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Simultaneous expression of stilbene synthase genes in Japanese red pine (*Pinus densiflora*) seedlings

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Abstract We analyzed sequences of the stilbene synthase (STS) cDNA in *Pinus densiflora*. Three novel STS cDNA clones (pdsts1, pdsts2, pdsts3) that carry full coding sequences were isolated from a cDNA library constructed from the roots. The homologies in their coding regions were about 95%, and they have a conserved STS motif. Their phylogenetic relation was also discussed. The 3′ end of the STS cDNA fragments was amplified by the polymerase chain reaction and subcloned. The 3′ untranslated region (3′UTR) of the fragments highlighted the structure diversity. Twelve STS cDNA clones were categorized into seven distinct subclasses according to 3′UTR sequences. We discussed the stability of the transcripts and their gene organization. The simultaneous expression of the STS members is one of the mechanisms for adaptation that pine trees have developed over time.

Key words Pinosylvin · Phytoalexin · Multigene family · Stilbene synthase · Chalcone synthase

Introduction

Pinosylvin, the stilbenoid phytoalexin, is an effective fungicide; and its monomethyl ether is a strong nematicide.[1] It is usually formed in pine heartwood but is inducible under biotic and abiotic stress.[2–6] Stilbenoids are distributed in higher plants without any direct phylogenic correlation,[7] whereas flavonoids are widely distributed in the plant kingdom. Stilbene synthase (STS) and chalcone synthase (CHS) are key enzymes in stilbenoid and flavonoid biosynthesis, respectively. Both enzymes catalyze the addition of three molecules of malonyl-coenzyme A (CoA) to a starter CoA ester, producing either a stilbenoid or a flavonoid, respectively. They are highly homologous (60%–70%) in amino acid sequence and STS genes are probably derived from CHS genes during plant evolution.[8] Some STS genes have been cloned and sequenced in *Arachis hypogaea*,[9] *Vitis vinifera*,[10,11] *Pinus sylvestris*,[12] and *Pinus strobus*.[13]

The pine genome is 10 times the size of the human genome.[14] During its evolution it might have been prominently amplified and dispersed to form complex families.[15] Southern analysis has revealed several STS genes in *Pinus densiflora*,[16] and 10 genomic STS genes from *Pinus sylvestris* were grouped into five subclasses according to the size of their introns.[17] However, diverse STS transcripts are not yet reported in the conifer. To clarify the STS members expressed in pine trees, we analyzed the 3′ untranslated region (3′UTRs). UTRs are rich in sequence diversity, whereas the coding sequences are generally well conserved. We took advantage of this diversity for identifying family members. In this study, we have isolated diverse STS genes expressed in pine seedlings and discuss their relation with the multigene family.

Materials and methods

Construction of a cDNA library

The 14-day-old seedlings were used in this study. We have already reported that the STS genes are expressed in roots of the seedlings rather than in their hypocotyls.[16] Accordingly, mRNA was prepared from the roots of pine seedlings elicited with salicylate according to a previous report.[18] cDNA was synthesized from 8.8 μg of the mRNA using the SuperScript Plasmid System (Life Technologies) according to the supplier's manual. The cDNA was size-fractionated by 1% agarose gel electrophoresis. Then cDNA larger and smaller than 1.0 kb was inserted into plasmid vector

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pSPORT1 (Life Technologies), respectively. The library containing the cDNA smaller than 1.0 kb was used to isolate 3′ end fragments (ca. 0.4 kb) of STS cDNA clones, and that containing the cDNA larger than 1.0 kb was used to isolate full-length STS cDNA (ca. 1.4 kb) clones.

Amplification of 3′-end STS cDNA

The cDNA library (smaller than 1.0 kb) was used as a template for the amplification of STS cDNA 3′ ends. The forward primer was based on the plasmid vector pSPORT1, and the reverse primer was based on the nucleotide sequence of a highly conserved block in STS. The vector specific primer sequence was 5′-ACGAGGAAGGCGTC TCTACAA-3′ (21mer). The STS cDNA specific primer sequence was: 5′-CCAGTGAATTGAATTTAGGTGAC-3′ (23mer). DNA fragments with sequences corresponding to STS were amplified using these primers. Thermal cycling consisted of preincubation at 94°C for 6 min and 35 cycles of denaturation at 94°C for 1 min followed by annealing/extension at 55°C for 2 min. The polymerase chain reaction (PCR) products were observed as a smear band (200–400 bp) on 1% agarose gel after electrophoresis. The bands were rescued from the gel using a Whatmann GF/C glass filter. The purified PCR products were subcloned into a plasmid pGEM-T Easy vector (Promega). The ligated DNA was transformed into *Escherichia coli* DH10B cells by electroporation. After the subcloning, 50 white colonies were arbitrarily checked for insertional DNA, and their nucleotides were sequenced.

Screening of full-length STS cDNA clones by colony hybridization

From the cDNA library (larger than 1.0 kb), 3×10^4 colonies were screened using the PCR-amplified probe for STS, 900 bp long. The colonies were transferred to a nylon membrane (Hybond N^+; Amersham). The colony hybridization was carried out using ECL direct nucleic acid labeling and detection systems (Amersham). Single colonies were obtained from positive spots by repeating the hybridization twice.

Nucleotide sequencing

The nucleotides of the cloned STS were sequenced for both strands by the dideoxy chain termination method[19] using the Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems, Japan).

Analysis of STS cDNA clones

The BLAST algorithm[20] was used to search the GenBank database for similarity of the STS cDNA nucleotide sequence with other reported sequences. Phylogenetic analysis was performed using PHYLIP Version 3.57c.[21]

Results and discussion

Analysis of 3′ ends in STS cDNA

Some 12 cDNA clones were assigned as STS for the following reasons. First, they showed higher homology to *Pinus sylvestris* STS than CHS. They had a highly conserved block for the STS coding sequence, followed by 3′ UTRs, which are similar to STS but not to CHS. Second, the three novel STS cDNA clones that carry full coding sequence were also isolated from the library and were identified as one of the 12 cDNA clones, respectively. Third, the recombinant STS expressed in *Escherichia coli* cells were confirmed by Western blotting analysis, and enzyme activity was successfully detected (A. Kodan, et al., unpublished results).

Three prime UTRs of the mRNA transcripts highlighted the sequence diversity. The mRNA transcripts with putative multiple polyadenylation sites, often observed in other plant mRNAs,[22] were present. We analyzed the phylogenetic relations among the diverse transcripts, but the sequence lengths were not enough to create a reliable phylogenetic tree. Thus we classified them by some characteristic sequences, for example the number of translation factors such as SBF-1,[23] HSF, AAUAAA, and AUUUA[24] motifs present in the 3′UTRs. Table 1 shows that 12 STS mRNA transcripts are organized by seven subclasses. Most of the STS are novel molecular species, except for pdsts1–40 (AB030141) and pdsts1–35 (AB030143). The former was identical to that isolated from *Pinus sylvestris*,[12] and the latter corresponded to that isolated from *Pinus strobus*.[13]

Each STS cDNA clone had one or two putative polyadenylation signals composed of AAUAAA or AAUAAA-like sequences, which are responsible for stabilizing the mRNA, preceding a polyadenylation tail. Some of the STS cDNA clones had multiple reiterations of the AUUUA motifs and adjacent AU-rich domain, which were identified as selective mRNA destabilizers.[24] Both the stability and instability sequences suggest that long- and short-lived mRNA transcripts are present and may control stilbenoid metabolism via the mRNA level. Examples of long-lived mRNA transcripts are pdsts1–40 (AB030141), pdsts1–25 (AB030142), and pdsts1–35 (AB030143), none of which had a destabilizing factor in the 3′UTR. Such mRNA transcripts may have the potential to increase STS production levels and upregulate pinosylvin biosynthesis.

Isolation of three STS cDNA clones with full coding sequences

Four positive clones were isolated from our cDNA library using the PCR-amplified probe for STS. The three clones were about 1.4 kb long and had one open reading frame coding for STS; but they were not identical. They were highly homologous in their coding regions but showed clear differences in nucleotide sequence among the 3′UTRs. Therefore, they were designated PDSTS1 (AB015489 in the GenBank database), PDSTS2 (AB030139 in the GenBank database), and PDSTS3 (AB030140 in the GenBank data-

Table 1. Characteristics of 3′-end STS cDNA fragments in *Pinus densiflora*

3′-End STS cDNA clones	Accession no. in GenBank	Coding region, length (bp)	3′UTR						Corresponding full-length STS cDNA clones
			Stop codon	Length (bp)	SBF-1	HSF	AAUAAA	AUUUA	
pdsts1–40	AB030141	126 (100%)[a]	UAA	106	0*	1*	3	0	
pdsts1–05	AB030153	126 (92%)	UAA	66	1*	2*	2	1	
pdsts1–14	AB030154	126 (92%)	UAA	124	1*	2*	3	1	PDSTS1
pdsts1–37	AB030155	126 (92%)	UAA	140	1*	2*	3	1	
pdsts1–16	AB030156	123 (91%)	UAG	121	1*	1*	3	1	
pdsts1–19	AB030157	123 (91%)	UAG	139	1*	1*	3	1	PDSTS2
pdsts1–42	AB030158	123 (91%)	UAG	90	1*	1*	2	1	
pdsts1–25	AB030142	126 (92%)	UAA	116	0*	1*	3	0	
pdsts1–35	AB030143	123 (89%)	UAA	173	0*	0*	1	0	
pdsts1–24	AB030144	123 (92%)	UAA	132	0*	2*	2	2	PDSTS3
pdsts1–18	AB030145	123 (92%)	UAA	85	0*	2*	2	3	
pdsts1–22	AB030146	123 (89%)	UAA	78	0*	2*	2	1	

The number of SBF-1, HFS, AAUAAA, and AUUUA motifs were examined for the classification of 12 STS cDNAs
SBF-1, silencer binding factor of chalcone synthase from bean; HSF, heat shock factor from *Drosophila*; AAUAAA and AUUUA, numbers of possible polyadenylation signals and selective mRNA destabilizing signals, respectively; broken lines, borders among seven subclasses; PDSTS1 (AB015489), PDSTS2 (AB030139), and PDSTS3 (AB030140), full-length STS cDNA clones isolated from *Pinus densiflora*; *Numbers of motifs present in the sequences aligned to pdsts1–05 whose 3′UTR was shortest (no asterisk represent numbers of motifs in the full sequence)
[a] Identities with pdsts1–40

Fig. 1. Alignment of deduced amino acid sequence for three PDSTS and PDCHSX. Amino acid sequences of PDSTS1, PDSTS2, PDSTS3, X60573, and PDCHSX are shown from the start (M) to stop codons (*). X60573 represents *P. sylvestris* pinosylvin synthase. *Points* show identical amino acids, and *hyphen* positioned at V-94 shows inserted deletion. Conserved cysteine residues (C), which are essential for activity, are indicated by an inverted triangle. A motif around the cysteine (Cys-167), which is probably the binding site for cinnamoyl-coenzyme A, is indicated by a *box*. The three amino acid residues, which is the most remarkable property of a pinosylvin synthase, are shown by large solid circles

base). This finding indicates that the gene has multiple copies. The other clone was about 1.7 kb long with an open reading frame coding for CHS, designated PDCHSX (AB015490 in the GenBank database). The CHS clone was screened at the same time because of its high homology (about 60%) with the three STSs. The CHS probably plays an important role in pinocembrin biosynthesis in the seedlings.

The three STS cDNAs were deduced to encode pinosylvin synthase for the following reasons. First, the amino acid sequence deduced from the cDNAs exhibited significant homology (92–98%) with that for the *Pinus sylvestris* STS (X60753). Second, they had three distinct amino acid residues (solid circles in Fig. 1) in the N-terminal area compared to the resveratrol synthase isolated from grape and peanut, which is the most remarkable property of a pinosylvin synthase. Third, they had a conserved cysteine residue located in the central section of these proteins (amino acid position 167). This residue is essential for the catalytic activity of both STS and CHS[25] and probably represents the binding site for cinnamoyl-CoA.[26,27] The amino acid residues around this active site were well conserved. The motifs in PDSTS1 and PDSTS2 are identical, whereas in PDSTS3 isoleucine replaces valine (amino acid position

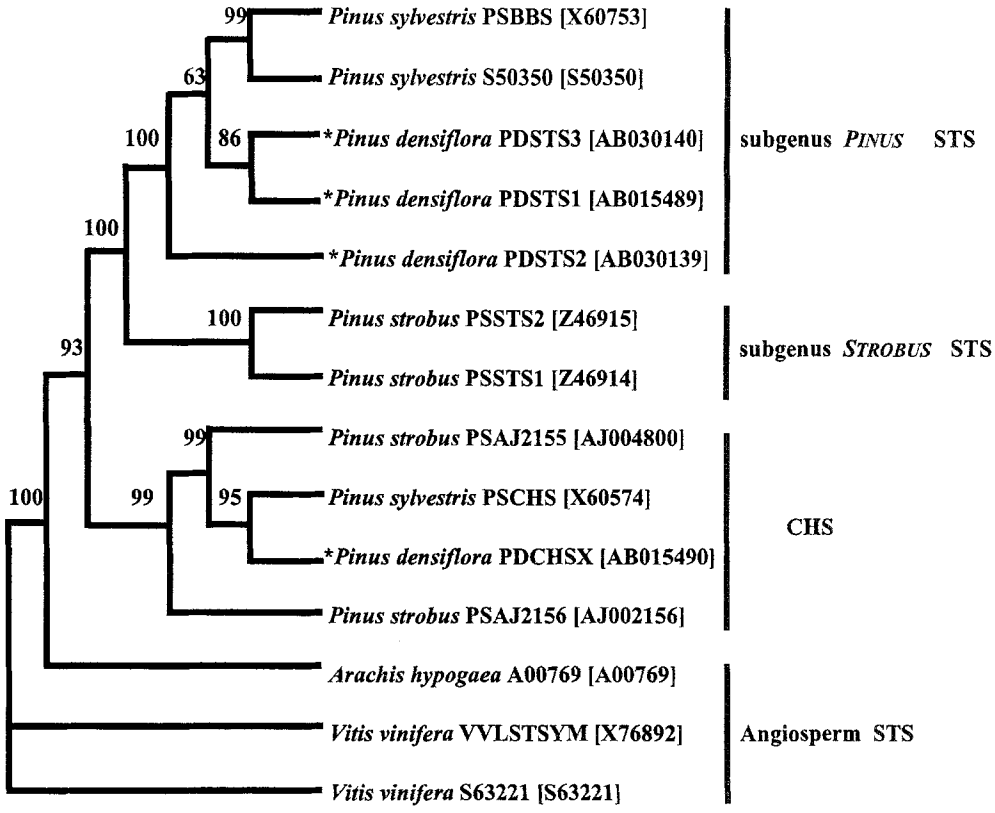

Fig. 2. Strict consensus tree of STS and CHS inferred by the neighbor-joining method. The tree was created from the amino acid sequences using PHYLIP. The numbers at the forks indicate percent bootstrap values for 1000 bootstraps. *Brackets* represent accession numbers in the GenBank database. Our cDNAs cloned are shown by asterisks. The sequence of PDSTS3, which lacks 82 amino acid residues in the C-terminal area due to a frame shift, is used in a gap-filling mode to align

162). PDSTS3 had a two-base deletion (nucleotide positions 879–880: AB030140 in the GenBank database) compared to the others, resulting in a shift in the reading frame, which lacks 82 amino acid residues in the C-terminal area.

Phylogenetic analysis of STS cDNA clones

To understand the sequence relations among the three STSs, we constructed a molecular phylogenetic tree by the neighbor-joining method with bootstrapping. The tree clearly shows four primary lineages corresponding to subgenus *PINUS* STS, subgenus *STROBUS* STS, CHS, and angiosperm STS (Fig. 2). According to the classification of genus *Pinus*,[28] the three STSs fell into a group of subgenus *PINUS* STS gene rather than subgenus *STROBUS* STS gene. This is in consistent with the classical phylogeny because our sample belongs to the subgenus *PINUS*.

The STS genes had higher orders of regulatory complexity, perhaps to ensure normal control by simultaneous expression of the family members rather than a single copy. The complexity of the gene family is probably due to the large genome size of pine, although another explanation for the simultaneous expression of the family members is also possible. Conifer species are not fully converged into a pure line, unlike vegetative crops, and are expected to contain genetic variation within a species. The STS gene family might be caused to some extent by variation in the pine population because the material used in this study was not a single tree but seedlings. At any rate, we demonstrated simultaneous STS mRNA transcript expression, with as many as 12 STS mRNA transcripts grouped into seven subclasses, in the conifer species.

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