

Expression of Stilbene Synthase Gene in Japanese Red Pine (*Pinus densiflora*) Seedlings*¹

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Abstract—The present paper shows that stilbene synthase gene (*sts*) expresses with or without salicylate (SA) treatment in Japanese red pine seedlings, the chemical that is known as a key substance of angiosperms against diseases. The effect of SA was diversified in those seedlings, that may reflect the divergent response within the species.

Keywords : stilbene synthase gene, gene expression, stress response, pine seedlings, *Pinus densiflora*

1. Introduction

The stilbene derivatives are typical extractives in pine heartwood and in the stressed tissues¹⁾. They show weak but well-defined anti-fungal activities and are clearly demonstrated as a phytoalexin because the gene introduced into tobacco plants gave the plants resistant to fungal attack²⁾. A few German groups have been studied for the stilbene biosynthesis in a pine, especially, in Scots pine (*Pinus sylvestris*). For example, the pine seedlings express stilbene synthase gene (*sts*) with a stress, e.g., UV irradiation, fungal attack, and ozone exposure³⁾. This gene is responsible for the direct formation of pinosylvin from three malonates and one cinnamate^{3,4)} (Fig.1).

We employed Japanese red pine (*Pinus densiflora*) as a plant material. Pine trees in Japan have suffered severely from pine wilt diseases⁵⁾. We are interested in *sts* expression of the red pine, because it is directly related to the phytoalexin formation, and may give an acquired resistance to the conifer.

Treatment with salicylic acid (SA) is able to mimic pathological stresses in many plants⁶⁾. The stresses induce systemic acquired resistance or several pathogenesis-related (PR) proteins in a wide range of angiosperms⁷⁾. Endogenous SA levels increase and several PR proteins are induced during the plant response to pathogen infection⁸⁾. These are strong evidences that SA in angiosperm plays an important role in signal transduction pathway leading to the systemic acquired resistance. However, not all plant-pathogen

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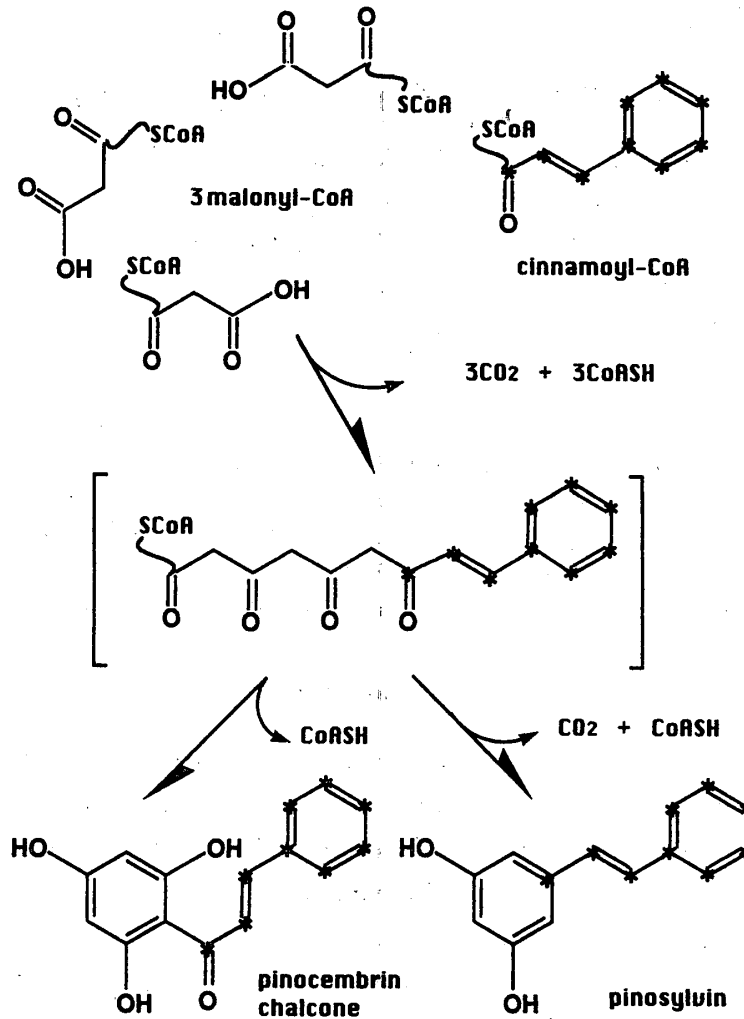


Fig. 1. Formation of a stilbene and a chalcone by the respective synthases. Both of the enzymes utilize same substrates, i.e., three malonates and a cinnamate to form tetraketide intermediate, followed by ring formation with or without decarboxylation. Astarisks in the compounds show cinnamate carbon skeleton.

systems respond to SA, nor report on the effect of SA on gymnosperm.

This paper focused on *sts* if it may or may not express with the SA treatment in a gymnosperm, *Pinus densiflora*.

2. Materials and Methods

The seeds of Japanese red pine, *Pinus densiflora*, were purchased from a local market. The seedlings were grown on vermiculite under dark at 28°C after soaking the seeds with tap water for 2 days. At the 15 days after seeding, 10mM of sodium salicylate had sprayed over the seedlings that were further incubated for 0 to 32 hours under the same condition. One gram fresh weight of the sample harvested was powdered with liquid nitrogen in a mortar

and extracted with guanidium salt. After removal of cell debris and polysaccharide by passing through a RNeasy spin column (Qiagen), one microgram of total RNA was immediately reverse-transcribed according to the manufacturer's instruction (Wako). To minimize experimental deviation, CAT mRNA (Gibco) was added as an internal standard besides constant total RNA contents. The single stranded cDNA was amplified by PCR that was performed at 94°C for 1 minute followed by at 65°C for 3 minutes by repeating the reactions for 26 and 28 cycles. The primer pairs are 21mers and designed on the *sts* from *Pinus sylvestris* (Accession No. X60753 in GenBank). Deviation of the cDNA synthesis and PCR among the samples were compensated by the CAT internal standard. The band amplified was purified with a QIAquick spin column (Qiagen) and directly cycle-sequenced with FITC-labeled primer (Fig. 2) and SequiTherm DNA polymerase (AR Brown) to confirm as a *sts* by an automated DNA sequencer.

		1480	1490	1500	1510	1520	
stsW1HcDNA	1451	CwCTGCCAAC	ATTGAAAAC	GTATGGTCGA	GGCGTTCAGT	CAGTWC AAAA	1500
sts (X60753)	1451	CTCTGCCAAC	ATTGAAAAC	GTATGGTCGA	GGCGTTCAGT	CAGTTC AAAA	1500
chs (X60754)	1451	CTCcAAgAAC	ATaGAgAAg	GtCtGGTtGA	GGCcTTCcG-	CAGTTCgGAA	1500
		1530	1540	1550	1560	1570	
stsW1HcDNA	1501	TATCCGACTG	GAACAAGTTG	TTCTGGGTTG	TTCATCCCGG	AGGACGTGCC	1550
sts (X60753)	1501	TATCCGACTG	GAACAAGTTG	TTCTGGGTTG	TTCATCCCGG	AGGACGTGCC	1550
chs (X60754)	1501	TcTCgGACTG	GAACcAGTTa	TTCTGGaTcG	caCATCCCGG	AGGtCcTGCC	1550
		1580	1590	1600	1610	1620	
stsW1HcDNA	1551	ATCCTTGATC	GGGTGGAGGC	CAAGCTCAAT	CTGGATCCCA	CAAAACTGAT	1600
sts (X60753)	1551	ATCCTTGATC	GGGTGGAGGC	CAAGCTCAAT	CTGGATCCCA	CAAAACTGAT	1600
chs (X60754)	1551	ATtCtGgATC	aGGTGGAGGC	CAAGCTaAAT	tTGGATCCCA	agAAACTGag	1600
		1630	1640	1650	1660	1670	
stsW1HcDNA	1601	ACCCACCAGG	CACGTTATGA	GCGAGTACGG	AAACATGTCC	AGTGCGTGCG	1650
sts (X60753)	1601	ACCCACCAGG	CACGTTATGA	GCGAGTACGG	AAACATGTCC	AGTGCGTGCG	1650
chs (X60754)	1601	tGCAACgAGG	CAaGTaCtGA	GCGActAtGG	AAACATGTcG	AGcGCGTGCG	1650
		1680	1690	1700	1710	1720	
stsW1HcDNA	1651	TCCACTTCAT	ATTGGATCAG	AaaAGGAAGc	-----	-----	1700
sts (X60753)	1651	TCCACTTCAT	ATTGGATCAG	ACGAGGAAGG	CGTCTCTACA	AAACGGATGT	1700
chs (X60754)	1651	TgCACTTCAT	cTTGGAcgAG	AtGAGGAAGt	CcTCTaaAga	gAAaGGATGT	1700
		1730	1740	1750			
stsW1HcDNA	1701	*****	*****	*****	1750		
sts (X60753)	1701	TCAACCACCG	GAGAGGGATT	GGAAATGGGA	1750		
chs (X60754)	1701	TcCAACCACCG	GAGAGGGACT	GGAtggGGGA	1750		

Fig. 2. Multiple Sequence Alignment for chalcon synthase gene family in pine trees. *stsW1HcDNA*: a sequenced stilbene synthase cDNA from *Pinus densiflora*. *sts (X60753)*: a stilbene synthase gene from *Pinus sylvestris*, and the sequence was retrieved from GenBank (accession no.: X60753). *chs (X60754)*: a chalcone synthase gene from the same species. The sequence was from the same DNA data bank (accession no.: X60754). Astarisks show the position where the backward primer locates. Small letters show mismatched bases with the sequence of X60753. The numbers show the base position from the 5' end of *sts (X60753)*. The Gaps(-) in those sequences were inserted for a maximum alignment.

3. Results and Discussion

The RT (reverse transcription)-PCR product amplified from the Japanese red pine, was ca.1.0 kbp in size, that is in good accordance with a *sts* expected (1036 bp) from the Scots

pine. Recently, the *sts* has recognized as a derivative from chalcone synthase genes⁹⁾, and both of the sequences and enzyme reactions are quite similar (Fig. 1). Thus, it has to demonstrate if the amplified fragment is real *sts*, although the fragment size is an expected one. The *sts* cDNA fragments from the Japanese red pine were partially sequenced (ca. 0.3 kbp) and aligned against those of *sts* and chalcone synthase gene from Scots pine (Fig. 2). The cDNA from the Japanese pine is 94% and 97% homologies in a *sts* from the Scots pine with amino acid and nucleotide sequences, respectively. On the other hand, it is 69% and 75% homologies in a chalcone synthase gene from Scots pine with amino acids and nucleotide sequences, respectively. Thus, the sequence obtained from the Japanese red pine clearly shows the one from *sts* but not from chalcone synthase gene.

Conifer species are not fully converged into a pure line comparing with vegetative crops, and are expected to contain genetic variations within a species. We selected two phenotypes observed in the Japanese pine seedlings. One has a reddish hypocotyle and the other was a pale one. The phenotypes under stress may respond differently. One of the stress applied was to uproot seedlings. The uprooted seedlings in the pine were diversely damaged after leaving them for 62 hours under moistened condition. There was a clear tendency that the reddish one is resistant while the pale one susceptible against the stress. The other stress was salicylate (SA) spray on the intact seedlings. It showed no apparent damage on the seedlings. However, the spray with or without SA induced *sts* expression that reached maximum level at 16 hours incubation. The pale seedlings with SA treatment showed higher *sts* level than ones with water control, while the expression level was almost same in the reddish ones. These observations suggest that individual seedlings may diverse responses to both of the stresses, i.e., uprooting and SA treatment. In conclusion, the susceptible seedlings to the uprooting show SA-sensitive *sts* expression, while resistant seedlings to the uprooting express *sts* with or without SA treatment.

References

- 1) Norin, T: "Stilbenes, Conioids, and Other Polyaryl Natural Products", in "Natural Products of Woody Plants I", (W.J. Rowe ed., Springer-Verlag), pp. 512-533, (1989).
- 2) R.HAIN et al.: *Nature*, **361**, 153-156 (1993).
- 3) A. SCHOEPPNER and H. KINDL: *FEBS Lett.*, **108**, 349-352 (1979), D. ROSEMAN, W. HELLER and H. SANDERMANN, Jr: *Plant Physiol.*, **97**, 1280-1286 (1991), A. SCHWEKENDIEK, G. PFEFFER and H. KINDL: *ibid.*, **301**, 41-44 (1992).
- 4) S. SCHANZ, G. SCHRODER and J. SCHRODER: *FEBS Lett.*, **313**, 71-74 (1992), S. TROPF, B. KARCHER, G. SCHRODER and J. SCHRODER: *J.Biol.Chem.*, **270**, 7922-7928 (1995).
- 5) Y. MAMIYA: "Origin of the pine nematode and its distribution outside the United States", in "Pathogenicity of the pine wood nematode", (M.J. Wingfield ed., APS Press, Minnesota), pp. 59-65 (1987).
- 6) D.F. KLESSIG and J. MALAMY: *Plant Mol. Biol.*, **26**, 1439-1458 (1994).
- 7) D. ROSEMAN et al.: *Plant Physiol.*, **97**, 1280-1286 (1991).
- 8) M.P. Van de REE et al.: *Plant Cell*, **2**, 357-366 (1990).
- 9) S. TROPF, T. LANZ, S.A. RENSING, J. SCHRODER and G. SCHRODER: *J. Mol. Evol.*, **38**, 610-618 (1994).