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PLANT GENETICS

Expression of the Stilbene Synthase Genes in the Needles of Spruce *Picea jezoensis*

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Abstract—Stilbenes are valuable plant phytoalexins, the biosynthesis of which is characteristic of different groups of phylogenetically unrelated plants. It is believed that all the stilbenes are the derivatives of resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) or compounds close to it (pinosylvin or piceatannol). The last stage of the resveratrol biosynthesis takes place with the involvement of stilbene synthase or resveratrol synthase (STS). The family Pinaceae is characterized by the presence of the derivatives of pinosylvin (genus *Pinus*) and piceatannol (genus *Picea*), the biosynthetic pathways of which are scarcely examined. Previously, in different species of the genus *Picea*, only two stilbene synthase genes were described. On the basis of RNA isolated from the needles of spruce *Picea jezoensis,* the full-length cDNAs of the four stilbene synthase genes, *PjSTS1a*, *PjSTS1b*, *PjSTS2*, and *PjSTS3*, were obtained. Then, using the clone frequency analysis and real-time PCR, expression of the *PjSTS1a*, *PjSTS1b*, *PjSTS2*, and *PjSTS3* genes was examined in the needles of *P. jezoensis* accessions of different age and sampled in different seasons (spring, summer, autumn, winter). Among the analyzed transcripts, the *PjSTS1a* and *PjSTS1b* genes were the most frequent, indicating their higher level of expression compared to other *STS* genes. The highest level of *PjSTS1a* and *PjSTS1b* expression was observed in autumn, while the level of *PjSTS2* and *PjSTS3* expression was the highest in spring and winter. Moreover, the highest *PjSTS* expression was detected in the young tissues of *P. jezoensis* in autumn, which may indicate a higher level of stilbene biosynthesis in these tissues.

Keywords: astringin, isorhapontin, piceid, resveratrol, *STS*, *Picea jezoensis*

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INTRODUCTION

Stilbenes, a kind of natural phytoalexins, are phenolic compounds with two benzene rings having the structure of C6–C2–C6. Stilbenes are found in a number of unrelated plant species from the families Fabaceae (e.g., peanuts), Pinaceae (e.g., spruce), and Vitaceae (e.g., grapes).

Most of the stilbenes are represented by the derivatives of resveratrol (3,5,4'-trihydroxystilbene), which in turn is a key precursor in the biosynthesis of stilbenes [1]. Resveratrol exists in two isomeric forms (*trans* and *cis*), but the most often found form in plants is *trans*-resveratrol (or *t*-resveratrol). This form has greater known biological activity than *cis*-resveratrol.

As a result of further modifications, *t*-resveratrol in plant cells may be converted into other stilbenes, such as viniferins (oxidation), pterostilbene (methylation), or piceid (glycosylation). *T*-resveratrol is a well-known molecule, a powerful antioxidant that is able to prevent the development of malignant tumors and has great potential to build on its base biologically active additives (BAA) and drugs [2, 3], which explains the great interest in *t*-resveratrol.

The biosynthesis of stilbenes, including *t*-resveratrol, follows the phenylpropanoid pathway [4] (Fig. 1). The phenylpropanoid pathway is an important component of the secondary metabolism of plants, in the course of which the phenylalanine (Phe) amino acid is converted into a large number of unique compounds (phenolic acids, flavonoids, lignins, stilbenes, and others) [5]. Stilbene synthase or resveratrol synthase (STS, EC 2.3.1.95) is an enzyme that synthesizes resveratrol. STS condenses three molecules of malonyl-CoA with one molecule of coumaroyl-CoA; the end product of this reaction (C2 $\rightarrow$ C7 aldol condensation) is resveratrol [6]. The subsequent ring closure of tetraketide intermediate, formed in this process, results in the generation of a new aromatic ring system [7]. The intermediates cannot be directly detected because of their instability. STS both structurally and functionally is closely associated with chalcone synthase (CHS, EC 2.3.1.74), a key enzyme in the synthesis of flavonoid compounds [7] (Fig. 1). For instance, STS and CHS from *Arachis hypogaea* have from 70 to

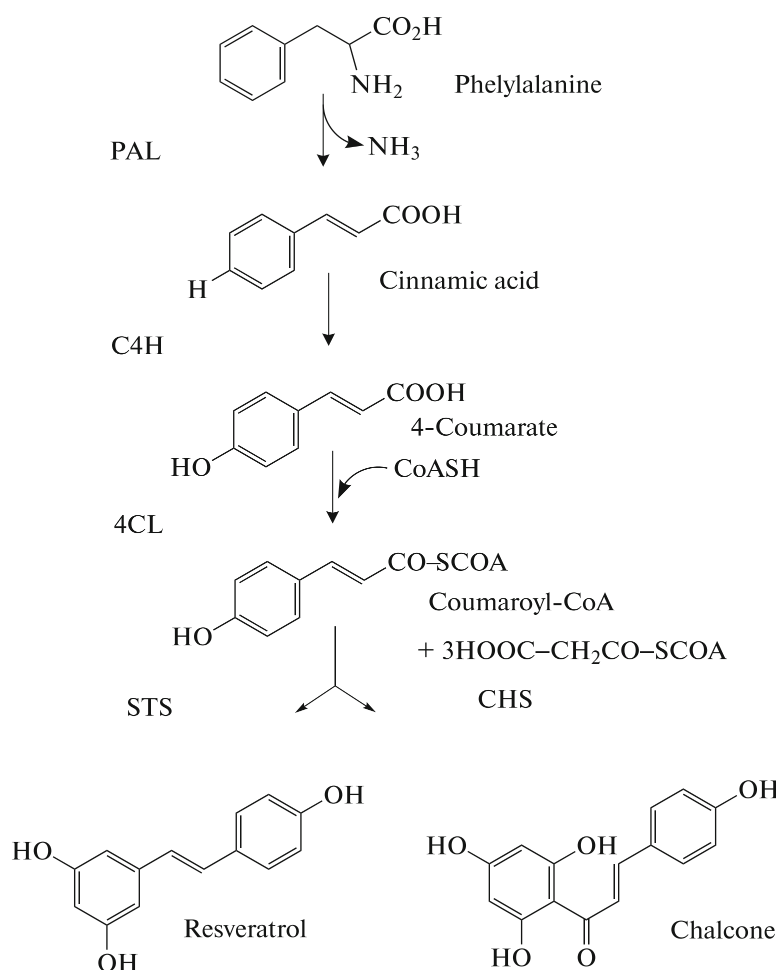

Fig. 1. Scheme of the resveratrol and chalcone biosynthesis [24]. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate-CoA ligase; STS, stilbene synthase; CHS, chalcone synthase.

75% identity on the protein level (their amino acid sequences differ only at 35 positions) [8].

Plant *STS* genes generally exist as small families. However, for example, in *Vitis vinifera* grapes, the *STS* genes are represented as multigene family consisting of 48 representatives. Moreover, it was demonstrated that 32 of them are full-length and functional genes [9]. Expression and activity of STS in the biosynthesis of *t*-resveratrol in grapes is studied quite well. However, in the literature, there is currently a lack of theoretical and experimental data on the biosynthesis of stilbenes in pine. It is known that, in Pinaceae, other forms of stilbenes, similar to resveratrol, i.e., astringin (the glycoside of piceatannol, 3,5,3',4'-tetrahydroxystilbene), isorhapontin (the glycoside of isorhapontigenin, 3,5,4'-trihydroxy-3'-methoxystilbene) and pinosylvin, 3,5-dihydroxystilben, are more frequent [10, 11].

In the needles and bark of spruce (*Picea* species), most of the stilbenes are represented by astringin and isorhapontin. It is suggested that there are three possible pathways of astringin and isorhapontin biosynthesis, i.e., through the formation of pinosylvin, resveratrol, or piceatannol [11]. It was demonstrated that recombinant *Picea abies* stilbene synthases, PaSTS1 and PaSTS2, synthesized resveratrol, and it was suggested that its further modifications could give rise to astringin and isorhapontin [11]. To date, the nuclear genome of the representatives of the genus *Picea* is not sequenced, and because of this, the number of *STS* genes in the members of this genus can hardly be assessed. Previously, two *STS* genes each were described in *P. abies, P. glauca*, and *P. sitchensis* [11]. However, these genes were identified by analyzing the available cDNA collections. Furthermore, the *PaSTS1* and *PaSTS2* genes were cloned from one type of tissues. Therefore, the possibility that not all *STS* genes of the genus *Picea* are found cannot be excluded.

The objective of this study was to clone all expressed *STS* genes from *Picea jezoensis* using the needles of different ages and sampled in different seasons and to examine their expression profiles.

MATERIALS AND METHODS

Plant material. The stilbene synthase gene expression was analyzed using the needles sampled from 15- to 20-year-old accessions of *Picea jezoensis* (Lindl. et Gord.) Fisch ex Carr. growing at the Botanical Garden-Institute of the Far Eastern Branch of the Russian Academy of Sciences, Vladivostok. The needle samples were divided into three groups: young (first year), middle (2–3 years), and old (4–5 years). The needles were sampled during all four seasons of 2014–2015.

Isolation of RNA and synthesis of complementary DNA (cDNA). Total RNA was extracted according to the method described earlier [12] and treated with DNase (Sileks M, Russia). cDNA was prepared from 1.5 μg of total RNA using the reverse transcriptase reagent kit (Sileks M, Russia) [13–15]. Reverse transcription polymerase chain reaction (RT-PCR) was carried out in 50 μL of a reaction mixture containing one-fold RT buffer; 0.25 mM of each of four dNTPs; 2 μM of primer, the sequence of which contained 15 deoxythymidine triphosphates (oligo-$(dT)_{15}$ primer); and the M-MLV reverse transcriptase (200 U) from the Moloney murine leukemia virus [16, 17]. The reaction was run for 1.5 h at 37°C. The obtained products (0.5 μL) were then PCR amplified. In the course of the experiment, M-MLV reverse transcriptase was replaced by ThermoScript reverse transcriptase (Invitrogen, United States), the use of which was described earlier [13].

DNA sequencing and analysis of nucleotide and amino acid sequences. PCR was performed using the DNA amplification kit with Pfu Plus polymerase (Sileks M, Russia) as recommended by the manufacturer. Degenerate primers for the beginning (5'ATG TCT GCA GRA ATG ACT GTT G) and the end (5'TGG AAG RAG AAC GCT CTT AAG A) of the protein-coding *STS* cDNA sequence were selected on the basis of the known stilbene synthase gene sequences from closely related species, including the *PaSTS1* (JN400048) and *PaSTS2* (JN400047) genes from *P. abies*, *PgSTS1* (JN400069) and *PgSTS2* (JN400070) from *P. glauca*, and *PsSTS1* (JN400059) and *PsSTS2* (JN400058) from *P. sitchensis*. The annealing temperature chosen for PCR was 50°C and extension was performed for 70 s.

In addition, in *P. jezoensis*, the cDNA fragments of the actin and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) genes were determined using a pair of primers selected for the actin gene of *P. abies* (FJ869868), 5'CAA ACA GAG AGA AGA TGA CTC A and 5'GAA CTG CTT TTA GCT GTC TCA A, and for *Gapdh* of *P. glauca* (BT116619), 5'CAT CTG AAG GGT CAC TAA AAG and 5'CGT GAA GCA ACT AAA GCC ATG.

The obtained amplicons were isolated from the gel using the Cleanup Mini kit (Evrogen, Russia) and cloned into the pJET1.2/blunt vector according to the protocol of the manufacturer (Fermentas, Lithuania). The presence of the insert in the plasmid was verified by PCR with primers 5'ACT CAC TAT AGG GAG AGC GGC and 5'AAG AAC ATC GAT TTT CCA TGG C. The reaction conditions consisted of denaturation at 95°C for 2 min, followed by 35 cycles of amplification (95°C for 20 s; 58°C for 10 s; 72°C for 80 s), and final extension at 72°C for 1 min. RT-PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Kit v3.1 as recommended by the manufacturer on a ABI 310 Genetic Analyzer (Applied Biosystems, United States) on the premises of the Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok.

The search for homologous amino acid sequences was performed with the help of BLAST, using the blastx algorithm [18] (www.ebi.ac.uk/blastx). Multiple alignment of the amino acid and nucleotide sequences was performed in the ClustalW software program [19] (www.ebi.ac.uk/clustalw/index.html). Analysis of amino acid sequence identity was carried out by a specialized NCBI BLAST software program (http://blast.ncbi.nlm.nih.gov), using the blastp (protein-protein BLAST) algorithm.

Quantification of the stilbene synthase PjSTS1a, PjSTS1b, PjSTS2, and PjSTS3 expression was performed with the help of real-time PCR (qRT-PCR). The selected regions of the *PjSTS1* transcripts were amplified in the presence of the EvaGreen fluorescent dye (Biotium, United States) using the PCR kit for real-time PCR according to recommendations of the manufacturer (Syntol, Russia). Amplification was carried out on a DTprime device with real time detection of PCR results (DNA-Technology, Russia) and using the method of relative analysis. Processing of the results was performed using the method of relative quantitation available in the RealTime_PCR v. 7.3 software package (DNA-Technology, Russia). The sample with the lowest C_t value was chosen as a reference tube.

Calculations were performed using the $\Delta\Delta C_t$ method [20] and the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ for each *PjSTS1* sample was normalized to actin and *Gapdh* according to the formulas (C_t, Target *PjSTS* – C_t, *Actin* or *Gapdh*)$_{\text{target sample}}$ – (C_t, Target *PjSTS* – C_t, *Actin* or *Gapdh*)$_{\text{control}}$. The average C_t values for all the autumn (when analyzed in different seasons) or for all young annual samples (when analyzing needles of different ages) were taken as the control. From the values thus obtained, we calculated the mean and standard errors. They represent the degree of change in the target gene expression relative to control conditions. The qRT-PCR data were calculated from two independent experiments in which for each DNA sample 16 technical replicates were made (8 were normalized to actin and 8 were normalized to Gapdh). The sequence and efficiency of amplification of the primers used for expression analysis, as well as the database accession

Table 1. Primers used in the study for determination of the stilbene synthase gene expression in *Picea jezoensis*

Gene (GenBank Acc. no.)	Primer sequence	Amplification efficiency, %	Size of PCR product, bp
PjSTS1a,1b (LT158484, LT158485)	5'AGG TAC CAT CGC TGG ACG T 5'GTA ATC TTA GAC TTG GGC TGC	104	115
PjSTS2 (LT158486)	5'TAC TGA AGG AGA ATC CCA ATC 5'TCC CAG CCG GGG CAC TG	103	95
PjSTS3 (LT158487)	5'GTA CCA TCC CTG GAC GC 5'GTA ATC TTA GAC TTC GGC TGT	98	113
PjActin (LT158488)	5'CTG GTA TTG TGC TAG ATT CTG 5'TCC TGT TCA TAG TCC AGT GC	112	232
PjGapdh (LT158489)	5'CTG ATG AAG ATG TCG TCT CG 5'AGT CCA CCA CTC GGT TGC TG	114	150

Table 2. Comparison of nucleotide and deduced amino acid sequences of the *STS* genes from *P. jezoensis* and *P. abies*

	PaSTS1	PaSTS2	PjSTS1a	PjSTS1b	PjSTS2	PjSTS3
PaSTS1 (AEN84236)	–		99 (99)	99 (99)	98 (98)	99 (99)
PaSTS2 (AEN84235)		–	97 (99)	98 (99)	97 (99)	98 (99)
PjSTS1a (LT158484)	99 (99)	97 (99)	–	99 (99)	97 (99)	98 (99)
PjSTS1b (LT158485)	99 (99)	98 (99)	99 (99)	–	97 (99)	98 (99)
PjSTS2 (LT158486)	98 (98)	97 (99)	97 (99)	97 (99)	–	97 (99)
PjSTS3 (LT158487)	99 (99)	98 (99)	98 (99)	98 (99)	97 (99)	–

Identity (%) of amino acid and nucleotide (in brackets) sequences was determined with the help of the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov), using the blastp algorithm (protein-protein BLAST).

numbers (GenBank, NCBI), are demonstrated in Table. 1. The amplification efficiency for each pair of primers used was assessed by the analysis of the slope of the calibration line in RealTime_PCR v. 7.3 software (DNA-Technology) using standard DNA dilutions. The cDNA dilutions of 1 : 1, 1 : 5, 1 : 25, 1 : 125, and 1 : 625 were used as standards.

Statistical analysis of the results was performed using the Statistica 10.0 software program. The results are expressed as mean ± standard error and tested by paired Student's *t*-test. The significance level of 0.05 was chosen as the minimum value of the statistical difference in all experiments.

RESULTS AND DISCUSSION

Cloning of the *STS* gene PCR products showed the presence of four full-length *STS* transcripts in *P. jezoensis*, including *PjSTS1a*, *PjSTS1b*, *PjSTS2*, and *PjSTS3*. It is noteworthy that, working with the first cDNA samples, we found a large number of short transcripts (about one-third of cloned samples). Similar transcripts were described by us earlier for the genes of calcium-dependent protein kinases [13, 21]. Moreover, similar short transcripts were described previously for the *PDSTS3* gene from *Pinus densiflora* [10]. However, these transcripts tend to disappear upon the replacement of the M-MLV by ThermoScript reverse transcriptase. Because of this, they can be attributed to the M-MLV reverse transcriptase errors, which previously was observed for other genes [13, 22].

The deduced amino acid sequences of the *PjSTS1a* and *PjSTS1b* genes differ only in two positions out of 396 amino acids, which indicates their high identity (Table 2), so they marked as 1a and 1b. Moreover, all obtained amino acid and nucleotide sequences of STS demonstrated a high percentage of identity between each other and with the previously described STS from other species of the genus *Picea* (97–99%, Identities, see Table 2), confirming the attribution of the obtained sequences to the stilbene synthase family.

Next, using frequency analysis of the PCR products [23] among the examined colonies, it was demonstrated that the *PjSTS1a* and *PjSTS1b2* genes were the most expressed among the four described *PjSTS* genes (Fig. 2a). The *PjSTS1a* and *PjSTS1b* expression depended on the season: it was the highest in spring and autumn (Fig. 2b). The *PjSTS2* and *PjSTS3* expression changed to a lesser extent, and the highest level was recorded for winter samples (Fig. 2a). The highest expression of all four *PjSTS* genes was observed in the young tissues of *P. jezoensis* during spring and autumn, suggesting a higher level of stil-

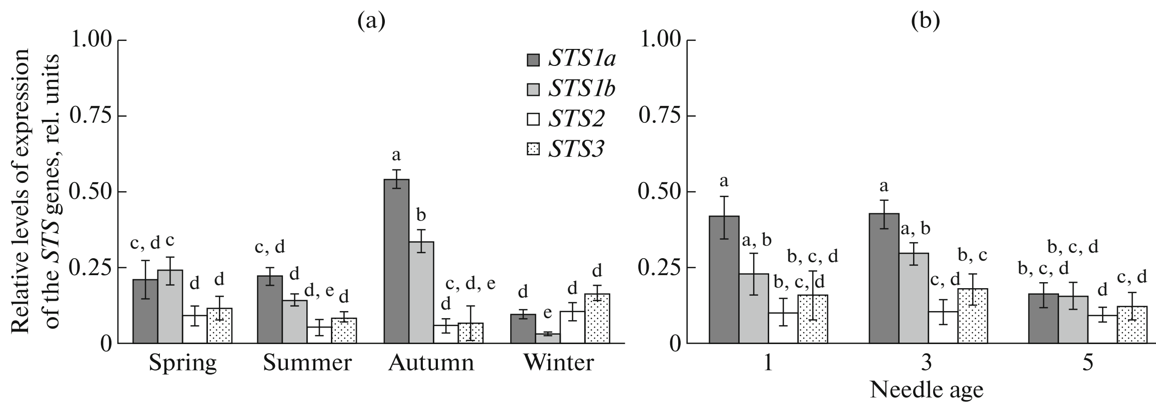

Fig. 2. Relative levels of expression of the *PjSTS* genes in the needles of *Picea jezoensis* sampled in different seasons (a) and of different age (b). Spring, the needles were sampled in March and April 2015; summer, in July and August 2015; autumn, in September and November 2014; winter, in December 2014. Needle age: 1, 1 year; 3, 2–3 years; 5, 3–5 years. Values marked with the same letters are not statistically different from each other ($P < 0.05$). (The same explanations apply to Fig. 3.) The data were obtained using frequency analysis of cloned RT-PCR products [23].

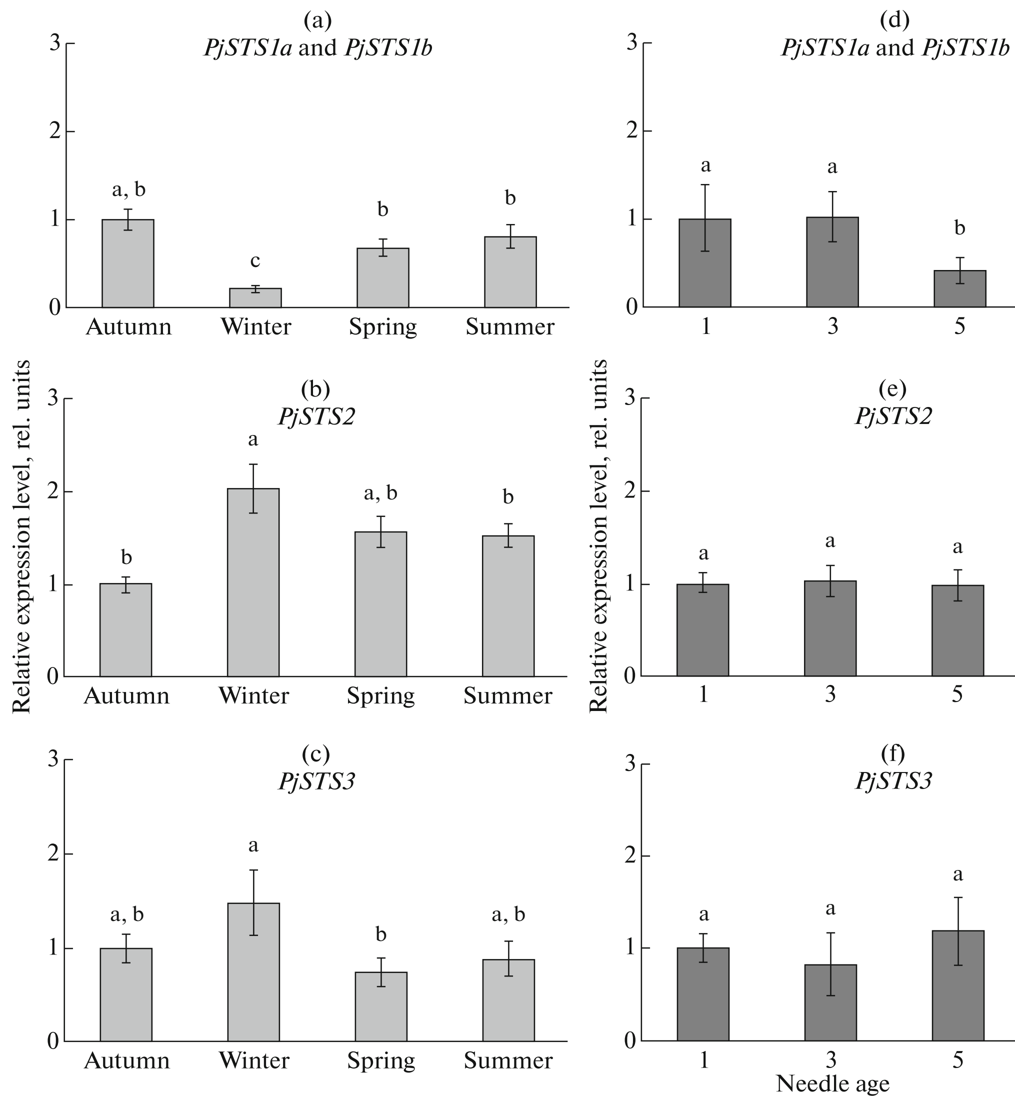

Fig. 3. Relative levels of expression of the (a, d) *PjSTS1a* and *PjSTS1b*, (b, e) *PjSTS2*, and (c, f) *PjSTS3* genes in the needles of *Picea jezoensis* sampled in different seasons (a, b, c) and of different age (d, e, f). The data were obtained using qRT-PCR. For other explanations, see caption to Fig. 2.

bene biosynthesis in these tissues. All data were verified using real-time PCR. In general, qRT-PCR data confirmed earlier results obtained using frequency analysis of cloned RT-PCR products. The highest *PjSTS1a* and *PjSTS1b* expression was observed in autumn and in young tissues of *P. jezoensis*, while the *PjSTS2* and *PjSTS3* expression was the highest in winter samples and depended little on the age of the sampled needles (Fig. 3).

Thus, we have described the presence of the four genes expressed in the needles of *P. jezoensis*, while previously only two such genes were described for the other members of the genus *Picea* [11].

In recent years, much has been learned about the biosynthesis of tetrahydroxystilbenes; however, a complete understanding of the biosynthesis of stilbenes in spruce (genus *Picea*) has yet to be gained. For example, in the needles and bark of spruce, most of the stilbenes are represented by astringin and isorhapontin. It is suggested that there are three possible pathways of astringin and isorhapontin biosynthesis, i.e., through the formation of pinosylvin, resveratrol, or piceatannol [11]. The pathway of stilbene biosynthesis in spruce through the formation of piceatannol is the first preference, because it leads to the formation of astringin and isorhapontin with fewer intermediate reactions. However, it was demonstrated that PaSTS1 and PaSTS2 stilbene synthase proteins from *P. abies* synthesized *t*-resveratrol, and it was suggested that its further modifications could give rise to astringin and isorhapontin [11]. Unfortunately, Hammerbacher et al. [11] described only two genes, while the results of our study demonstrated the presence of at least four spruce *STS* genes that differed in the nucleotide sequence and the expression profile from one another. It is possible that the protein products of the newly described *STS* genes can synthesize piceatannol, but this supposition requires further investigations.

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