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A chalcone synthase/stilbene synthase DNA probe for conifers

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Abstract A probe for chalcone synthase (CHS) was generated by PCR using chalcone synthase conserved sequences. The cloned PCR product has high similarity to both chalcone synthase and stilbene synthase sequences. The probe was used to examine the organization of chalcone synthase and stilbene synthase genes in *Abies procera*, *Pinus lambertiana*, *P. monticola*, *Picea glauca*, *P. sitchensis*, *Pseudostuga menziesii*, *Taxus brevifolia*, and *Thuja plicata*. A large number of hybridizing bands were found in all species except *T. plicata* which did not cross hybridize. The hybridization patterns are highly polymorphic between the species and are also polymorphic within several of them.

Key words *Pinus monticola* D. Don · Chalcone synthase · Stilbene synthase · DNA probes · Conifers

Introduction

Chalcone synthase (CHS) and stilbene synthase (STS) are closely related polyketide synthases which are key enzymes in the biosynthesis of flavonoids and stilbenes, repectively. Chalcone synthase catalyzes the first and key regulatory step in flavonoid biosynthesis in plants. Flavanoids constitute a diverse group of plant secondary metabolites, derived from the phenylpropanoid pathway, which are essential for a variety of biochemical processes. Much work has focused on the role of CHS in the nodulation, pigmentation, UV-protection, and defense responses of angiosperms (e.g., Firmin et al. 1986; Hahlbrock and Scheel 1989; Dixon and Harrison 1990). CHS is encoded by a single gene per haploid genome in some angiosperms (e.g., parsley, Hahlbrock and Scheel 1989; *Arabidopsis thaliana*, Feinbaum and Ausubel 1988; and snapdragon, Sommer and Saedler 1986), but as a small multigene family in beans (Ryder et al. 1987), soybean (Wingender et al. 1989), chickpea (Daniel and Barz 1990), pea (Harker et al. 1990), mustard (Batschauer et al. 1991), *Petunia hybrida* (Koes et al. 1989), grape (Sparvoli et al. 1994), and subterranean clover (Arioli et al. 1994). Stilbene synthases catalyze the reaction leading to the formation of the stilbene backbone. Stilbene phytoalexins possess antifungal and antibacterial properties and contribute to plant defense reactions (Hart 1981; Kemp and Burden 1986). Stilbene synthases from angiosperm crop plants are rare but the gene has been cloned from peanut (Schroder et al. 1988) and grape (Melchior and Kindl 1991; Sparvoli et al. 1994). STS is encoded by a small gene family in peanut (Schroder et al. 1988) but by a large gene family (15–20 copies per haploid genome) in grape (Sparvoli et al. 1994). There is evidence that stilbene synthases have independently evolved from chalcone synthases several times (Tropf et al. 1994).

Recently, these enzymes were reported from gymnosperms. Fliegmann et al. (1992) cloned and sequenced a CHS and a STS gene from *Pinus sylvestris* L. Raiber et al. (1995) screened a *Pinus strobus* L. cDNA library with the *P. sylvestris* STS probe and isolated eight clones representing two closely related STS genes. Rutledge and Xue (1993, personal communication), using the polymerase chain reaction, found that there are many CHS genes in the genome of *Picea mariana* (Mill.) BSP.

During the course of developing defense-response-related DNA probes to study defense reactions of *Pinus monticola* D. Don, a probe for chalcone and stilbene synthase sequences was generated using the polymerase chain reaction with degenerate oligonucleotides targeted to highly conserved regions within the chalcone synthase gene. The purpose of the present study was to investigate the organization of these genes in several conifer species.

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Materials and methods

Plant materials

Six native and two non-native conifer species from British Columbia were included in this study. These were: *P. monticola*, *Picea glauca* (Moench) Voss, *Picea sitchensis* (Bong.) Carr., *Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*, *Taxus brevifolia* Nutt., *Thuja plicata* D. Don, *Pinus lambertiana* Doug., and *Abies procera* Rehder. Branches were collected from two trees of each species growing at the Pacific Forestry Centre, Victoria, B.C., Canada. The probe was constructed using DNA from *P. monticola* needles collected in the Victoria watershed on southern Vancouver Island, British Columbia.

Probe construction and sequencing

Total DNA was isolated from 5–10 g of needles from *P. monticola* using the method described by White (1990) without sucrose gradient-separation of chloroplasts. Fifty to one-hundred nanograms of the extracted DNA was used as template in an amplification reaction employing CHS consensus-based primers [CS1: CGGAATTCACIACI T/A C/G IGGIGTIGA T/C ATG, and CS4: GCTCTAGAGCICC G/A TCICC G/A AAIA G/A IGC T/C TG (kindly supplied by Dr. R. Rutledge and Dr. B. Xue, CFS NRC, Petawawa, Ontario)]. The template DNA was denatured at 95°C for 10 min prior to amplification. The DNA was amplified in a 25-μl reaction mixture containing the template DNA, 250 ng of each primer, 2.5 units of Promega *Taq* DNA polymerase, 0.25 mM each of dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton-X-100, 1% DMSO, 2 mM $MgCl_2$, and than overlaid with mineral oil. Amplification was performed in a Perkin-Elmer/Cetus thermal cycler programmed for 24 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by one cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 5 min. The 265-bp product (PwCHS1) was cloned into the phagemid vector pTZ18 and its identity confirmed by automated DNA sequencing performed at the Biotechnology Laboratory, University of British Columbia.

DNA preparation and RFLP analysis

For Southern analysis total DNA was isolated from 5–10 g of needles collected from container-grown seedlings using the same method as described above. Five to ten micrograms of the total DNA extracted was digested with *Hin*dIII, *Bam*HI, AvaII, and *Eco*RI and resolved in 0.8% agarose (Boehringer Mannheim) or 0.6% agarose (BioRad)/0.2% Synergel (Diversified Biotech) gels. DNA fragments were transferred to a Hybond N (Amersham) nylon membrane after depurination, denaturation and neutralization. After an overnight upwards transfer with 10 × SSC the blots were oven dried at 80°C for about 10 min and UV fixed.

The 265-bp PwCHS1 insert was cut from the cloning vector and gel-purified. The insert was collected using glassmilk and then labelled with DIG-dUTP using a PCR protocol with a DIG-dUTP/dTTP ratio of 1/10 as described by Panaud et al. (1993) with some modifications. Briefly, 0.5 ng of heat-denatured PwCHS1 was amplified in a 50-μl reaction mixture containing 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25°C), 0.01% Tween-20, 2.5 mM $MgCl_2$, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dCTP, 0.09 mM dTTP, 0.01 mM DIG-dUTP, 500 ng each of CS1 and CS4, and 1.25 U of ID-POL (ID Labs Inc.). The thermocycle profile was: 29 × (95°C for 1 min, 55°C for 1 min, 72°C for 2 min); 1 × (95°C for 1 min, 55°C for 1 min, 72°C for 4 min). The DIG-labelled PCR product was purified using the QIA Quick-spin PCR kit (Qiagen).

Blots were pre-hybridized for at least 2 h at 42°C in 20 ml of hybridization solution [5 × SSC, 2% blocking agent (Boehringer Mannheim), 0.1% SLS, 0.02% SDS and 50% formamide] in Falcon tubes in a Robbins Scientific hybridization oven. For hybridization the pre-hybridization solution was removed and replaced with 3 ml of hybridization solution containing 10 μg of DIG-labelled PwCHS1. Hybridization was carried out overnight at 42°C. The blots were then washed as follows: 2 × (2 × SSC/0.1% SDS RT 5 min), 2 × (2 × SSC/0.1% SDS 55°C 1 h). *P. monticola* blots were given an additonal wash of 1 × (0.5 × SSC/0.1% SDS 65°C 10 min). Chemiluminescent detections were carried out essentially as recommended by Boehringer Mannheim for their DIG system except that the blocking step was performed for at least 1 h and the washes in washing buffer were lengthened to 30 min each. AMPPD [(3-2′-spiro-adamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt], a chemiluminescent substrate for alkaline phosphatase, was applied to the blots and the blots were exposed to X-ray film (XOMAT-AR) for about 65 h.

Results

The nucleotide sequence of the 265-bp CHS/STS probe generated using PCR (PwCHS1) is presented, and aligned with the homologous conserved regions of other published sequences in Fig. 1. PwCHS1 matches very closely (90%) the sequence for the same region from the *P. sylvestris* CHS gene (position 557–797). Matches to angiosperms are not as close though the area is conserved (77–70%). Based on the whole-gene data published for the *P. sylvestris* CHS gene, and assuming that the sequence length found in *P. monticola* would be similar, then PwCHS1 represents 14% of the complete chalcone synthase gene in *P. monticola*. High homology scores were also obtained for the nucleotide alignments of PwCHS1 with *P. strobus* (position 448–682) and *P. sylvestris* (position 414–649) stilbene synthase, 77 and 76%, respectively. PwCHS1 cross-hybridizes (data not shown) with the cloned *P. sylvestris* chalcone synthase and stilbene synthase obtained from Dr. J. Schroder (Fliegmann et al. 1992). An amino-acid alignment revealed a 95% match between the *P. monticola* sequence and the *P. sylvestris* chalcone synthase sequence. Other amino-acid sequence alignments to angiosperm chalcone synthases gave matches of 91–85%. The amino-acid identity between the *P. monticola* CHS/STS sequence and the *P. strobus* and *P. sylvestris* stilbene synthases was lower at 73%.

We investigated the nature of the CHS/STS genes first in *P. monticola* by Southern-blot analysis (Fig. 2). In each *Bam*HI digest (lanes 1–8) 9–11 bands are visible. *Hin*dIII digests of *P. monticola* DNA reveal 18 bands when probed with PwCHS1 (data not shown). Polymorphisms were found between individual *P. monticola* trees using *Bam*HI (compare Fig. 2 lanes 4 and 5 between 2.3 and 6 kb), and also with *Eco*RI (data not shown). *Hin*dIII did not show polymorphisms in *P. monticola* with PwCHS1 in this study. Southern blots were also prepared from total DNA of other conifer species and these blots were probed with PwCHS1 (Fig. 3). Bands were present for all species studied except *T. plicata*. With *Ava*II-digested DNA (Fig. 3a, lanes 1–8) ten bands were detected with *P. menziessii* DNA, none with *T. plicata*, ten with *P. sitchensis*, and ten bands with *A. procera*. Seventeen bands were observed with *Ava*II-digested *P. monticola*, 13 with *P. glauca* DNA, six with *P. lambertiana*, and seven with *Taxus brevifolia* (data not shown). With *Hin*dIII-digested DNA (Fig. 3b, lanes 1–8) there were 18 bands with *P. monticola*, 12 with *P. lambertiana*,

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PWCHS1  ACGACGTCGGGGGTGGACATGCCCGGAGCTGACTATCAGCTAACAAAGCTTCTAGGTCTCCGCTCCAGCGTTAACAGAGTGATGATGT
PSCHS   ---------..A..T........A..C..C...........G...........T..C......C..........G.....C.......
PSSTS   ---------------..TT.A........C....T.G..G..G.C...T.G..G..G..G.A.C....T..G..G......GGCG...
PSYSTS  --------------...TC.A..T.....A....T.G..G..G.C...T.G..G..G..G.A.C.G..T..G..G......GGCG...
PHCHS   ---------..T..........T..GTG.........A..C..T.....A..T..G..T..TC.ATCG..C..G..GC.C.......
PSCHS1  ---------..T..A..............C..T..C..A..C..C..A..CT.......T...C.ATAT..G..G...TAC.......

PWCHS1  ACCAACAGGGCTGCTTCGCTGGAGGCACCGTCCTCCGCGTGGCTAAAGATCTGGCAGAGAACAATCGGGGAGCTCGGGTTCTGGTCGT
PSCHS   .........T...................T.......................T..........C.................T..
PSSTS   T......T...........C..........T..T...C....G.....C..T..C..A.........A...........G........
PSYSTS  T......T...........C..........T..T..AA....G.....C..T..C..A.........A...........G......A.
PHCHS   .......A..T.....T.....T.....G..T..T..GT.A..C..G..CT....G..A.....CAA...C.....A..C..T..T..
PSCHS1  .......A..A.....T..A..T..G..G..G..T..TT.......G..CT....T........CAAA..C.....T..GT....T..

PWCHS1  CTGCAGCGAAATCACGGCTGTTACCTTCCGTGGCCCCTCCCACACTCACCTCGACAGCATGGTCGACCAAGCCCTTCGGCGACGGCGCT
PSCHS   T.....T........T.....C..A...............G.........................G.......--------------- 90%
PSSTS   G.....T....A...C..C...........C..A......G.G........G..TG..C.A..G.G..T.....--------------- 77%
PSYSTS  ...T..T....C...C..C........T.....A......G.G........G......C....G.GG.......--------------- 76%
PHCHS   T..TTCA........C..G..C...........G..AAATG.T.....TT.G..T..TT.A..T.G.......--------------- 70%
PSCHS1  T..TTCT...G.G..T..A.....A........T..AAG.G.T......T.G.....TC.T..T.GA.......--------------- 70%
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Fig. 1 Alignment of the nucleotide sequence of PwCHS1 and several other plant CHS sequences from the same region of the gene. Only the differences to PwCHS1 are shown in PSCHS, PSSTS, PSYSTS, PH, and PS1; the *dots* indicate sequence identity. *PWCHS1*, *P. monticola* CHS/STS probe; *PSCHS*, *P. sylvestris* chalcone synthase (Fliegmann et al. (1992); *PSSTS*, *P. strobus* stilbene synthase (Raiber et al. 1995); *PSYSTS*, *P. sylvestris* stilbene synthase (gbS50350); *PH*, *Petunia hybrida* chsA (embX14591); *PS1*, *Pisum sativum* PSCHS1 (embX63333)

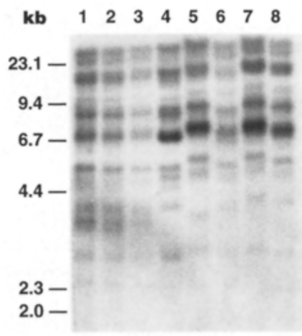

Fig. 2 Southern analysis of *P. monticola* chalcone synthase/stilbene synthase sequences. Total DNA from eight *P. monticola* trees were cut with the restriction endonuclease *Bam*HI (lanes 1–8), and the Southern blot was hybridized with PwCHS1. *Hin*dIII-digested lambda DNA was used for size markers

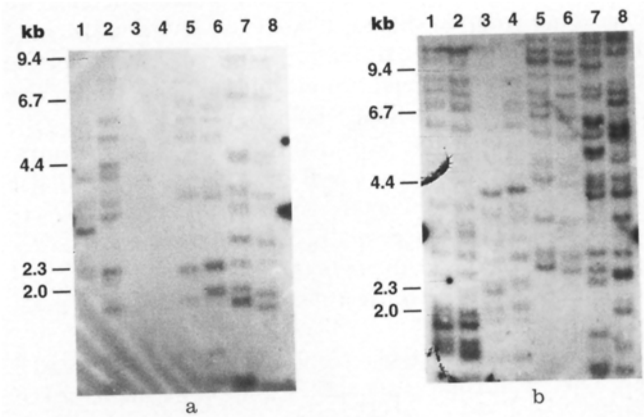

Fig. 3 a, b Southern analysis of several conifers using the PwCHS1 probe. Total DNA from two trees each of eight species were cut with *Ava*II (**a**) and *Hin*dIII (**b**) and the Southern blots were hybridized with PwCHS1. **a** (bands above 9.4 kb not shown) *Lanes 1 and 2*, *P. menziesii*; *lanes 3 and 4*, *T. plicata*; *lanes 5 and 6*, *P. sitchensis*; *lanes 7 and 8*, *A. procera*. **b** (bands below 1 kb not shown) *Lanes 1 and 2*, *P. monticola*; *lanes 3 and 4*, *P. lambertiana*; *lanes 5 and 6*, *T. brevifolia*; and *lanes 7 and 8*, *P. glauca*. *Hin*dIII-digested lambda DNA was used for size markers

14 with *T. brevifolia*, and 19–20 with *P. glauca*. With *Hin*dIII-digested *P. menziessii* DNA, 13 bands were detected with PwCHS1, none with *T. plicata*, 11 with *P. sitchensis*, and nine with *A. procera* (data not shown). Each species displayed a unique CHS/STS fingerprint. Polymorphisms were found between individuals of *P. glauca* using *Hin*dIII (Fig. 3b, lanes 7 and 8).

Discussion

In preliminary trials, a 800 bp *Hin*dIII fragment of the heterologous CHS probe from *P. sylvestris* did not hybridize to *P. monticola* Southern blots. Consequently a homologous probe directed to a highly conserved region of the gene was sought.

The probe developed in this study displays a high degree of homology with both chalcone synthase and stilbene synthase sequences. Since it hybridizes to both chalcone synthase and stilbene synthase clones isolated from *P. syl-*

vestris and has a similar sequence, PwCHS1 should detect both chalcone synthase and stilbene synthase sequences if both genes are present in the genome. Chalcone synthases occur in many plant species including conifers (Tropf et al. 1994). Stilbene synthases are found in only a few widely unrelated plants but stilbenes are wide-spread in conifers (Raiber et al. 1995). Both CHS and STS have been found to occur as multigene families. Multiple hybridizing bands in all those species exhibiting signals in the present study indicate that the CHS and/or STS genes of these species occur as members of gene families. The intensity of the bands in many of the tree species investigated indicates that there are many copies of these genes in some conifer genomes. Further studies are required to differentiate CHS- and STS-specific bands in these species. PwCHS1 could be used to screen cDNA or genomic libraries to isolate full-length CHS or STS genes. The identity of the gene product could be confirmed by expressing the isolated genes in *E. coli* as has been done in other systems (Schanz et al. 1992).

The multigenic nature of these genes in conifers may mean that only some of them are important to defense. In *Phaseolus vulgaris* different members of the CHS gene family are known to be differentially regulated in response to different environmental stimuli (Ryder et al. 1987). The gene dosage of a particular member of the CHS multigene family in soybean may play an important role in determining the plants response to UV-B (Akada et al. 1993). It has yet to be determined whether or not CHS or STS genes in gymnosperms are differentially regulated or if the gene doseage of any particular member of the gene families in gymnosperms plays a specific role in any physiological response.

Our data reveal extensive polymorphism for CHS/STS genes between conifer species. Variation in intensities of hybridizing bands from different species may indicate that sequences of individual CHS/STS family members have diverged or that the gene copy number differs in different species. Chalcone synthase genes have been used to study evolutionary relationships within the plant kingdom (Niesbach-Klosgen et al. 1987). The probe developed in the present study could be used in phylogenetic studies of gymnosperms.

The results also show that there is considerable within-species variation in the chalcone synthase/stilbene synthase families in conifers. Polymorphisms have been observed in *P. monticola* and *P. glauca* even with the very limited sample size employed. This indicates the potential usefulness of the PwCHS1 probe in studies of genetic diversity in these species.

Studies are in progress to determine whether or not selection for resistance to white pine blister rust in *P. monticola* alters the frequency of polymorphisms detected with this probe. Correlation with a resistance phenotype would provide a valuable tool for marker-aided selection in the tree-improvement program for resistance (Meagher et al. 1993).

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References

Akada S, Kung S, Dube SK (1993) Nucleotide sequence of a soybean chalcone synthase gene with a possible role in ultraviolet-B sensitivity, Gmchs6. Plant Physiol 102:699–701

Arioli T, Howles PA, Weinman JJ, Rolfe BG (1994) In *Trifolium subterraneum*, chalcone synthase is encoded by a multigene family. Gene 138:79–86

Batschauer A, Ehmann B, Schafer E (1991) Cloning and characterization of a chalcone synthase gene from mustard and its light-dependent expression. Plant Mol Biol 16:175–185

Daniel S, Barz W (1990) Elicitor-induced metabolic changes in cell cultures of chickpea (*Cicer arietinum* L.) cultivars resistant and susceptible to *Ascochyta rabiei*. Planta 182:279–286

Dixon RA, Harrison MJ (1990) Activation, structure, and organization of genes involved in microbial defense in plants. In: Scandalios JG (ed) Advances in genetics vol 28. Genomic responses to environmental stress. Academic Press, New York, pp 165–234

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol 8:1985–1992

Firmin JL, Wilson KE, Rossen L, Johnston AWB (1986) Flavonoid activation of nodulation genes in *Rhizobium* reversed by other components present in plants. Nature 324:90–92

Fliegmann J, Schroder G, Schanz S, Britsch L, Schroder J (1992) Molecular analysis of chalcone and dihydropinosylvin synthase from Scots pine (*Pinus sylvestris*), and differential regulation of these and related enzyme activities in stressed plants. Plant Mol Biol 18:489–503

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 40:347–369

Harker CL, Ellis NT, Coen ES (1990) Identification and genetic regulation of the chalcone synthase multigene family in pea. Plant Cell 2:185–194

Hart JH (1981) Role of phytostilbenes in decay and disease resistance. Annu Rev Phytopathol 19:437–458

Kemp MS, Burden RS (1986) Phytoalexins and stress metabolites in the sapwood of trees. Phytochemistry 25:1261–1269

Koes RE, Spelt CE, Van den Elzen PJM, Mol JNM (1989) Cloning and molecular characterization of the chalcone synthase multigene family of *Petunia hybrida*. Gene 81:245–257

Meagher MD, Hunt RS, White EE, Ekramoddoullah AK, Jensen GD, Dronzek J (1993) Western white pine improvement program for British Columbia. In: Lavereau J (ed) Proc 24th Meet Can Tree Improvement Assoc, Fredericton, New Brunswick, pp 147–149

Melchior F, Kindl H (1991) Coordinate- and elicitor-dependent expression of stilbene synthase and phenylanine ammonia-lyase genes in *Vitis* cv Optima. Arch Biochem Biophys 288:552–557

Niesbach-Klosgen U, Barzen E, Bernhardt J, Rhode W, Schwarz-Sommer Z, Reif HJ, Wienand U, Saedler H (1987) Chalcone synthase genes in plants: a tool to study evolutionary relationships. J Mol Evol 26:213–225

Panaud O, Magpantay G, McCouch S (1993) A protocol for non-radioactive DNA labelling and detection in the RFLP analysis of rice and tomato using single-copy probes. Plant Mol Biol Rep 11:54–59

Raiber S, Schroder G, Schroder J (1995) Molecular and enzymatic characterization of two stilbene synthases from Eastern white pine (*Pinus strobus*). A single Arg/His difference determines the activity and the pH dependence of the enzymes. FEBS Lett 361:299–302

Ryder TB, Hedrick SA, Bell JN, Liang X, Clouse SD, Lamb CJ (1987) Organization and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in *Phaseolus vulgaris*. Mol Gen Genet 210:219–233

Schanz S, Schroder G, Schroder J (1992) Stilbene synthase from Scots pine (*Pinus sylvestris*). FEBS Lett 313:71–74

Schroder G, Brown JWS, Schroder J (1988) Molecular analysis of resveratrol synthase cDNA, genomic clones and relationship with chalcone synthase. Eur J Biochem 172:161–169

Sommer H, Saedler H (1986) Structure of the chalcone synthase gene of *Antirrhinum majus*. Mol Gen Genet 202:429–434

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C (1994) Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol Biol 24:743–755

Tropf S, Lanz T, Rensing SA, Schroder J, Schroder G (1994) Evidence that stilbene synthases have developed from chalcone synthases several times in the course of evolution. J Mol Evol 38:610–618

White EE (1990) Chloroplast DNA in *Pinus monticola*. 2. Survey of within-species variability and detection of heteroplasmic individuals. Theor Appl Genet 79:251–255

Wingender R, Rohrig H, Horicke C, Wing D, Schell J (1989) Differential regulation of soybean chalcone synthase genes in plant defence, symbiosis and upon environmental stimuli. Mol Gen Genet 218:315–322