* leads to the accumulation of stilbenes identified as *trans*-piceid. To our knowledge, this is the first report that an *STS* gene from *F. multiflora* transferred to other plants leads to stilbene accumulation. Considering the relevance of the *FmSTS1* expression pattern with the distribution of THSG reported in our previous research (Sheng *et al*. 2010), *FmSTS1* may play a crucial role in stilbenes biosynthesis in *F. multiflora* and may directly affect THSG biosynthesis. Further research will focus on its transformation into *F. multiflora* cell line or hair root to confirm whether it can improve THSG production.

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