Tolerance of *Arabidopsis thaliana* plants overexpressing grapevine *VaSTS1* or *VaSTS7* genes to cold, heat, drought, salinity, and ultraviolet irradiation

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Abstract

Stilbene synthases (STS) are plant enzymes that are responsible for the biosynthesis of stilbenes, which are plant phenolic compounds with valuable biological properties. Stilbenes also play important roles in plant tolerance to biotic and abiotic stresses. Therefore, plants that overexpress STS genes can be more resistant to various stresses. This paper investigated the effects of STS gene overexpression in *Arabidopsis thaliana* (L.) Heynh. Columbia-0 plants on stilbene content and tolerance to the following abiotic stresses: low and high temperatures, salinity, drought, and ultraviolet irradiation (UV-B and UV-C). We used *VaSTS1* and *VaSTS7* genes from grapevine (*Vitis amurensis* Rupr.) expressed under the double cauliflower mosaic virus 35S (CaMV35S) promoter. This study firstly demonstrated that overexpression of the *VaSTS1* and *VaSTS7* genes in *A. thaliana* plants considerably increased plant tolerance to UV-B and UV-C, while the tolerance to the low and high temperatures, salinity, and drought was not affected. We showed that the highest trans-piceid and trans-resveratrol total content was in ST1 *A. thaliana* plants that overexpressed the *VaSTS1* gene in the range 8.28 - 22.66 µg g⁻¹(f.m.). ST7 plants that overexpressed the *VaSTS7* gene showed only trans-resveratrol at 0.02 - 0.08 µg g⁻¹(f.m.). Stilbene content and UV tolerance in transgenic *A. thaliana* plants correlated with STS transgene expression. STS expression, UV tolerance, and stilbene content was higher in *VaSTS1* transgenic plants compared with that in *VaSTS7* transgenic plants.

Keywords: glucosyltransferase, peroxidase, piceid, resveratrol, stilbene synthase, viniferin.

Introduction

Stilbenes are natural phenolic compounds that occur in several unrelated plant families, including *Pinaceae* (spruce, pine), *Fabaceae* (false acacia, peanut), *Polygonaceae* (knotweed, rhubarb), and *Vitaceae* (grapevine) (Dubrovina and Kiselev 2017). Stilbenes are biologically active (Kiselev 2011, Shankar et al. 2011, Suwalsky et al. 2015), and they also play a positive role in plant tolerance to abiotic and biotic stresses. Stilbenes are involved in constitutive and inducible plant defense responses as phytoalexins and have also attracted interest due to their protective properties against plant fungal pathogens, nematodes, and herbivores (Chong et al. 2009, Jeandet et al. 2010, Laavola et al. 2015, Weiskirchen and Weiskirchen 2016). Stilbenes directly affect pathogens by inhibiting their growth and protect the plant host cells from excessive destructive oxidative stress, which always appears under pathogen attack.

Stilbenes are synthesized *via* the phenylpropanoid
pathway (Langcake and Pryce 1977), where stilbene synthase (STS, EC 2.3.1.95) directly catalyzes the resveratrol (monomeric stilbene) formation by condensing three molecules of malonyl-CoA and one molecule of p-coumaroyl-CoA to form the stilbene backbone. It has been shown that some stress conditions caused considerable induction in stilbene biosynthesis in plants (e.g. grapes), which indicates the stilbene participation in the defense against the stress conditions (reviewed in Dubrovina and Kiselev 2017).

There are several approaches to establish plants more tolerant to abiotic stresses. One of them is the obtaining of transgenic plants, overexpressing different STS genes. For example, He et al. (2018) transformed Nicotiana benthamiana using the Polygonum cuspidatum PcSTS1 gene, and they showed that the transgenic plants accumulate resveratrol [21.1 μg g\(^{-1}\) (f.m.)]. In addition to an increase in resistance to the pathogen Monilinia fructicola, the transgenic plants exhibit improved tolerance to salt and osmotic stresses (He et al. 2018). Overexpressing the SSvin gene from grapevine in kiwifruit Actinidia delicosa lead to the piceid production in the transgenic kiwifruit [182 μg g\(^{-1}\) (f.m.)], but those kiwifruit plants are not resistant against the gray-mold disease caused by Botrytis cinerea (Kobayashi et al. 2000).

In 2004, Giorelli et al. overexpressed the Vitis vinifera StSy gene in Populus alba. Two new compounds were detected in transgenic poplar plants: the piceid trans- and cis-isomers together up to 615 μg g\(^{-1}\) (leaf f.m.). However, poplar transgenic plants are not resistant against the pathogen Melampsora pulcherrima, which causes rust diseases (Giorelli et al. 2004). Another paper (Yu et al. 2006) described overexpression of the Sorghum bicolor ShSTSI gene in Arabidopsis thaliana tt4 mutants, which are defective in chalcone synthase activity. Firstly, the authors found cis-piceid up to 580 μg g\(^{-1}\) (f.m.) in the Arabidopsis transgenic lines (Yu et al. 2006). Later, three additional resveratrol-related metabolites are found in the transgenic Arabidopsis plants, including a resveratrol diglucoside up to 2.8 μg g\(^{-1}\) (f.m.) and trans- and cis-resveratrol acetylated and hexosylated up to 0.07 and 10.4 μg g\(^{-1}\) (f.m.), respectively (Lo et al. 2007). Liu et al. (2011) also used A. thaliana for experiments, but they overexpressed the P. cuspidatum PcRS gene. The transgenic Arabidopsis plants produced a new compound in both the leaves and seeds, which was identified as trans-piceid up to 15.2 -183.7 μg g\(^{-1}\) (f.m.). Moreover, the transgenic Arabidopsis plants show enhanced resistance to its fungal pathogen Colletotrichum higginsianum, modified seed coat pigmentation, and a significant reduction in anthocyanin content (Liu et al. 2011).

There are some data regarding the content of stilbenes and the pathogen resistance of transgenic plants. It has been shown previously that stilbene content considerably increased after UV irradiation in grapevine (Duan et al. 2015, Tyunin and Kiselev 2016, Kiselev et al. 2019b), but no one analyzed the tolerance of STS transgenic plants to the UV irradiation, cold, and heat stresses. Therefore, we decided to obtain STS transgenic Arabidopsis plants and study the content of stilbenes and their tolerance for abiotic stresses (UV radiation, cold, heat, drought, and salt stresses). We used two STS genes from wild-growing grapevine that were not previously used in other papers for plant transformation.

**Materials and methods**

**Plant cultivation and stress induction**: Plants of Arabidopsis thaliana (L.) Heynh. ecotype Columbia-0 (stored by our laboratory) were grown in pots filled with commercially available rich soil in a controlled environmental chamber (Sanyo MLR-352, Panasonic, Osaka, Japan) kept on a 16-h photoperiod, an irradiance of ~120 μmol m\(^{-2}\) s\(^{-1}\), and a temperature of 22 °C. Wild-type and transgenic sterilized seeds were germinated on 1/2 Murashige and Skoog medium (1962; MS), pH 5.6, solidified with 0.8 % (m/v) agar. Then, the seedlings grown on the MS medium for 7 - 8 d were transferred to commercially available soil. The plants were treated with different stresses (cold, drought, salt, and heat stresses) as described in Dubrovina et al. (2017). Briefly, after planting in the soil the plants were subjected to drought by culturing without additional irrigation for 5 weeks and then re-watered. For salt stress treatments, the transferred seedlings were cultivated without additional irrigation for 2 weeks and then the plants were irrigated with 350 mM NaCl solution. One week after irrigation with NaCl the pots were placed in fresh water for 3 - 4 h to leach the salt from the soil. For cold stress assay, normally cultured A. thaliana plants (3-week-old) were stressed at -10 °C in freezer for 1 h and then cultured at 8 °C for 2 h and after that transferred to normal conditions. For heat stress assays, normally cultured plants (3-week-old) were stressed at 45 °C in a controlled incubator for 3 h. The survival rates were determined as the number of visibly green plants 3 d after re-watering (drought), 1 week after heat and cold stress treatments and 1 week after salt leaching (salt stress). Two pots of plants (10 seedlings per pot) were grown for each transgenic line and each treatment. The experiments were repeated at least eight times.

We did not test the effect of UV irradiation on the survival of Arabidopsis plants, because the treatment killed the overground part of plants. However, all the plants produced new leaves after a week, because the underground part of all plants remained alive. Therefore, we analyzed leaves viability of the irradiated plants. Four-week-old Arabidopsis plants were exposed to UV-B (312 nm) or UV-C (254 nm) using a UV lamp VL-215 MC provided by Vilber Lourmat Company (Marne-la-Vallee, France). The plants were irradiated for 10 min at a distance of 15 cm above pots and peak output being 254 or 312 nm as described (Tyunin and Kiselev 2016). According to the manufacturer’s manual, we used 1800 μW cm\(^{-2}\) of UV-B and 930 μW cm\(^{-2}\) of UV-C irradiation. The leaf survival rates were determined as the number of visibly green leaves 1 d after the end of stress.

**Obtaining VaSTS1 or VaSTS7 overexpressing Arabidopsis plants**: To create transgenic Arabidopsis
plant lines overexpressing the VaSTS1 (Genebank GG167204) or VaSTS7 (EU659868) genes from *Vitis amurensis*, we used pZP-RCS2-(VaSTS1 or VaSTS7)-nptII plasmid construction (Aleynova et al. 2016, Kiselev and Aleyanova 2016). The constructs pZP-RCS2-(VaSTS1 or VaSTS7)-nptII or empty vector pZP-RCS2-nptII were introduced into the *Agrobacterium tumefaciens* strain GV3101:pMP90 and transformed by floral dip method as described previously into wild-type *A. thaliana* Col-0 plants (Zhang et al. 2006) for generating the VaSTS1- or VaSTS7-overexpressing lines.

Transgenic plants were selected by kanamycin resistance on half-strength MS medium supplemented with 50 mg dm−3 kanamycin and confirmed by PCR using the primers for *VaSTS1*, *VaSTS7*, and nptII genes listed in the Table 1 Suppl. The PCR products were verified by DNA sequencing (ABI 3130, Applied Biosystems, Foster City, CA, USA). Three representative independent T1 homozygous lines with single copy transgene insertion (ST1-1, ST1-2, ST1-3 and ST7-1, ST7-2, ST7-3) with different mRNA amounts of VaSTS1 or VaSTS7 genes were chosen for detailed analyses.

**RNA, DNA analysis, and real-time quantitative PCR (qPCR):** Total RNA isolation from plant leaves was performed using the cetyltrimethylammonium bromide-based extraction (Kiselev et al. 2013, Ogneva et al. 2019). Complementary DNA was synthesized using 1.5 μg of total RNA by the RNA PCR kit (Silex M, Moscow, Russia) as described (Kiselev et al. 2007). The real-time qPCRs were performed using a HS polymerase and 50X SYBR Green I (Evrogen, Moscow, Russia). Expression was calculated by the 2−ΔΔCT method (Livak and Schmittgen 2001), where the value 1 in the relative mRNA calculation in each qPCR reaction was assigned for transgenic plants overexpressing only selective marker nptII gene (KA0), AtActin2 (GB acс. No. NM_112764) and AtGAPDH (GB acс. No. NM_111283) genes were used as endogenous controls to normalize variance in the quality and the amount of cDNA of genes were used as endogenous controls to normalize variance in the quality and the amount of cDNA of *A. thaliana* used in each real-time qPCR experiment (Czechowski et al. 2005, Dubrovina et al. 2020).

The primers used for analyzing the *VaSTS1* and *VaSTS7* transcriptions in the transgenic *Arabidopsis* plants (Table 1 Suppl.) were designed to the 3’ end of the protein coding region of the *VaSTS1* or *VaSTS7* mRNA (the cloning *VaSTS1* and *VaSTS7* genes have the same nucleotide sequence in the end of the protein coding region) and to the CaMV 35S terminator in the pSAT1 vector. Real-time qPCR data were obtained from at least three independent experiments and are averages of eight technical replicates for each independent experiment (Dubrovina et al. 2020).

Total DNA was purified from 20 mg of dried *A. thaliana* tissues (mixed rosette leaves) using the EZ DNA methylation-Gold kit (Zymo Research, Irvine, CA, USA) from the eight-week-old *A. thaliana* plants as described (Kiselev et al. 2015). Bisulfite sequencing was performed as stated in Ogneva et al. (2016). After DNA conversion, 352-bp nptII fragments were amplified using primers listed in Table 1 Suppl. We sequenced 16 clones for each DNA region from the 2 biological replicates (8 clones per each individual plant).

**Analytical chromatography:** All solvents were of high performance liquid chromatography (HPLC) grade. Analytical standards: trans-resveratrol and trans-piceid were obtained from Sigma-Aldrich (St. Louis, MO, USA); ε-viniferin was obtained from Panreac AppliChem (Darmstadt, Germany); trans-piceatannol was obtained from Enzo Life Sciences (Farmingsdale, NY, USA). The aboveground part of 35-d-old plants was dried using hot air flow (50 °C for 2 h), weighted and used for stilbene detection. Samples containing 100 mg of dried tissues were extracted with 2 cm³ of 96 % (v/v) ethanol at 60 °C for 2 h. Stilbene content was analyzed at least three times. The HPLC was carried out using an LC-20 analytical HPLC system (Shimadzu, Kyoto, Japan), equipped with a SPD-M20A photodiode array detector, LC-20ADXR pump, Shim-pack XR-ODS II column and SIL-20ACXR auto sampler as described (Aleynova et al. 2016).

**Analysis for statistical differences and correlation:** The statistical analysis for significant differences and correlation in experiments was carried out using the Statistica 10.0 program (StatSoft, Boston, USA). The data are presented as means ± standard errors (SEs) and were tested by paired Student’s t-test.

**Results and discussion**

After selection on kanamycin, we obtained three lines of *Arabidopsis* homozygous T3 plants that overexpressed *VaSTS1* (ST1 lines) gene and three lines that overexpressed *VaSTS7* gene (ST7 lines). All lines were obtained as a result of independent transformations. Next, we analyzed the expression of transferred STS transgenes. We used...
the same primers for the analysis of VaSTS1 and VaSTS7 genes, and we compared STS gene expression between them and with KA0 transgenic Arabidopsis plants that overexpressed only *nptII* gene, a selective marker for transgene plant selection (Fig. 1).

In all transgenic plants, the STS gene amplification was significantly higher compared with the background level in control KA0 Arabidopsis plants (Fig. 1). Thus, all STS transgenic Arabidopsis plants expressed STS genes. Moreover, we showed that VaSTS1 gene expression in all ST1 lines was 6.5 - 13.3 times higher than the VaSTS7 gene expression in all ST7 lines (Fig. 1). These results were unexpected because VaSTS1 and VaSTS7 genes are under the control of the same strong constitutive promoter cauliflower mosaic virus 35S and therefore might have approximately the same expression.

Transgenes in the plants are known to be inhibited by cytosine DNA methylation gene silencing (Rajeevkumar et al. 2015). Perhaps in our case, STS gene sequences undergo different levels of methylation. Thus, we analyzed the cytosine methylation of the VaSTS1 and VaSTS7 gene sequence in the ST1-1 and ST7-1 lines using bisulfite sequencing (Rajeevkumar et al. 2015). We demonstrated that the cytosine methylation of the 3'-end of the protein coding region of the VaSTS1 gene in line ST1-1 was 33.3 ± 9.0 %, while methylation of the VaSTS7 gene in the ST7-1 line was 2.1-times higher (70.1 ± 9.4 %, Fig. 2A). Thus, the VaSTS7 gene was hypermethylated and this is the reason for the low VaSTS7 gene expression in transgenic ST7 Arabidopsis plants.

5-Azacytidine (5A), a chemical analogue of the nucleoside cytidine, is an inhibitor of DNA methylation (Kiselev et al. 2019a). We showed that treatment with 5A increased the VaSTS1 gene expression only in ST1-2 line by 1.3 times (Fig. 2B). However, this elevation of the VaSTS1 gene expression was not significant. At the same time 5A increased hypermethylated VaSTS7 gene expression by 1.2 - 1.9 times in all the ST7 lines of the transgenic Arabidopsis plants (Fig. 2C). Thus, 5A decreased total DNA methylation, which leads to increased expression of the more methylated VaSTS7 genes (Fig. 2C). Expression of the less methylated VaSTS1 gene after 5A treatment did not change significantly (Fig. 2B).

Then, we found that no stilbenes were detected using HPLC in KA0 *A. thaliana* plants that did not express grapevine STS genes (Fig. 1A Suppl., Table 1), while transgenic *A. thaliana* plants overexpressing the VaSTS1 gene produced two stilbenes: trans-piceid and trans-resveratrol (Fig. 1B Suppl., Table 1). Plants overexpressing the VaSTS7 gene produced only trans-resveratrol (Table 1). The highest trans-piceid and trans-resveratrol content was in *A. thaliana* plants that overexpressed the VaSTS1 gene: 8.28 - 22.66 µg g⁻¹(f.m.) (Table 1). The highest stilbene content among the VaSTS1 transgenic plant lines was in ST1-3, while the lowest was in ST1-2 (Table 1). This stilbene content positively correlated with VaSTS1 gene expression (*r* = 0.96). Total stilbene content in ST7 plants overexpressing the VaSTS7 gene was in 103 - 1133 times lower than the stilbene content in the VaSTS1 transgenic plant lines, we found only trans-resveratrol: 0.02 - 0.08 µg g⁻¹(f.m.) (Table 1) and this result also correlated (*r* = 0.98) with low VaSTS7 transgene expression.

There are several papers in which different transgenic plants overexpressing different STS genes have been obtained. Thus, analyzing the available papers, we have shown that the content of stilbenes in our ST1-3 Arabidopsis plants (highest stilbene content in our experiments) was 50.4-times higher compared with Ziziphus jujuba. [0.45 µg g⁻¹(f.m.)] that overexpressed the *P. cuspidatum* PcPKS5 gene (Luo et al. 2015). However, the content of stilbenes in the transgenic white poplar overexpressing the *V. vinifera* StSy gene was 27.1 times higher [up to 615 µg g⁻¹(leaf f.m.)] of *trans- and cis- isomers of piceid* compared with stilbene content in ST1-3 Arabidopsis plants (Giorcelli et al. 2004). There were also no stilbenes in transgenic strawberries that overexpressed *NS-Vitis*3 gene encoding STS from *Vitis riparia* (Hanhineva et al. 2009).

Unfortunately, stilbenes in transgenic Arabidopsis plants overexpressing STS genes were not analyzed (Liu et al. 2011) or STS genes were transferred to Arabidopsis
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**tt4** mutants plants, which could not make flavonoids (Buer and Muday 2004). Flavonoids are competitors of stilbenes in the biosynthesis pathway because flavonoids and stilbenes use the same precursors (Shumakova et al. 2011). Therefore, precursors of phenolic compounds were used in the stilbene biosynthesis. In those plants, the content of stilbenes reaches 600 μg g\(^{-1}\) (cis-piceid, resveratrol diglucoside, and trans- and cis-resveratrol acetylhexosides), which is one of the highest contents, and this may be because there is an excess of the stilbene precursors in these plants (Yu et al. 2006, Lo et al. 2007).

Thus, our ST1 lines for the production of stilbenes were closer to the average level in transgenic plants, and our ST7 lines had one of the lowest stilbene content.

Transgenic *Arabidopsis* plants were exposed to the main abiotic stresses: low and high temperatures, salinity, drought, and UV radiation (UV-B, 312 nm and UV-C, 254 nm). Our results showed that overexpression of the *VaSTS1* and *VaSTS7* gene did not increase tolerance to salinity, drought, and low temperatures (Fig. 3 A, C, D). We showed that viability of the all *STS* overexpressed plants after heat treatment was on 1 - 14 % higher compared with KA0 plants, but this enhancement was not statistically significant (Fig. 3B).

We detected increased tolerance to UV radiation: *VaSTS1* gene overexpression elevated the quantity of green leaves remained after UV-B treatment (1.2 - 1.3 times) (Fig. 3E, Fig. 4). This enhancement was statistically significant for all *VaSTS1*-transgenic *Arabidopsis* plants (Fig. 3E). *VaSTS7* gene overexpression also slightly

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**Table 1. Content of trans-piceid and trans-resveratrol in the KA0, ST1-1, 2, 3, ST7-1, 2, 3 Arabidopsis plants, overexpressing the *VaSTS1* or *VaSTS7* genes.** The plant samples were harvested from the 35 d-old plants. Means ± SEs, \( n = 8; * - P < 0.05, ** - P < 0.01 \) versus values in the empty vector-transformed KA0 plants.

| Cell lines | Trans-piceid [μg g\(^{-1}\) (f.m.)] | Trans-resveratrol [μg g\(^{-1}\) (f.m.)] | Total stilbenes [μg g\(^{-1}\) (f.m.)] |
|------------|---------------------------------|---------------------------------|---------------------------------|
| KA0        | 0                               | 0                               | 0                               |
| ST1-1 (*VaSTS1*) | 12.42 ± 4.11*                   | 0.32 ± 0.23                     | 12.73 ± 4.34*                   |
| ST1-2 (*VaSTS1*) | 8.01 ± 2.02**                   | 0.28 ± 0.19                     | 8.28 ± 2.19*                    |
| ST1-3 (*VaSTS1*) | 21.43 ± 4.98**                  | 1.24 ± 0.55*                    | 22.66 ± 5.66**                  |
| ST7-1 (*VaSTS7*) | 0                               | 0.08 ± 0.03                     | 0.08 ± 0.03                     |
| ST7-2 (*VaSTS7*) | 0                               | 0.02 ± 0.02                     | 0.02 ± 0.02                     |
| ST7-3 (*VaSTS7*) | 0                               | 0.02 ± 0.02                     | 0.02 ± 0.02                     |

Fig. 3. Survival rates of *Arabidopsis* transgenic plants (A-D) and leaves (E,F) after different stresses: cold (A), heat (B), salinity (C), drought (D), ultraviolet B irradiation (UV-B, E), and ultraviolet C irradiation (UV-C, F). KA0 - plants overexpressing only selective marker *nptII* gene; ST1-1, -2, or -3 - lines overexpressing *VaSTS1* gene; ST7-1, -2, or -3 - lines overexpressing *VaSTS7* gene. Means ± SEs, \( n = 160; * - P < 0.05, ** - P < 0.01 \) versus values of plant or leaves survival rate in KA0 plants (paired Student's t-test).
increased the viability of the UV-B irradiated plants, but this enhancement was not statistically significant (Fig. 3E).

Similar results were obtained when UV-C was applied, but the negative impact on the survival of the leaves under the same conditions (time, distance to plants from the UV-C lamp) was much stronger (Fig. 3F, Fig. 4), although the UV-B radiation intensity was almost 2 times greater (1 800 µW cm–2 of UV-B versus 930 µW cm–2 of UV-C). This confirms earlier findings that UV-C exposure is more damaging for plants than UV-B exposure (Nawkar et al. 2013). Under more severe conditions of UV-C irradiation, the protective effect of the VaSTS gene overexpression was manifested more strongly. Thus, overexpression of the VaSTSI gene significantly increased the quantity of green leaves after UV-C treatment by 2.0 - 2.3 times compared with KA0 Arabidopsis plants (Fig. 3F). VaSTS7 gene overexpression also increased the viability of the UV-C irradiated leaves by 1.3 - 1.8 times, but this enhancement was statistically significant for only two of the three Arabidopsis ST7 plants lines that were used in the experiments (for ST7-1 and ST7-3, Fig. 3F).

Conclusions

We showed that the tolerance to UV-B and UV-C radiation in transgenic A. thaliana plants positively correlated with total stilbene content and STS transgene expression. Therefore, STS expression led to stilbene accumulation, which had a positive effect on plant tolerance of UV radiation. Thus, we firstly experimentally showed stilbene participation in plant protection from UV radiation. For obtaining VaSTSI and VaSTS7 transgenic plants, we used the same genetic constructs and methods of transformation, but we detected significant differences in VaSTSI and VaSTS7 expression, methylation of the DNA of the VaSTSI and VaSTS7 gene protein coding region, and different stilbene accumulation and stress tolerance. Thus, stilbene accumulation and stress tolerance in Arabidopsis plants depends on the nucleotide sequences of the selected STS gene, which requires further study.

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