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. multiflora* (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...
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 STSG 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'-AAAAAATTCTTAAAGATGGGCACACTTC-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) strain THSG 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⁻²). 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 EcoRI 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 cm³ 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 cm³ of 80 % methanol. After being filtered through a 0.22 μm film, 0.01 cm³ 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 cm³ 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. 2A). Fifteen independent hygromycin-resistant transgenic *Arabidopsis* lines named L1 - L15 were regenerated, and the presence of *FmSTS1* was confirmed by PCR (Fig. 2B). Putative transgenic and control plants were selected for...
further studies.

Genomic DNAs from representative transformants and control plants were digested with EcoRI. 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. 3A). Northern blot analysis demonstrated no hybridization signal in the untransformed control (Fig. 3B). 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-C6H10O5]- 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.) (Delaunois et al. 2009). Other STS-encoding genes, such as AhRS gene from peanut (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.) (Delaunois et al. 2009). Other STS-encoding genes, such as AhRS gene from peanut (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.) (Delaunois et al. 2009). Some of the transgenic FmSTS1 gene from F. multiflora (Liu et al. 2011), and an STS gene from Polygonum cuspidatum (Liu et al. 2011). These studies 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 of 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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