Volume 301, number 1, 41–44 FEBS 10913 April 1992

Pine stilbene synthase cDNA, a tool for probing environmental stress

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Received 13 February 1992

Stilbene synthase cDNAs were isolated from a pine (*Pinus sylvestris*) cDNA library. Poly(A)$^+$RNA required for the preparation was obtained from young seedlings challenged with *Botrytis cinerea*. A full-length cDNA encoding pinosylvin-forming stilbene synthase was sequenced, and the deduced amino acid sequence was compared with sequences of resveratrol-forming stilbene synthases. The cDNA coding for the key enzyme of pinosylvin formation is a valuable tool for detecting early effects of environmental stress in pine needles.

Phytoalexin; Pinosylvin; Stilbene synthase sequence; *Pinus sylvestris*

1. INTRODUCTION

Pinosylvin and its methyl ether were first described from the heartwood of the Scots pine (*Pinus sylvestris* L.) by Erdtman [1]. Later, the importance of heartwood constituents for resistance against fungal and insect attacks became evident [2,3]. In recent years, the enzyme responsible for the formation of pinosylvin (Fig. 1) from malonyl-CoA and cinnamoyl-CoA has been isolated and characterized [4–6]. The induced formation of the enzyme is an early, selective and sensitive process elicited by fungi or environmental stress. Especially, young pine seedlings respond quickly to the attack of various fungi by synthesizing the enzymes required for the formation of phytoalexins typical of pine species [6]. Thus, rapidly activated stilbene synthase-encoding genes are responsible for plant resistance [7].

Stilbene synthases occurring in a small number of plant genera have been grouped into two types. The enzymes from peanut [8] or grapevine [9] use *p*-coumaroyl-CoA and malonyl-CoA as substrates and form resveratrol while the pine enzyme [6] acts with cinnamoyl-CoA and malonyl-CoA and leads to pinosylvin (Fig. 1). Ring A of the stilbene is formed by the polyketide pathway. Ring B stems from the phenylpropane branch of the pathway. As a hydroxylation in position 4′ does not occur at the level of stilbenes, the branching point for the synthesis of the two subclasses of stilbenes is at the conversion of cinnamic acid (Fig. 1).

We isolated and characterized a full-length pinosylvin synthase cDNA. The homology between pinosylvin-forming stilbene synthase and resveratrol-forming stilbene synthases is less pronounced than the homology between resveratrol synthase and chalcone synthase both using *p*-coumaroyl-CoA as a substrate.

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2. MATERIALS AND METHODS

Poly(A)$^+$RNA was isolated from 3-week-old seedlings of *Pinus sylvestris* L. challenged with fungus [6]. The method of Hughes and Galau [10] in a slightly modified form was used to separate RNA from phenols and polysaccharides. Following extraction with 8.5 M KAc in the presence of 1 mM aurintricarboxylic acid, the solution was clarified by centrifugation and carefully separated from mucilage. Crude RNA was precipitated for 2 h at −20°C by adding 0.1 Vol. 3 M NaAc and 0.5 Vol. isopropanol. The pellet was resuspended, and the RNA precipitated with 0.25 Vol. 10 M LiCl. The following steps included precipitation with 5 M KAc and extraction with phenol/chloroform. After removal of polysaccharides by precipitation with 0.1 Vol. ethanol, total RNA was obtained by sedimentation with 2 Vol. ethanol. Poly(A)$^+$RNA was isolated as described [11].

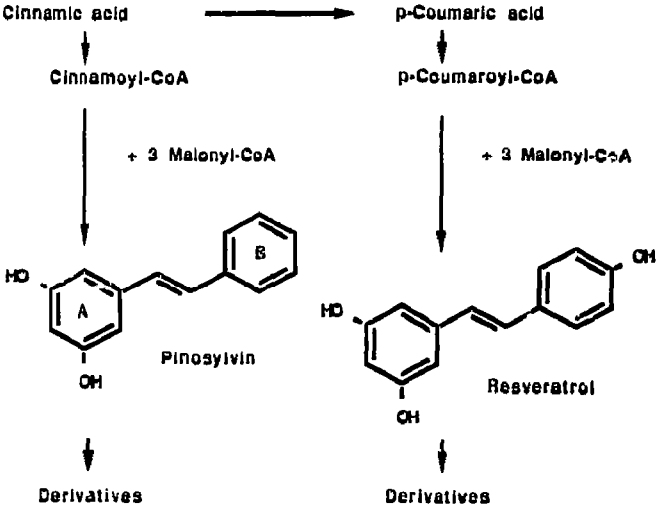

Fig. 1. Two different pathways leading to stilbene derivatives. The metabolic sequences corroborated by work with different plants include steps catalyzed by two distinct stilbene synthases. The structure of the pinosylvin-forming enzyme is the subject of this paper.

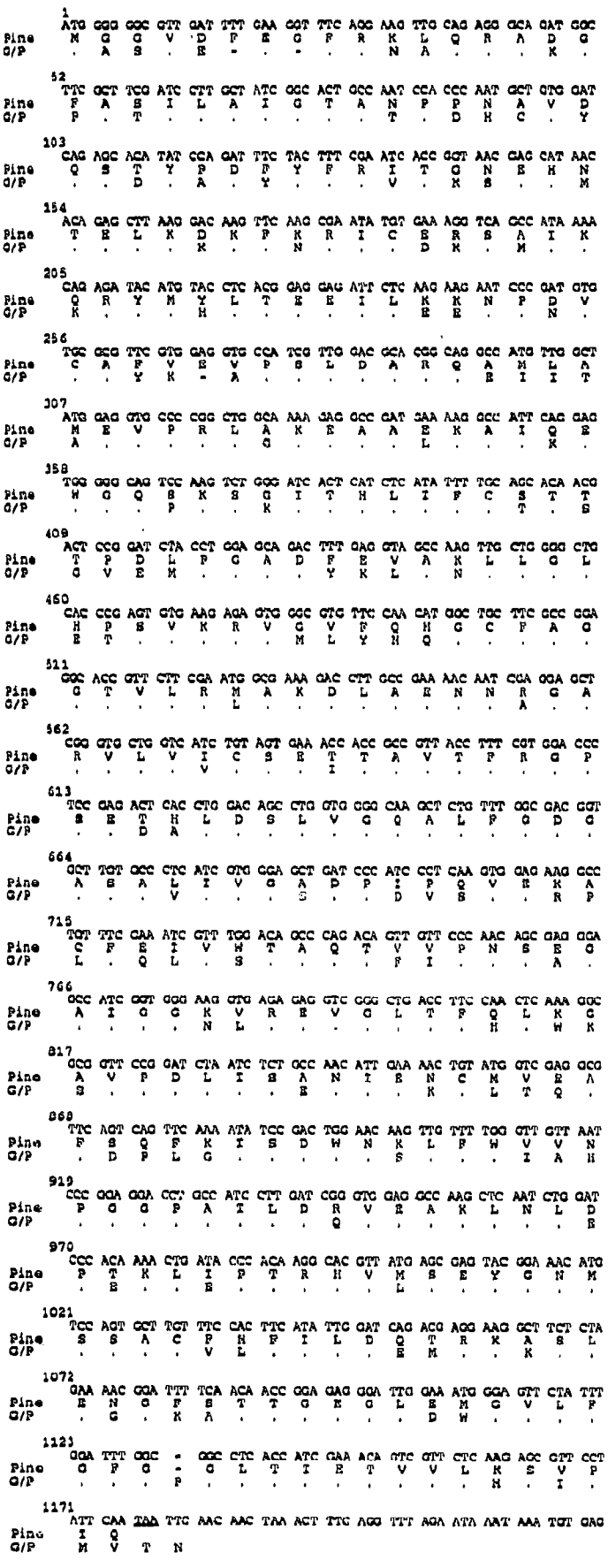

Fig 2. cDNA sequence of pine stilbene synthase and the amino acid sequence deduced. For comparison, an approximate consensus sequence for resveratrol-forming stilbene synthases from grapevine and peanut is underneath (denoted as G/P). The numbering of nucleotides refers to the sequence in pSP-54.

A cDNA library in *E. coli* NM522 (Stratagene) using the vector pT7T3-18U was constructed following the supplier's recommendations (Pharmacia). Positive clones were identified by screening with cDNA fragments derived from various parts of grapevine stilbene synthase cDNA [12]. Subsequently, the sequence of both DNA strands was determined by the dideoxy chain termination method using synthetic oligonucleotide primers which were 0.3 kb apart.

Treatment on 3-week-old pine seedlings grown in continuous light was performed with conidia suspensions (10^4 spores/ml) at 20°C and 90% humidity for the time indicated [6]. Total RNA was isolated as described above. For Northern blot analysis, 20 μg of total RNAs were denatured at 50°C in the presence of glyoxal and separated electrophoretically in 1.5% agarose gel. After blotting onto Hybond-N filters (Amersham), prehybridization and hybridization with the insert of pSP-54 was performed as recommended by the manufacturer. Hybridization was done for 16 h at 37°C.

The mRNA activity was assayed by translation in vitro. Total RNA (5 μg) was translated in a reticulocyte lysate (20 μl) in the presence of [^{35}S]L-methionine. From the translation mixture, stilbene synthase protein was isolated by immunoabsorption of the antigen–antibody complex on protein-A Sepharose [11]. Subsequently, the products were analyzed on SDS-polyacrylamide gel electrophoresis and fluorography. In parallel, the enzyme activities of stilbene synthase, chalcone synthase and L-phenylalanine ammonia-lyase were monitored during the period of fungal treatment. The determination of enzyme activities was as described earlier [6,11].

3. RESULTS

3.1. *Isolation and characterization of clone pSP-54 containing full-length stilbene synthase cDNA*

A plasmid (pT7T3-18U) cDNA library constructed from poly(A)$^+$RNA from fungus-challenged pine seedlings was screened with various cDNA fragments encoding grapevine stilbene synthases. By screening of approximately 7×10^5 clones, we obtained 28 positive clones with inserts of 0.4–2.0 kb. They were further analyzed by restriction enzyme mapping and sequencing. The main portion of the nucleotide sequence and the derived amino acid sequence of the pSP-54 cDNA are shown in Fig. 2. The sequence presents a 1176 bp open reading frame which encodes a peptide with 392 amino acid residues. The molecular mass of the peptide was calculated to be 42.6 kDa which closely corresponds with the 43 kDa described earlier for the resveratrol forming stilbene synthases of grapevine. On the basis of comparisons with resveratrol-forming stilbene synthases and due to the fact that several stop codons in frame are upstream of the open reading frame, we conclude that the insert of pSP-54 encodes a full-length stilbene synthase cDNA.

Inspection of the critical amino acid residues between positions 212–218 shows that an enzyme of the stilbene

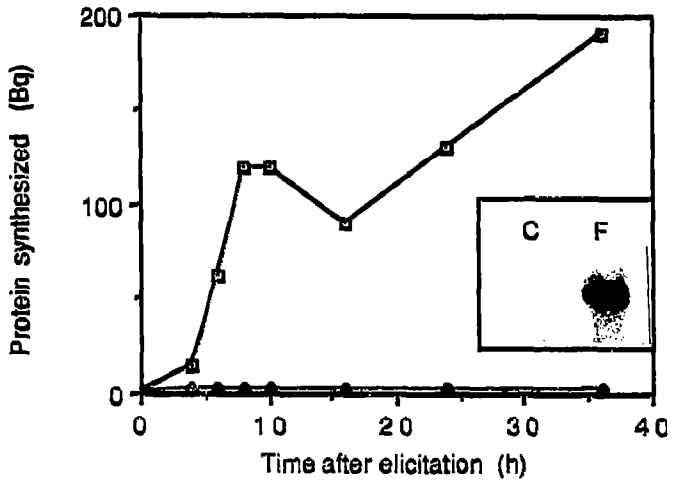

Fig. 3. Time course for the induction of extractable stilbene synthase mRNA activity. The mRNA was assayed by translation in vitro and quantitation of immunoabsorbed protein. (□-□) sample from seedlings treated with fungus; (◆-◆) untreated plants. Insert: Northern hybridization analysis of the mRNAs encoding pine stilbene synthase in needles from control (C) and fungus-treated (F) seedlings.

synthase type and not a chalcone synthase was cloned in pSP-54.

3.2. *Northern blot analysis of RNA from treated and untreated pine seedlings*

According to the electrophoretic analysis of poly(A)$^+$RNA from induced seedlings and hybridization with pSP-54 cDNA on Northern blots a size of 1.6 kb was determined for the pine stilbene synthase mRNA. This value agrees with a coding region of 1.2 kb and 0.1 kb 5′ and 0.3 kb 3′ non-coding region. A time course experiment was performed using fungus-treated seedlings and water treated controls to investigate the onset of stilbene synthase gene expression. This kind of study was paralleled by examination of both the in vitro testable mRNA activity governing the translation of stilbene synthase and the protein synthesis monitored by in vivo labelling. Fig. 3 provides both the analysis of mRNA activity and a representative example of gene expression as obtained by Northern blot analysis of total RNA. The samples of seedlings were taken at different times after onset of treatment with fungus.

Enzyme activity was induced 70-fold within 10 h of exposure to *Botrytis cinerea* conidia (data not shown). Stilbene synthase mRNA increased substantially within 8 h after induction (Fig. 3; insert). The needles contained a constant very low level of stilbene synthase mRNA throughout the time course. This level was virtually undetectable on Northern blots but within the range of detection if the translation activity of the RNA isolated was determined in vitro. By means of immunoabsorption, gel electrophoresis and fluorography, the level of mRNA coding for stilbene synthase was estimated (Fig. 3).

4. DISCUSSION

4.1. *Comparison of the deduced amino acid sequence with the structure of other stilbene synthases*

The structure of pinosylvin synthase for the first time allows a comparison between enzymes converting phenolic or unsubstituted aromatic substrates. All stilbene synthases and chalcone synthases described act on *p*-coumaroyl-CoA. The most remarkable properties of pinosylvin synthase are, besides the homologies to resveratrol synthases and chalcone synthases, the insertions of 3 amino acid residues in the N-terminal part. The insertion of a glutamyl residue at position 90 and the changes in charged residues in the region 81–89 may reflect the fact that pinosylvin synthase acts on a non-hydroxylated substrate and should, therefore, lack interactions to the dipol of a phenolic OH-group.

4.2. *Pine stilbene synthase cDNA as tool to detect environmental stress*

As the pinosylvin-forming stilbene synthase cDNA differs in substantial parts from the cDNA sequence of the known resveratrol-forming stilbenen synthases [12–14] it was important to obtain a reliable tool to analyze the gene activation responsible for the stilbene formation in pine species. Low concentrations of fungal spores, during states of high humidity, substantially activate the expression of stilbene synthase. The quick response of young seedlings to fungal attack seems to be an important means for defence [7,15]. In addition, even slightly enhanced concentrations of ozone cause a substantial increase of stilbene synthase activity [16]. In this respect, the level of pinosylvin synthase mRNA is an excellent indicator of environmental stress. Preliminary experiments [17] provided hints that attacks by insects lead to a systemic response in pine plants. Furthermore, it was found that wood impregnated with 0.01% of pinosylvin monomethyl ether was not eaten by the dry-wood termite *Cryptotermes brevis*. These data stress the correlation between defined ecological situations and the expression of pinosylvin-forming stilbene synthase.

Acknowledgements: This investigation was supported by the Deutsche Forschungsgemeinschaft (SFB 305) and the Fonds der Chemischen Industrie.

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