

## CHAPTER 3:

### *A. thaliana* does not encode stilbene synthase.

#### ABSTRACT:

The molecular architecture of an enzyme complex is postulated to facilitate the channeling of intermediates and partitioning of metabolites among competing biosynthetic pathways. In an attempt to test this hypothesis we have developed a screen for genes encoding the enzyme stilbene synthase (STS). This enzyme competes with chalcone synthase (CHS) for coumaroyl-CoA and malonyl-CoA and could be useful in domain-swapping experiments with CHS to determine how enzyme-enzyme interactions affect the partitioning of substrates at this biochemical branch point. We report here the use of a PCR assay designed to detect and amplify novel loci encoding STS. This assay was used to amplify the previously-identified STS gene from *Arachis hypogaea* (peanut) and was shown to be able to discriminate between STS and CHS sequences in this plant species. The results of this screen also indicate that *A. thaliana* does not contain an STS locus.

## INTRODUCTION:

Living cells commonly use simple molecules to manufacture complex derivatives. To do this cells must control how much of a common substrate is consumed at biosynthetic branch points in the manufacture of various products. One hypothesis to explain how cells control the specificity of the end products produced is that the kinetic values of competing enzymes at branch points determine the relative amounts of products synthesized (Metzler, 1977). The more efficient the catalyst, the more substrate is consumed by that metabolic branch. The relative amounts of end products produced by branch pathways are thereby determined by the affinity of competing enzymes for common substrates. An alternative explanation is that soluble enzymes form complexes that direct intermediates toward the manufacture of specific derivatives. In this model the identity and amount of specific end products is determined by the combination of enzymes assembled into the complex and the number of different complexes assembled. One way to distinguish these possibilities is to perform domain-swapping experiments with competing enzymes to examine the effects of enzyme-enzyme interaction on end product synthesis.

Higher plants are characterized by unique metabolic systems that convert the products of central metabolism into a wide variety of so-called secondary products (Hahlbrock, 1989). Secondary metabolism has been extensively studied in many plants due to the involvement of these compounds in a variety of essential functions (Hahlbrock & Scheel, 1989; Shirley, 1996). Several secondary pathways, including flavonoid and stilbenoid biosynthesis, compete for substrates derived from phenylalanine (Figure 1) (Stafford, 1990). Flavonoids are synthesized by most plants but stilbenoids have only been identified in a much more limited, but diverse group of plant species, including *Pinus sylvestris* (Scots pine), *Vitis vinifera* (grape), and *Arachis hypogaea* (peanut) (Langcake, 1976; Gorham, 1980; Stein and Blaich, 1985; Pont and Pezer, 1990). Stilbenoids are a class of aromatic compounds that have antifungal properties and are believed to serve as defense molecules (Gorham, 1980).

Stilbene synthase (STS) and chalcone synthase (CHS) catalyze the first committed steps in stilbenoid and flavonoid synthesis, respectively (Schöppner and Kindl, 1984) (Figure 1). These enzymes catalyze virtually identical condensations, except that STS catalyzes an additional decarboxylation (Schöppner & Kindl, 1984). CHS and STS also share extensive conservation at the amino acid level (Schröder et al., 1988). These genes are believed to have arisen from duplication and modification of a single ancestral gene (Schröder and Schröder, 1990; Tropf et al., 1994). We hypothesize that, although CHS and STS share considerable amino acid sequence homology, the functional divergence of these two enzymes makes it unlikely that STS can interact with chalcone isomerase (CHI), the second enzyme in the flavonoid pathway. The similarities between STS and CHS make these enzymes good candidates for domain-swapping experiments in order to define possible protein interaction domains.

Here we describe the use of a PCR approach for amplifying STS sequences from genomic DNA. This technique uses degenerate primers to amplify all possible nucleotide sequences that encode amino acid residues conserved between known CHS and STS enzymes. STS-encoding PCR products are then selectively re-amplified from this pool using internal primers that anneal to nucleotide sequences encoding amino acid residues conserved specifically in STS. We describe the use of this system to clone a genomic DNA fragment encoding the *A. hypogaea* STS gene and to screen the *A. thaliana* genome for STS-encoding sequences.

## **MATERIALS and METHODS:**

### **Plant Material and Growth Conditions.**

*Arabidopsis thaliana* ecotype Columbia and *A. hypogaea* cultivar NC7 were used in these studies. Approximately 1000 (25 mg) wild-type *A. thaliana* seeds and 100 *A. hypogaea* seeds were stratified on Murashige and Skoog-sucrose medium in the dark at 4°C for four days (Kubasek et al., 1992). Seeds were transferred to soil and germination induced with constant light at 22°C. Plants were grown in soil at 22°C under a 16-hr-light / 8-hr-dark cycle for 3-weeks. Whole plants were harvested and stored at -70°C.

### **DNA isolation.**

*A. thaliana* genomic DNA was isolated according to Watson and Thompson (1986). These samples were further purified through a CsCl equilibrium centrifugation gradient (Maniatis et al., 1982) and stored in 10 mM Tris pH 7.2, 1 mM EDTA at 4 °C. *A. hypogaea* genomic DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) technique. Ten grams of *A. hypogaea* tissue was homogenized with 35 ml of extraction buffer (0.1M Tris pH 7.5, 0.5 M EDTA, 20 mM Na-bisulfate, 6.4 % sorbitol) and filtered through 2 layers of cheesecloth and 1 layer of miracloth. Filtrate was centrifuged at 1200 x g for 15 min at 4 °C. The pellet was resuspended in 5 ml of extraction buffer then 5 ml of nuclei lysis buffer (0.2 M Tris pH 7.5, 0.05 EDTA, 2 M NaCl, 2 % CTAB, and 1ml of 10 % sarcosyl) were added. The sample was mixed gently and incubated at 60 °C for 1 hour. Fifteen microliters of chloroform/IAA (24:1) was used to extract the sample once. The aqueous phase was isolated and 1 volume of isopropyl alcohol was used to precipitate high molecular weight DNA. Purified DNA was stored at 4°C.

### **Probe synthesis.**

T3 and T7 primers were used to amplify a CHS cDNA clone in the presence of 1:6 dUTP-digoxigenin:dTTP (Boehringer Mannheim). Reactions contained 0.1 ng pCHS.CR2 (Pelletier and Shirley, 1996) template, 0.2 µM T3 and T7, 0.2 mM each dATP, dCTP, and dGTP, 0.167 µM dTTP, 33 µM dUTP-digoxigenin, 1 X buffer salts, and 2.5 units of *Taq* polymerase in a volume of 100 µl. Reactions were denatured (94°C, 30 s), annealed (46°C, 30 s), and extended (72°C, 120 s) for 50 cycles. The probe was purified from unincorporated nucleotides by Sephadex G-50 (Sigma) gel filtration (Ausubel et al., 1989). Purified probe was quantified according to manufacturer's instructions (Boehringer Mannheim).

### **DNA gel blot analysis.**

Six micrograms of genomic DNA was digested with 40 units of either EcoRI, HindIII, or BglII (Promega, Madison) in 50 µl at 37°C for 18 hours. Digestions were stopped with the addition of EDTA to a final concentration of 1 mM. Samples were stored at 4°C until gel analysis. Two micrograms of digested genomic DNA were loaded per lane. Samples were fractionated through a 0.8 % agarose gel in Tris-borate-EDTA buffer (Maniatis et al., 1982). DNA fragments were blotted from the gel onto 0.2 µm nylon membrane (Boehringer Mannheim) as described in Shirley (1992). DNA was covalently attached to the nylon membrane using a FB-UVXL 1000 cross linker (Fisher). Hybridization was performed as described in Shirley (1992) at 55°C for 16 hours using a probe concentration of 1 ng/ml. Bound probe was detected using alkaline phosphatase-conjugated monoclonal anti-digoxigenin Fab' fragments (Boehringer Mannheim). Alkaline phosphatase activity was detected with X-OMAT film (Kodak) using the chemiluminescent substrate Lumiphos 530 (Boehringer Mannheim) according to the manufacturer's recommendations.

### **PCR assay for STS.**

Degenerate PCR primers were designed to amplify all nucleotide sequences that encoded conserved amino acid residues in the active site and termini of CHS and STS (Figure 2). Primary PCRs were 100 µl. Each contained 100 ng genomic DNA, 1.25 mM dNTP's, 5 µg of each

appropriate degenerate primer, 1.5 mM MgCl<sub>2</sub>, 1 X buffer salts, and 2.5 units *Taq* DNA polymerase (Promega). Reactions were denatured (94°C, 60 s), annealed (58°C, 120 s), and extended (72°C, 180 s) for 50 cycles concluding with an additional 5 min. at 72°C. 10 µl of sample was analysis by agarose gel electrophoresis and DNA was visualized with ethidium bromide staining (Maniatis et al., 1982). The DNA in the remaining 90 µl was purified from unincorporated nucleotides and primers using a Wizard PCR preps column (Promega). One fiftieth of the purified products from each of the first reactions was used as template in a second round of amplification using STS-specific nested primers. These PCRs contained 1.25 mM dNTP's, 5 µg of each primer, 1.5 mM MgCl<sub>2</sub>, 1 X buffer salts, and 2.5 units *Taq* DNA polymerase in 100 µl. Samples were denatured (94°C, 90 s), annealed (58°C, 120 s), and extended (72°C, 180 s) for 50 cycles. Ten microliters of each reaction were fractionated by agarose gel electrophoresis and DNA was visualized by ethidium bromide staining (Maniatis et al., 1982).

#### **Primers used during this study.**

- 1: ayscngaytwytaytymg
- 2: tcngcnavrctytngc
- 3: ccggaattcggnggnacngtn(c/t)tn(c/a)g
- 4: cccaagctna(g/a)nccnggncc(a/g)aanc
- a: ggnacytcnvbnrynad
- b: swrtc nadnksnkyyc
- c: swdatnccnarnggrtc

#### **Restriction enzyme analysis.**

Plasmid DNA was isolated using a modification of the alkaline lysis miniprep method (Birboim and Doly, 1979) in which the DNA and protein precipitate was pelleted at 50,000 rpm in a TLA-100 ultracentrifuge (Beckman, Palo Alto, CA). Ten µl of miniprep plasmid DNA was digested with EcoRI and HindIII (Promega) at 37°C for 4 hours, fractionated on a 1 % agarose gel, and visualized by staining with ethidium bromide (Maniatis et al., 1982).

For the digestion of PCR products the entire sample was incubated with 40 units of either EcoRI or HindIII with 1 X buffer salts in 50 µl at 37°C for 12 hours. One twentieth of each digestion was fractionated on a 1 % agarose gel. DNA was visualized by ethidium bromide staining.

#### **Cloning vector preparation.**

For cloning of PCR products using restriction enzymes, 5 µg pBluescript KS (+) (Stratagene) was digested with EcoRI and HindIII (Promega) at 37°C for 4 hours. Linearized vector was purified using a Wizard PCR preps column and eluted in 50 µl of distilled water. For direct cloning of PCR products 5 µg pBluescript KS (+) was digested with SmaI (Promega) at 37°C for 4 hours. Linearized vector was purified using a Wizard PCR preps column and eluted in 50 µl of distilled water. The vector was then incubated with 0.2 mM dTTP, 1.5 mM MgCl<sub>2</sub>, 1 X buffer salts, and 2.5 units of *Taq* DNA polymerase at 72°C for 4 hours to attach dTMP to the 3' termini of the vector. The vector was then purified using a Wizard PCR preps column and eluted in 50 µl of distilled water.

#### **Cloning and sequence analysis.**

Primers 3 and 4 contain 5' EcoRI and HindIII recognition sites, respectively. Products synthesized using these primers can be cloned by digestion with EcoRI and HindIII and ligation with vector. The products amplified from *A. thaliana* using primers 3 + 4 were fractionated in a 1% low-melt agarose gel in Tris-acetate-EDTA buffer (Maniatis et al., 1982) and the 720 bp and the 650 bp fragments were excised and purified from agarose using Wizard gel purification columns. Purified PCR products were digested with EcoRI and HindIII at 37°C for 4 hours and purified from enzyme mixture using Wizard gel purification columns. One hundred nanograms of EcoRI +

HindIII cut pBluescript KS (+), prepared as described above, was incubated with 20 ng of PCR product inserts with 5 units of T4 DNA ligase (Boehringer Mannheim) in 20  $\mu$ l at 18°C for 18 hours.

Recombinant *Taq* polymerase lacks endogenous 3' to 5' proofreading activity (Mead et al., 1991). As a result products contain a single adenosine at each 3' terminus. The products of selected PCRs were fractionated on a 1% low-melt agarose gel in Tris-acetate-EDTA buffer. DNA fragments were excised from the gel, purified using Wizard DNA preps columns and stored at 4 °C. PCR products were cloned by direct ligation into linearized pBluescript KS(+) containing 3' single thymine termini prepared as described above. One hundred ten nanograms of linear 3'-thymine pBluescript KS(+), 2.5  $\mu$ l purified PCR product, 1 X buffer salts, and 5 units of T4 DNA ligase was incubated in a total volume of 10  $\mu$ l at 18 °C for 16 hours.

Two microliters of each ligation product was used to transform 50  $\mu$ l JM109 *E. coli* using the Gene-Pulser (model #1652076, Bio-Rad). Transformants were recovered on LB medium containing 100  $\mu$ g/ml ampicillin, 0.1  $\mu$ M IPTG and 0.1 mg 1-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside.

Plasmid DNA for sequence analysis was isolated as described above. Sequencing was performed as described in Shirley (1992).

## RESULTS:

### Genomic DNA gel blot analysis.

*A. thaliana* has been previously shown to have a single gene encoding CHS (Feinbaum and Ausubel, 1988). As a first approach to determine whether *A. thaliana* encodes STS we hybridized a CHS cDNA probe to a DNA gel blot of digested *A. thaliana* genomic DNA under low stringency conditions (Figure 3). Restriction fragments corresponding to the known CHS genomic locus as well as several other cross-hybridizing fragments were identified in this experiment. This suggests that other sequences in the genome are partially complementary to the CHS probe. STS may be coded for by some of these cross-hybridizing sequences.

### PCR assay for stilbene synthase.

A PCR-based assay was developed in an effort to determine if *A. thaliana* contained an STS gene. *A. thaliana* and *A. hypogaea* genomic DNA was used as templates to amplify sequences encoding amino acid residues conserved between known STS and CHS proteins (Figure 4). An *A. thaliana* CHS genomic clone was used as a positive control. The molecular weight of products amplified from the *A. hypogaea* genomic template were consistent with the known sequence for the STS locus (Figure 5). Products of the expected size were also amplified from the *A. thaliana* CHS genomic clone using either primer pairs 1 + 2 or 3 + 4. Products of identical size were also amplified from *A. thaliana* genomic DNA, consistent with the known single CHS gene. Two potential STS candidates of 850 bp and 720 bp were amplified from the *A. thaliana* genomic template using primer pairs 1 + 2 and 3 + 4, respectively. Other PCR products were obtained from *A. thaliana* using primers 3 and 4, but these fragments were less than 600 bp in size and thus too small to encode STS. These products were excluded from further study.

To isolate STS-encoding DNA fragments, the first round of products were purified and used as templates in a second round of PCR using the STS-specific primers a, b, and c together with primers 1 or 3. This second round of PCR produced products consistent with the size of the known STS gene from *A. hypogaea* (Figure 5). In contrast, PCR products were not generated, when the products of the CHS genomic clone were used as templates in the second round of nested PCR. However, products were produced from the *A. thaliana* genomic template using primers 1 and a. Although products were also amplified from *A. thaliana* using primer pairs 3 + b and 3 + c these are not expected to be identical in size and thus likely represent artifacts.

### Analysis of the STS PCR products.

To determine whether the amplified DNA fragments coded for STS, the PCR products were subcloned into pBluescript KS (+). Digestion of the 720 bp PCR product amplified from *A. thaliana* using primers 3 and 4 revealed an internal EcoRI site (Figure 6). This shows that the 720 bp product was not amplified from the CHS locus because there is no internal EcoRI site at this location in the CHS gene (Feinbaum & Ausubel, 1988). PCR products corresponding to potential STS coding fragments amplified from *A. thaliana* using primers 1 and a (Figure 5), and the 350 bp fragment resulting from the EcoRI and HindIII digestion of the 720 bp PCR product were cloned. The PCR products amplified from *A. hypogaea*, using primers 1 and a, 3 and b, and 3 and c, were consistent with the size of the published STS locus (Figure 5).

This PCR assay specifically amplified a segment of the STS gene from *A. hypogaea*. The sequences of the three PCR products isolated from *A. hypogaea* were identical to the published STS gene. Moreover the orientation of the cloned DNA insert was consistent with that predicted by primer design. The sequence of the 650 bp fragment amplified from *A. thaliana* using primers 3 + 4 (Figure 5) was also identical to the published *A. thaliana* CHS sequence and consistent with that predicted by primer design.

The sequence of the 3 potential STS candidates amplified from *A. thaliana* revealed that none encoded STS. The sequence of these clones did not correspond to any cloned gene in the

Genbank database. These sequences might correspond to several possible identities predicted by the Genbank software.

## DISCUSSION:

Southern blot analysis indicated that the *A. thaliana* genome contains sequences related to CHS that are potential candidates for a STS gene. The identification of the expected genomic restriction fragments encoding CHS indicated that the cDNA probe can detect genomic CHS sequences under the conditions used in these experiments. The hybridization of this probe to numerous additional fragments in the genome indicated that *A. thaliana* contains DNA sequences that share homology with the CHS coding region.

Primer pairs 1 + 2 and 3 + 4 amplified products consistent with the size of the known *A. thaliana* CHS loci from both genomic DNA and the cloned CHS gene. This shows that these degenerate primers can detect and selectively amplify CHS-coding sequences from templates as complex as genomic DNA. Moreover, these primer pairs also amplified products consistent with the size of the published STS genomic locus from the *A. hypogaea* template. This suggests that STS sequences can also be selectively amplified using these same primer combinations.

The second round of PCR produced products of the predicted size for the known STS locus from *A. hypogaea*. This result indicates that these degenerate nested primers can specifically amplify STS sequences from a complex template. Because these primers did not amplify sequences of the *A. thaliana* CHS genomic clone, this method can discriminate between CHS and STS. Therefore, if *A. thaliana* does encode an STS locus we predict that this technique would identify it.

The sequence of the cloned PCR products amplified during the course of this study confirms that this technique is sensitive enough to detect a single-copy STS locus in the genome of *A. hypogaea*. None of the likely STS DNA fragments amplified from *A. thaliana* encode STS. However, based on these results we conclude that *A. thaliana* probably does not contain an STS gene. Fragments of *A. thaliana* genomic DNA that hybridize with the CHS cDNA probe under low-stringency conditions represent other related sequences that are not STS. In support of this conclusion, to date no clear STS clone has been identified in the *A. thaliana* expressed sequence tag (EST) database.

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**Figure 1. Plant secondary metabolism.**

Many plant secondary metabolites are derivatives of the aromatic amino acid phenylalanine via general phenylpropanoid metabolism. CHS and STS catalyze the first committed steps in flavonoid and stilbenoid biosynthesis, respectively. These enzymes compete for 4-coumaroyl-CoA and malonyl-CoA. Phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl-CoA ligase (4CL) catalyze the reactions of the general phenylpropanoid pathway.

**Figure 2. Alignment of known CHS and STS proteins.**

Boxes enclose conserved amino acid sequences that were used to design degenerate primers. Large boxes enclose sequences conserved between CHS and STS. Small boxes enclose sequences conserved exclusively in STS. Arrows indicate the 5' to 3' direction of the degenerate primer. Numbers indicate specific primers used in the primary PCR. Letters indicate internal primers used during the secondary PCR. The star designates the active site cysteine in these enzymes.

**Figure 3. Low-stringency DNA gel blot analysis of *A. thaliana* genomic DNA.**

**A.** Map of the *A. thaliana* CHS genomic locus. The transcribed region is indicated by the arrow. Numbers indicate the position in bp relative to the start of transcription (position 1). The black box represents the coding region. Restriction enzymes recognition sites: H, HindIII; E, EcoRI; B, BglII.

**B.** Low-stringency gel blot of *A. thaliana* genomic DNA. Arrows indicate bands that correspond to fragments the size of the published CHS genomic locus. Probe was hybridized at 55 °C under low-salt phosphate buffer conditions.

**Figure 4. Schematic of PCR assay for STS.**

Diagram of the PCR assay for the identification of STS coding sequences. Hatched boxes represent coding region sequences. Arrows represent primers. During the primary round of PCR, primer combinations 1 + 2 and 3 + 4 amplify all templates that encode amino acid residues conserved between CHS and STS. This is because there are few STS protein sequences in the data base, and annealing to these highly conserved sequences should increase the chances of amplifying the target. This pool of products is then used as template to amplify sequences encoding amino acid residues conserved exclusively to STS using internal primers, a, b, or c in combination with primers 1 or 3. Products of these nested PCRs can then be directly cloned and sequenced.

**Figure 5. PCR assay for STS.**

**A.** Resolution of primary PCR products. Lane 1 contains the products of the primary PCRs using primers 1 + 2. Lane 2 contain the products of the primary PCRs using primers 3 + 4. The template DNA for each pair of reactions is indicated at the top of the figure. Size is indicated in kb. PCR products amplified from *A. thaliana* and subsequently cloned (720 bp + 650 bp) are indicated by the arrows.

**B.** Resolution of secondary PCR products. The templates used in these reactions were the products of the primary reactions. The templates used are indicated at the top of the figure (CHS refers to the genomic clone). The internal primers used for these reactions are indicated at the top of each lane. Size is indicated in kb. PCR products amplified from *A. hypogaea* and *A. thaliana* and subsequently cloned are indicated by the arrows.

**Figure 6. Digestion of purified PCR products.**

Agarose gel resolution of digested PCR products. Lane 1 contains the 340 bp EcoRI / HindIII fragment clone digested with EcoRI and HindIII. Lane 2 contains the 720 bp and 650 bp PCR products from the *A. thaliana* primary PCRs digested with EcoRI and HindIII. Lane 3 contains the 650 bp fragment amplified from *A. thaliana* digested with EcoRI and HindIII.