A Stilbene Synthase Gene (*SbSTS1*) is Involved in Host and Non-host Defense Responses in *Sorghum bicolor*[1]

Christine K.Y. Yu[2], Karin Springob, Jürgen Schmidt, Ralph L Nicholson, Ivan K Chu, Wing Kin Yip, and Clive Lo*

Departments of Botany (C.K.Y.Y., W.K.Y., C.L.) and Chemistry (IKC), The University of Hong Kong, Pokfulam Road, Hong Kong, China; Leibniz-Institut für Pflanzenbiochemie (IPB), Weinberg 3, 06120 Halle/Saale, Germany (K.S., J.S.); Department of Botany and Plant Pathology, Purude University, West Lafayette, IN 47907 (RLN)

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* Corresponding author; e-mail clivelo@hkucc.hku.hk; fax +852-2858-3477.

ABSTRACT

A chalcone synthase (CHS)-like gene, *SbCHS8*, with high expressed sequence tag abundance in a pathogen-induced cDNA library was identified previously in sorghum. Genomic Southern analysis revealed that *SbCHS8* represents a single copy gene. *SbCHS8* expression was induced in sorghum mesocotyls following inoculation with *Cochliobolus heterotrophus* and *Colletotrichum sublineolum*, corresponding to non-host and host defense responses, respectively. However, the induction was delayed by approximately 24 h when compared to the expression of at least one of the other *SbCHS* genes. In addition, *SbCHS8* expression was not induced by light and did not occur in a tissue-specific manner. *SbCHS8*, together with *SbCHS2*, was over-expressed in transgenic *Arabidopsis tt4* mutants defective in CHS activities. *SbCHS2* rescued the ability of these mutants to accumulate flavonoids in seed coats and seedlings. In contrast, *SbCHS8* failed to complement the mutation, suggesting that the encoded enzyme does not function as a CHS. To elucidate their biochemical functions, recombinant proteins were assayed with different phenylpropanoid-CoA esters. Flavanones and stilbenes were detected in the reaction products of SbCHS2 and SbCHS8, respectively. Taken together, our data demonstrated that *SbCHS2* encodes a typical CHS that synthesizes naringenin chalcone necessary for the formation of different flavonoid metabolites. On the other hand, *SbCHS8*, now

re-termed as *SbSTS1*, encodes an enzyme with stilbene synthase activities, suggesting that sorghum accumulates stilbene-derived defense metabolites in addition to the well-characterized 3-deoxyanthocyanidin phytoalexins.

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is well known for its adaptability to adverse environments such as hot and dry conditions. The plant is also a rich source of distinct natural products. For example, sorghum seedlings accumulate high levels of dhurrin, a cyanogenic glycoside derived from tyrosine (Busk and Møller, 2002). To preclude competition for resources, sorghum roots exude sorgoleone and derivatives, a group of hydrophobic *p*-benzoquinone compounds, which inhibit electron transfer in PSII (Czarnota et al., 2001). In response to pathogen infection, sorghum synthesizes a unique class of flavonoid phytoalexins, the 3-deoxyanthocyanidins, as an essential component in the plant's active defense mechanisms (Lo et al., 1999).

Chalcone synthase (CHS) catalyzes the first committed step in flavonoid biosynthesis. The enzyme is the prototype of the plant type III polyketide synthase (PKS) family including the closely related stilbene synthases (STSs), pyrone synthases, acridone synthases, valerophenone synthases, and benzalacetone synthases (Springob et al., 2003), giving rise to the diversity of type III PKS-derived phytochemicals throughout the plant kingdom (Austin and Noel, 2003). Particularly interesting is the STS enzymes which utilize the same starter phenylpropanoid-CoA esters as the CHS enzymes and perform three condensations with malonyl-CoA

generating a common tetraketide intermediate, but result in the formation of the stilbene backbone following a completely different cyclization mechanism (Fig. 1). In different public databases, hundreds of plant DNA sequences are annotated as CHS genes based on sequence homology. However, these PKS genes may in fact have different metabolic roles, such as stilbene-forming activities, which can only be uncovered by experimental characterizations (Springob et al., 2003).

We have previously described a family of eight CHS genes, *SbCHS1-SbCHS8*, in sorghum (Lo et al., 2002). *SbCHS1* to *SbCHS7* (AF152548-AF152554) are highly conserved (at least 97.5% sequence identity at amino acid level) and closely related to the maize *C2* and *Whp* genes encoding CHS enzymes. *SbCHS8* (AY069951), on the other hand, is only 81-82% identical to *SbCHS1-SbCHS7* at amino acid level and appears to be more distantly related as revealed by phylogenetic analysis (Lo et al., 2002). These findings suggested that *SbCHS8* was duplicated from the ancestral form of *SbCHS1-SbCHS7* and diverged in protein coding sequence. In *in silico* analysis, *SbCHS8* was found to have significantly higher expressed sequence tag (EST) abundance in a pathogen-induced library (Lo et al., 2002). This EST library was prepared from 2-week-old seedlings 48 h after inoculation with the anthracnose pathogen *Colletotrichum sublineolum* (University of Georgia). Accumulation of

3-deoxyanthocyanidin was consistently detected in sorghum tissues inoculated with this fungal pathogen (Lo et al., 1999; Snyder and Nicholson, 1990), leading to our speculation that *SbCHS8* is involved in the biosynthesis pathway (Lo et al., 2002).

In this study, we used the well-established mesocotyl inoculation system (Hipskind et al., 1996; Lo and Nicholson, 1998) to investigate *SbCHS8* gene expression in sorghum. In addition, we attempted to define the biochemical functions of the encoded protein through analysis of transgenic Arabidopsis flavonoid mutants and *in vitro* activity assays of recombinant proteins. Our data demonstrate that *SbCHS8*, in fact, encodes a STS enzyme and gene expression was activated during host and non-host defense responses. Possible metabolites derived from the activity of the sorghum STS enzyme are discussed.

RESULTS

Genomic Southern analysis of *SbCHS* genes

For genomic Southern analysis, total DNA samples from 3 different sorghum cultivars (BTx623, Sc748-5, and DK46) were digested to completion with *Eco*R I, *Hind* III, or *Xba* I. A *SbCHS8*-specific PCR fragment containing part of the coding sequence and 3'-untranslated (UTR) region was used as a hybridization probe.

Results indicated that *SbCHS8* is a single copy gene and there are no RFLPs among the different cultivars examined (Fig. 2A). In contrast, a number of signals with varying intensities and sizes were detected when the digested DNA samples were hybridized with a CHS universal probe (Fig. 2B), which was derived from a conserved region in the *SbCHS1*-*SbCHS7* coding sequences. RFLPs were observed among the different cultivars following *Hind* III digestion. For example, Sc748-5 displayed a hybridization pattern distinct from the other two cultivars (Fig. 2B).

Northern analysis of *SbCHS* gene expression

Sorghum cultivar DK46 accumulates anthocyanin pigments in mesocotyls of etiolated seedlings upon light induction (Lo and Nicholson, 1998). Total RNA samples were prepared from mesocotyl tissue at various time points following light exposure. Northern analysis revealed that *SbCHS8* gene expression was not inducible by light (Fig. 3A). In contrast, expression of at least one of the *SbCHS1*-*SbCHS7* genes was detected when the universal probe was used in the hybridizations. These data indicated that *SbCHS8* is not involved in the light-induced anthocyanin biosynthesis pathway. The expression of *SbCHS8* was then investigated in different sorghum tissues. RNA samples were collected from roots and leaves of 6-d-old etiolated seedlings and 1-month old plants, as well as

developing panicles. As shown in Fig 3B, *SbCHS8* transcripts were not detectable in any of these tissues during normal growth conditions, indicating that the gene is not expressed in a tissue-specific manner.

To study the expression of *SbCHS8* during defense responses, etiolated seedlings of DK46 were inoculated with either *Cochliobolus heterostrophus*, a maize pathogen but nonpathogenic to sorghum, or *Colletotrichum sublineolum*, the causal agent of sorghum anthracnose. The inoculated seedlings were either kept in the dark or placed under constant light. Total RNA samples from various time points were analyzed by northern hybridizations. Transcripts of *SbCHS* genes, including *SbCHS8*, were detected in all the inoculation conditions examined (Fig. 3C-H). However, pathogen-induced accumulation of *SbCHS8* transcripts was delayed compared to transcripts detected by the universal *SbCHS* probe. For example, transcripts of *SbCHS8* were not detected until 24 h after inoculation with *C. heterotrophus* under dark conditions while transcripts of at least one of the other *SbCHS* genes were detected within 3 h (Fig. 3D). Similarly, *SbCHS8* gene expression was not observed until 72 h after inoculation with *C. sublineolum* under dark conditions while the expression of at least one of the other *SbCHS* genes was observed within 36 h (Fig. 3F). Although *SbCHS8* is not light inducible, the

pathogen-induced gene expression appeared to be enhanced under light. Thus, transcripts of *SbCHS8* were detected 12 h earlier in *C. heterotrophus*-inoculated plants and 24 h earlier in *C. sublineolum*-inoculated plants under light compared to the respective infected plants kept in the dark (Fig. 3C-F).

The expression of *SbCHS8* was also examined in two sorghum inbred lines, BTx623 and Sc748-5, with differential physiological and biochemical responses to the anthracnose pathogen *C. sublineolum* (Lo et al., 1999). Transcripts of *SbCHS8* were detected in Sc748-5 (resistant) plants with an accumulation pattern (Fig. 3G) similar to that observed in DK46 plants after inoculation with *C. sublineolum* (Fig. 3E). In contrast, *SbCHS8* transcript accumulation was delayed and less intense in the inoculated BTx623 (susceptible) plants (Fig. 3H). On the other hand, the patterns of the accumulation of *SbCHS* transcripts detected by the universal probe were similar in both cultivars following fungal inoculation (Fig. 3G-H).

Transgenic analysis of *Arabidopsis tt4* mutants

The complementation of *Arabidopsis* transparent testa (*tt*) mutants by maize genes demonstrated the convenience of this system to establish the function of uncharacterized coding sequences with homology to flavonoid structural genes (Dong

et al., 2001). Arabidopsis *tt4* mutants are deficient in CHS activities, resulting in the absence of flavonoid-derived metabolites in different tissues. *SbCHS2* and *SbCHS8* genes were expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in *tt4* plants. *SbCHS2* was selected as a representative of the highly conserved *SbCHS1-SbCHS7* genes. Expression of the sorghum genes in transgenic *tt4* mutants was confirmed by northern analysis in 10 to 14-day-old T1 seedlings (data not shown). Three independent lines with strong expression for each transgene were selected for phenotypic studies.

Transgenic *tt4* mutants expressing *SbCHS2* produced T1 seeds with brown pigmentation characteristic of wild type seeds (Fig. 4A), indicating the accumulation of tannins in seed coats. In addition, these transgenic seedlings showed anthocyanin pigments in cotyledons and hypocotyls when germinated in medium devoid of nitrogen sources, a sensitive condition previously employed to induce the anthocyanin biosynthesis pathway in Arabidopsis (Dong et al., 2001; Hsieh et al., 1998). In contrast, seed coats of *SbCHS8*-expressing *tt4* plants remained yellow in both T1 and T2 generations and the transgenic seedlings failed to accumulate anthocyanin under nitrogen deficiency (Fig. 4A). These results demonstrated that *SbCHS2* was able to fully complement the *tt4* mutation in *Arabidopsis* and hence the gene product is a

functional CHS enzyme. In contrast, *SbCHS8* does not encode CHS that could otherwise rescue the deficiencies in flavonoid biosynthesis in the *tt4* mutants.

To further characterize the flavonoids synthesized by the transgenic Arabidopsis *tt4* mutants, HPLC experiments were performed using acid hydrolyzed methanol extracts prepared from 14-d-old seedlings. Expression of *SbCHS2* in transgenic *tt4* plants resulted in the accumulation of the flavonols quercetin and kaempferol which were not present in the extracts prepared from non-transformed mutants (Fig. 4B). The flavonoid profile, monitored at A_{360}, of these transgenic plants was near identical to that of the wild type plant, Landsberg *erecta* (*Ler*), confirming the complete complementation of *tt4* mutation by *SbCHS2*. In contrast, accumulation of these flavonols was not detected in the *SbCHS8*-expressing *tt4* plants, further suggesting that this sorghum enzyme does not function as a CHS *in planta*.

Biochemical analysis of SbCHS recombinant proteins

SbCHS2 and *SbCHS8* were over-expressed in *E. coli* and purified by immobilized metal affinity chromatography to generate electrophoretically homogenous recombinant proteins (data not shown). Purified protein samples were incubated with ^{14}C-malonyl CoA and different phenylpropanoid-CoA esters.

Recombinant proteins of *Cassia alata* CHS (CalCHS1; Samappito et al., 2002) and *Rheum tataricum* STS (RtSTS1; Samappito et al., 2003) were included as reference enzymes in our assays. The resulting radioactive products were resolved by reversed-phase thin layer chromatography (RP-TLC). With cinnamoyl-CoA and *p*-coumaroyl-CoA as start substrates, the radiolabeled RP-TLC profiles of the SbCHS2 reaction were the same as those of CalCHS1 (Fig. 5A). Surprisingly, the SbCHS8 reaction profiles were almost identical to those of RtSTS1 (Fig. 5A). SbCHS2 and SbCHS8 assays resulted in the production of flavanones (pinocembrin and naringenin) and stilbenes (pinosylvin and resveratrol), respectively. Flavanones were presumably detected due to spontaneous isomerization of the respective chalcones. *Bis*-noryangonin (BNY)-type and *p*-coumaroyltriacetic acid lactone (CTAL)-type derailed pyrone byproducts were also identified in most of the assays (Fig. 5A). In addition, SbCHS8 was found to produce small amounts of flavanones (Fig. 5B, pinocembrin to pinosylvin ratio = 5.5: 100; naringenin to resveratrol ratio= 2.0: 100). The CHS side activity of SbCHS8 was lower than that of RtSTS1 as reflected from their product ratios. Cross-reaction between CHS and STS enzymes has been demonstrated in *in vitro* reactions previously (Samappitto et al 2002, 2003; Yamaguchi et al, 1999). Similarly, trace levels of pinosylvin were detected in the SbCHS2 and CalCHS1 assays with cinnamoyl-CoA while no resveratrol was detected

with *p*-coumaroyl-CoA (Fig. 5B). We also used caffeoyl-CoA and feruloyl-CoA in the assays but the BNY-type and CTAL-type pyrones were formed predominantly (data not shown), suggesting that these starter-CoAs are not physiologically relevant substrates (Samappito et al., 2003).

To unambiguously identify the reaction products, recombinant proteins were incubated with unlabeled malonyl-CoA and starter CoA esters in scaled-up reactions. The product mixtures obtained in these experiments were analyzed by combined LC/electrospray ionization (ESI)-MS/MS in selected reaction monitoring (SRM) mode using the reactions leading to key ions. Under positive ESI conditions, flavanones were detected by reactions leading to a key ion at *m/z* 153 (trihydroxybenzoyl moiety) as well as the respective phenylpropanoyl cations: cinnamoyl cation at m/z 131 and *p*-coumaroyl cation at m/z 147 (Fig. 5C; Samappito et al., 2002). The mass spectral behavior of stilbenes under negative ESI conditions is characterized by the loss of ketene units. Resveratrol was confirmed by measuring the two key reactions *m/z* 227 $[M-H]^-$ to *m/z* 185 $[M-H-CH_2CO]^-$ and *m/z* 227 $[M-H]^-$ to *m/z* 143 $[M-H-2CH_2CO]^-$, respectively (Fig. 5C, Stecher et al., 2001; Samappito et al., 2003). Similarly, pinosylvin was identified by the reaction *m/z* 211 $[M-H]^-$ to *m/z* 169 $[M-H-CH_2CO]^-$ (Fig. 5C). Taken together, our results clearly demonstrated that SbCHS8 encodes an enzyme with STS activities.

DISCUSSION

SbCHS8 was initially annotated as a CHS-like gene having high EST abundance in a cDNA library prepared from infected sorghum plants with the accumulation of 3-deoxyanthocyanidin phytoalexins (Lo et al., 2002). However, we demonstrated *SbCHS8* is not involved in flavonoid biosynthesis *in planta* as it failed to complement the *tt4* mutation in Arabidopsis (Fig. 4). Instead, the recombinant SbCHS8 protein synthesized pinosylvin and resveratrol as major products *in vitro* using cinnamoyl-CoA and *p*-coumaroyl-CoA as starter molecules, respectively (Fig. 5) and the sorghum gene was, therefore, re-termed as *SbSTS1*.

To our knowledge, *SbSTS1* represents the first example of a STS gene in monocots. The gene is not constitutively expressed, but inducible following fungal inoculation. Related enzymes performing STS-like cyclizations, e.g. bibenzyl synthases, have been isolated from a *Phalaenopsis* orchid (Preisig-Muller et al., 1995). STS enzymes occur only in limited numbers of unrelated plant species. Resveratrol STS enzymes were originally described in grapes and peanuts, which accumulate elevated levels of the stilbene following pathogen inoculations and elicitor treatments (Schröder et al., 1988; Wiese et al., 1994). Recently, a root-specific STS cDNA was

reported in *Rheum*, a medicinal plant in the Polygonaceae family, with resveratrol accumulation in roots (Samappito et al. 2003). A second category of STS, pinosylvin STS enzymes, is largely associated with pine trees. These enzymes utilize cinnamoyl-CoA as the starter ester to synthesize pinosylvin which is found in the heartwood or serving as phytoalexins in sapwoods and needles (Preisig-Müller et al., 1999). In *Psilotum nudum*, two pinosylvin STS enzymes were recently identified through *in vitro* enzyme assays of the recombinant proteins, although stilbenes or their derivatives have not been isolated from this primitive vascular plant (Yamazaki et al., 2001).

The expression of STS genes is often induced by a variety of abiotic and biotic stresses, such as elicitor treatment, pathogen inoculation, wounding, UV irradiation, and post-harvest wilting procedures (Preisig-Müller et al., 1999; Verari et al., 2001). Constitutive expression of STS genes was described in young seedlings of grapes, presumably representing a pre-existing defense mechanism (Sparvoli et al, 1994). In sorghum, *SbSTS1* gene expression was not detected under non-induced conditions in all tissues examined (Fig. 3). Our results also revealed that *SbSTS1* is a late component during both non-host (against *C. heterotrophus*) and host (against *C. sublineolum*) defense responses, comparing to the expression of at least one of the

SbCHS genes (Fig. 3). In inoculated plants kept in the dark, a condition in which flavonoid metabolism was not induced by light, transcripts of *SbSTS1* gene were not detected until 24 h or 72 h after inoculation *with C. heterotrophus* or *C. sublineolum*, respectively (Fig.3D, F), during which significant amounts of 3-deoxyanthocyanidins had accumulated (data not shown). The late induction of *SbSTS1* expression provided further evidence that the enzyme is not involved in the biosynthesis of 3-deoxyanthocyanidins in sorghum. Nevertheless, *SbSTS1* and *SbCHS* genes are components involved in both non-host and host defense responses. Interestingly, earlier and stronger induction of the *SbSTS1* gene was detected in cultivar Sc748-5 compared to cultivar BTx623, following inoculation with *C. sublineolum* (Fig. 3G-H). In the mesocotyl inoculation system, we have previously observed that fungal development in cultivar Sc748-5 (resistant host) was essentially contained during early stages of pathogenesis (Lo et al., 1999). In contrast, the fungal pathogen was able to colonize cultivar BTx623 (susceptible host) with the proliferation of primary and secondary hyphae. The differential expression of *SbSTS1* in the incompatible interaction suggests that the enzyme plays a key role in the expression of resistance against *C. sublineolum*.

An intriguing question remains regarding the identities of the sorghum defense

metabolites derived from SbSTS1 enzyme activities. In members of the Poaceae, resveratrol has been isolated from endophyte-infected grasses such as fescue, ryegrass, barley, sleepygrass, and bluegrass (Powell et al., 1994). Piceatannol, with an additional hydroxyl group at the 5'-position, was identified as a sugarcane phytoalexin after stalk inoculation with *Colletotrichum falcatum* (Brinker and Seigler, 1993). However, neither resveratrol nor piceatannol were detected in acid-hydrolyzed extracts prepared from transgenic Arabidopsis or infected sorghum under our standard LC/MS-MS conditions (data not shown). It is likely that the immediate product(s) of SbCHS8 had been further modified *in planta*. The most common stilbene derivative piceid is a 3-*O*-glucoside of resveratrol, but the aglycone would be easily detected following acid hydrolysis. In Scots pine, pinosylvin is modified by an SAM dependent *O*-methyltransferase (PMT) to pinosylvin 3-*O*-methyl ether following ozone or fungal elicitor treatment (Chiron et al., 2000). The recombinant PMT protein showed *in vitro* activities toward a broad range of substrates including resveratrol (Chiron et al., 2000). *O*-methyl ethers are common derivatives of flavonoid-related secondary metabolites. In fact, the two major 3-deoxyanthocyanidin components luteolinidin and apigeninidin also exist as *O*-methyl ethers in sorghum (Lo et al., 1996; Lo and Nicholson, 1998). Whether a stilbene-*O*-methyl ether accumulates in inoculated sorghum plants is now under

investigation. Alternatively, SbSTS1 may utilize substrates other than phenylpropanoid-CoA esters leading to the formation of a more complex secondary metabolite in sorghum. Dayan et al. (2003) demonstrated recently that an STS-type reaction is involved in the biosynthesis of the sorghum root exudate sorgoleone. Sorgoleone and its derivatives are benzoquinone containing aliphatic tails of 15 or 17 carbons with various degrees of unsaturation (Netzly et al., 1988). Thus, the "STS" involved would accept acyl-CoA esters of C16 and C18 fatty acids as starter molecules (Dayan et al., 2003). Examination on the activities of the recombinant SbSTS1 enzyme towards CoA esters of different fatty acids as well as other phenylpropanoids should help define its precise biochemical role in nature. Furthermore, recent advances in metabolic profiling technologies (von Roepenack-Lahaye et al., 2004) should allow one to identify novel natural products in plants in a more robust and efficient manner.

MATERIALS AND METHODS

Sorghum growth conditions and fungal inoculations

All sorghum seeds and fungal strains used in this study were described previously (Lo and Nicholson, 1998; Lo et al., 1999). For genomic DNA isolation, sorghum plants were grown in a greenhouse (16 h light, 8 h dark). For inoculation

experiments, sorghum seed were planted in rolls of germination paper and kept in the dark for 4 days at 28ºC as described previously (Lo et al., 1996). Etiolated seedlings with elongated mesocotyls were then inoculated with conidial suspensions of *C. heterotrophus* or *C. sublineolum* at 5.5×10^4 or 1.0×10^6 conidia ml^{-1}, respectively. Tween 20 was used as a wetting agent (100 µl 100 ml^{-1}) in the inoculum. The resulting suspensions were misted onto the etiolated seedlings with an atomizer, and the plants were incubated at 100% RH at room temperature for at least 24 h.

DNA isolation and Southern blotting

Genomic DNA samples were extracted from 4-week-old sorghum plants. Leaf tissues (1 g) were ground to a fine powder in liquid nitrogen and transferred to microfuge tubes containing the DNA extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 10 mM mercaptoethanol). 20% (w/v) Sodium dodeceyl sulfate (SDS) (1 ml) was added to each tube and the mixtures were incubated at 65ºC for 10 minutes. 5 M potassium acetate (5 ml) was then added and the tubes were incubated at 4ºC for 20 min. The final mixtures were centrifuged at 4,000 rpm for 20 min and the supernatants were transferred into tubes containing 10 ml of isopropanol. After incubation at -20ºC for 30 min, DNA samples were centrifuged at 14,000 rpm for 20 min. The pellets were washed in 70% ethanol,

air-dried, and resuspended in 0.5 ml of TE buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0). DNA samples (20 µg) were digested to completion with selected restriction enzymes. The digested DNA was separated by electrophoresis on a 0.8% agarose gel, depurinated, denatured, and blotted in 20× SSC (3 M NaCl, 0.3 M sodium citrate) by downward capillary transfer for at least 16 h onto a GeneScreen Plus nylon membrane (PerkinElmer, Boston, MA) then covalently cross-linked to the membrane with a UVP CL-1000 UV crosslinker (UVP, Cambridge, England).

RNA extraction and northern blotting

Sorghum tissues (1 g) were ground into a fine powder with liquid nitrogen and extracted with 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) in microfuge tubes. Chloroform (200 µl) was added to each tube and the resulting mixtures were centrifuged at 14,000 rpm for 10 min. The supernatants were transferred to new tubes containing 500 µl of isopropanol and 60 µl of 3 M sodium acetate. The mixtures were then centrifuged at 14,000 rpm for 10 minutes. The pellets were washed with 70% ethanol, air-dried, and re-suspended in 30 µl of RNase-free water. Fifteen micrograms of total RNA from each sample were denatured and fractionated on a 1% formaldehyde gel in 1× FA buffer, pH 7.0 (20 mM MOPS; 5 mM sodium acetate; 1 mM EDTA) and transferred to nylon membranes as described above.

Equal loading of RNA on gels was confirmed by ethidium bromide staining.

Southern and northern hybridizations

Individual membranes were pre-hybridized in hybridization buffer (1 M sodium chloride; 0.1% dextran sulfate; 1% SDS; 100 μg ml^{-1} salmon sperm DNA) for 1 h at 65ºC. The membranes were then hybridized in the same buffer containing different denatured ^{32}P-labeled DNA probes for at least 16 h at the same temperature. The hybridized membranes were washed twice in 2× SSC, 0.1% SDS for 20 min at 65ºC, and twice in 0.2× SSC, 0.1% SDS for 20 min at 65ºC. High stringency washes in 0.1× SSC at 65ºC were performed when necessary. After washing, the membranes were exposed to FUJI 100NIF X-ray films (Fuji Photo Ltd., Tokyo, Japan) with intensifying screens at -80ºC.

Hybridization probes

PCR fragments were generated for use as probes in the hybridization experiments. The CHS8 probe (394 bp) was amplified from a full-length cDNA clone (Lo et al., 2002) using primers derived from the 3'-UTR as well as part of the coding region (Forward: 5'-GGC AAC ATG TCA AGC GTT TG-3'; Reverse: 5'-CCA CTG CAC TGT GTT GAC TTG-3'). The CHS-U probe (643 bp) was amplified

from a full-length *SbCHS2* cDNA clone (L Pratt, University of Georgia, Athens, GA) using primers derived from a region conserved in *SbCHS1-SbCHS7* (Forward: 5'-CGC TGG ACG CCC GCC AGG ACA -3'; Reverse: 5'- GG GTG CGC CAC CCA GAA GAT). The hybridization probes were gel-purified (Qiagen, Valencia, CA) and labeled with ^{32}P-dCTP using the Rediprime II kit following the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Complementation of Arabidopsis *tt4* mutants

Full-length *SbCHS2* and *SbCHS8* cDNA fragments were each cloned into the *Bam*H I and *Xho* I sites of 103c-SK (E. Lam, Rutgers University, New Brunswick, NJ), an over-expression vector containing the CaMV 35S promoter and the nopaline synthase 3'-terminator. The resulting plasmids were cloned into the *EcoR* I and *Hind* III sites of the binary vector pCAMBIA 1300 (CAMBIA, Australia) to generate pCAM1300-*SbCHS2* and pCAM1300-*SbCHS8* for *Arabidopsis* transformation.

The Arabidopsis *tt4* mutants (CS8605) were obtained from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus, OH). They are of the *Ler* genetic background and have a yellow seed coat color. The plant expression vectors were transformed into the mutants by the floral dip method

(Clough and Bent 1998). For selection of transformants, harvested seeds were surface-sterilized with 70% ethanol and 100% chlorox, followed by rinsing three times in sterilized water. The sterilized seeds were germinated on Murashige and Skoog (MS) (Sigma, MO, USA) agar plates containing 3% (v/v) sucrose, 25 μg ml^{-1} of hygromycin, and 500 μg ml^{-1} of carbenicillin. After 2 weeks of selection, hygromycin-resistant plants (T_0 plants) were transplanted to soil and placed in a growth chamber (25ºC, 16 h light, 8 h dark). T_1 seeds from individual T_0 lines were collected and examined for seed coat color. For nitrogen deficiency assays, T_1 seeds were germinated on MS plates without nitrogen sources. Accumulation of anthocyanins on cotyledons was observed in 4 to 5 days.

HPLC analysis of transgenic Arabidopsis *tt4* mutants

T_1 and T_2 lines expressing *SbCHS2* or *SbCHS8* were grown on MS agar containing 3% (v/v) sucrose and hygromycin (25 μg ml^{-1}). Plant materials (0.5-1.0 g) were collected from 10 to 14 d-old seedlings and ground to a fine powder in liquid nitrogen. Methanol (300 μl) containing 1% (v/v) HCl was then added to the tissue powder. Acid hydrolysis was carried by addition of an equal volume of 2N HCl, followed by incubation at 70ºC for 1 h. The hydrolyzed samples were evaporated to dryness under nitrogen and resuspended in 100 μl of acidified methanol. Final

sample preparations (20 µl) were injected onto a HP 1100 series HPLC system (Agilent Technologies, USA) equipped with a Nucleosil 100-5 C18 column (5 µm, 250×4 mm, Agilent Technologies). Chromatographic separation was performed using a solvent system of 1% acetic acid (v/v) (A) and acetonitrile (B) with a linear gradient of 20-60% B over 25 min. Flow rate was maintained at 0.6 ml min^{-1} and the elution was monitored by a diode-array detector (200-600 nm). Flavonol standards were obtained from Sigma (St Louis, MO).

Over-expression of SbCHS proteins in *E. coli* and enzyme assays

To express the sorghum proteins in *E. coli*, cDNAs were cloned into the *Nde* I and *Bam*H I sites of the pET14b vector (Novagen, San Diego, CA) containing a hexahistidine N-terminal fusion tag. To engineer the restriction sites in the inserts, PCR amplifications were performed using gene-specific primers [SbCHS2-F (5'-AGT CAT ATG GCC GGC GCG ACT GTG ACC-3') and SbCHS2-R (5'-AGT GGA TCC TCA GGC GGT GAT GGC CGC-3'); SbCHS8-F (5'-AGT CAT ATG ACG ACT GGG AAG GTA ACA-3') and SbCHS8-R (5'-GAT GGA TCC TCA TGC AGC CAC TGT GGT-3')] with the corresponding full-length cDNA clones as templates and the enzyme *Pfu* polymerase (Promega, Madison, WI). The resulting plasmids were each transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). After 20 h

induction with 0.4 mM isopropyl -1-thio- β -D –galactopyranoside at 28°C, the recombinant proteins were purified from the bacterial cultures following procedures essentially as described previously (Samappito et al., 2002). The reference enzymes CalCHS1 and RtSTS1 were expressed and purified according to Samappito et al. (2002 and 2003).

The standard enzyme assays contained 100 mM HEPES buffer (pH 7.0), 20 µM starter CoA, 15 µM [2-^{14}C] malonyl-CoA (24,000 dpm), and 1.0 µg protein in a 50-µl reaction. Starter CoAs (cinnamoyl-CoA, *p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA), prepared essentially as described (Stöckigt and Zenk 1975), were kindly provided by Dagmar Knöfel (Department of Secondary Metabolism, IPB, Halle, Germany). The assay mixtures were incubated for 30 min at 30ºC. The reactions were stopped by addition of 5 µl of 50% (v/v) acetic acid and extracted with 200 µl of ethyl acetate. The organic phase was dried and separated by TLC (RP18) and developed in MeOH-H_2O-acetic acid (75:25:1). The ^{14}C-labelled products were visualized by phosphoimaging and quantification was performed with the ImageQuant software (Molecular Dynamics). Reaction products were identified by the use of authentic standards as well as comparison to published profiles of CalCHS1 and RtSTS1 reactions (Samappito et al., 2002, 2003). To confirm the identities of flavanone and stilbene products, scaled-up reactions were performed containing 10 µg

recombinant proteins, 50 µM starter CoA, and 100 µM malonyl-CoA in a total volume of 200 µl for LC/ESI-MS/MS analysis in SRM mode. Positive and negative ESI mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage, 4.5 kV; heated capillary temperature, 220 °C; sheath and auxiliary gas, nitrogen) coupled with a Surveyor MicroLC system equipped with an RP18-column (5 µm, 1x100 mm, SepServ, Berlin). For all measurements a gradient system ranging from H_2O:CH_3CN 90:10 (each containing 0.2% acetic acid) to 10:90 over 15 min, followed by isocratic elution with a 10:90 mixture of both solvents for 10 min, was used at a flow rate of 50 µl min^{-1}. Argon was used as collision gas and the collision pressure was at 1.8 x 10^{-3} Torr.

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FIGURE LEGENDS

Figure 1. Reaction steps catalyzed by CHS and STS. Cinnamoyl-CoA and *p*-coumaroyl-CoA are the common start substrates for CHS and STS enzymes. Chalcones are usually converted to flavanones spontaneously *in vitro*.

Figure 2. Genomic Southern analysis of *SbCHS* genes. Total DNA samples were prepared from the indicated cultivars and digested to completion with *Eco*R I (E), *Hind* III (H), or *Xba* I (X). A, Southern blot containing the digested DNA samples were hybridized with a *SbCHS8*-specific probe, CHS8. A single hybridization signal was detected in each digested sample following film exposure for 3 d. B, A universal CHS fragment, CHS-U, for the *SbCHS1*-*SbCHS7* genes was used to probe a blot containing *Hind* III digested DNA samples. A number of hybridization signals of varying intensities were detected following overnight film exposure and RLFPs were observed among the cultivars.

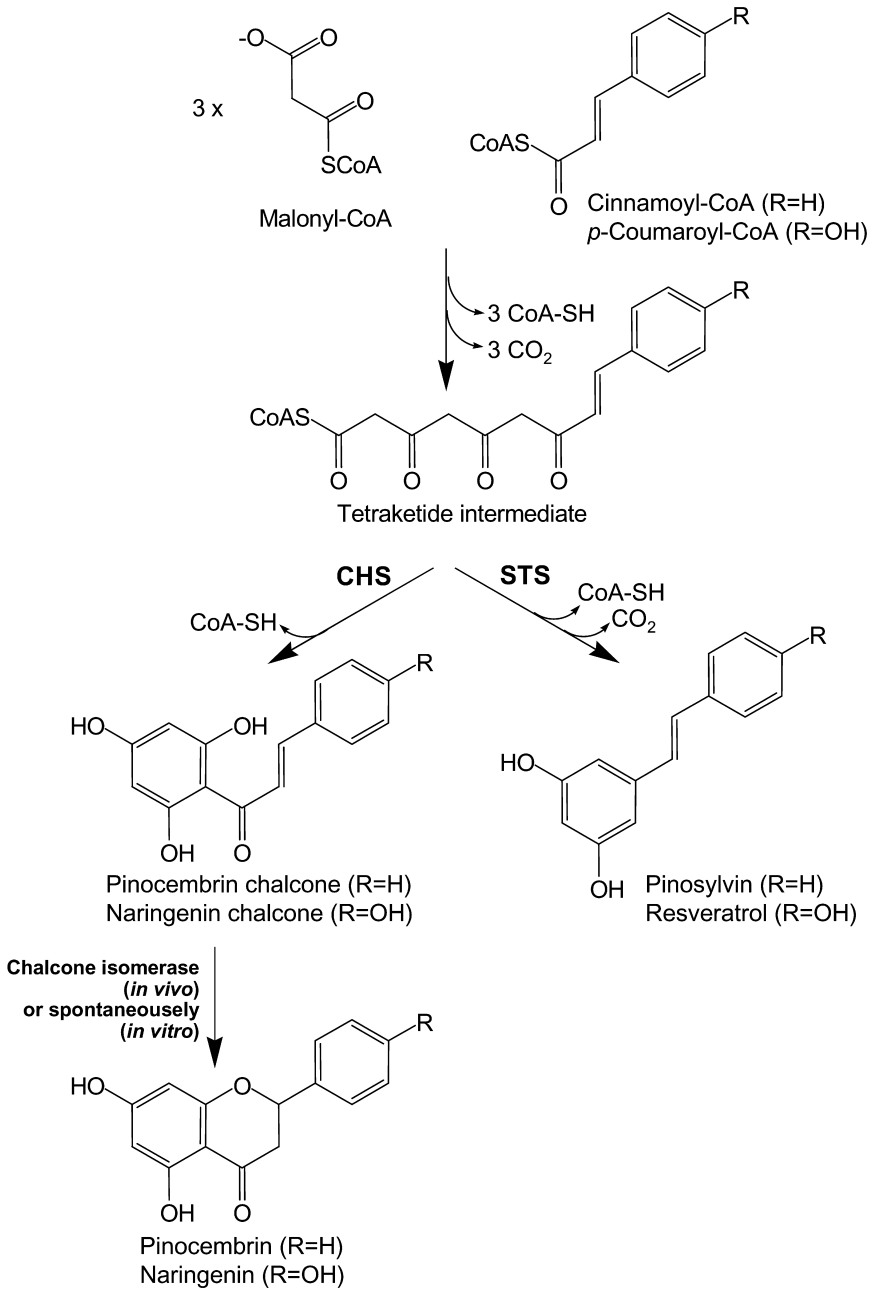
Figure 3. Northern analysis of *SbCHS* gene expression. A, Etiolated seedlings (4-d old) of cultivar DK46 were exposed to light and RNA samples were prepared from tissues collected at the indicated time point (h). B, To examine tissue specific expression (B), RNA samples were collected from 6-d-old roots (YR), 1-month-old

roots (MR), 6-d-old leaves (YL), 1-month-old leaves (ML), and developing panicles (P). For infection experiments, 4-d-old etiolated seedlings were inoculated and RNA samples were prepared from tissues collected at the indicated time points (h). C-D, DK46 plants were inoculated with *C. heterotrophus* and kept under light or in the dark. E-F, DK 46 plants were inoculated with *C. sublineolum* and kept under light or dark. G-H, Inbred cultivars BTx623 (susceptible) and Sc748-5 (resistant) were inoculated with *C. sublineolum*. Hybridization probes (CHS8 and CHS-U) were the same as those used in the Southern experiments.

Figure 4. Analysis of transgenic Arabidopsis *tt4* mutants. A, Complementation of seed coat color and anthocyanin pigmentation. Seed coats of wild type plants (*Ler*) are brown due to tannin deposition. *Ler* seedlings accumulate anthocyanin in cotyledons and hypocotyls when germinated on MS medium without nitrogen sources (MS-N). Arabidopsis *tt4* mutants produced seeds with yellow color and failed to accumulate anthocyanin under nitrogen deficiency. Note the complementation of *tt4* phenotypes in T1 lines of SbCHS2-expressing plants (*tt4* + *SbCHS2*). On the other hand, *SbCHS8* did not restore the *tt4* mutations in T1 transgenic plants (*tt4* + *SbCHS8*). Same phenotypes were observed in the T2 lines of *SbCHS8*-expressing plants (data not shown). B, HPLC profiles of transgenic *tt4* plants.

Acid-hydrolyzed extracts were prepared from T1 lines and analyzed by HPLC with elution monitoring at A_{360}. Note the detection of peaks representing quercetin (Q, 19.0 min) and kaempferol (K, 23.0 min) in *Ler* and *tt4 + SbCHS2* plants.

Figure 5. Enzyme assays of recombinant CHS proteins. A, RP-TLC analysis of products extracted from enzyme assays of recombinant proteins (CalCHS1, SbCHS2, SbCHS8, and RtSTS1). Assays were performed with 1.0 µg of purified protein, radiolabeled malonyl-CoA and either cinnamoyl-CoA or *p*-coumaroyl-CoA. Positions of flavanones (Pc, pinocembrin; N, naringenin), stilbenes (Ps, pinosylvin; R, resveratrol), and the BNY-type and CTAL-type pyrone byproducts (BNY-P and CTAL-P) are indicated. Inset: SDS-PAGE analysis of recombinant proteins visualized with Coomassie Brilliant Blue R250. Lane 1, SbCHS2 crude cell lysate; Lane 2, purified SbCHS2; Lane 3, SbCHS8 crude cell lysate; Lane 4, purified SbCHS8. B, Ratios of flavanone to stilbene products in the assay reactions. ^{14}C –labelled products were quantified after phosphoimaging and ratios were calculated based on average values from three independent assays. C, LC/ESI-SRM analysis of reaction products. Flavanone and stilbene products were confirmed by LC/MS-MS in SRM mode. RT, retention time. CE, collision energy. Structures of the starter-CoAs, flavanones, and stilbenes are shown in Fig. 1.



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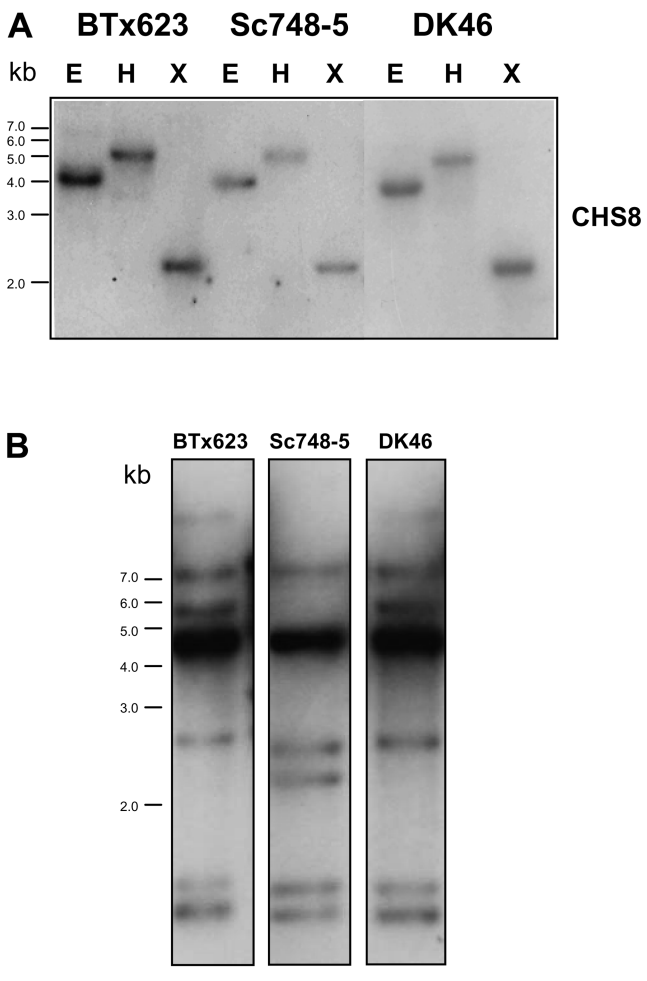

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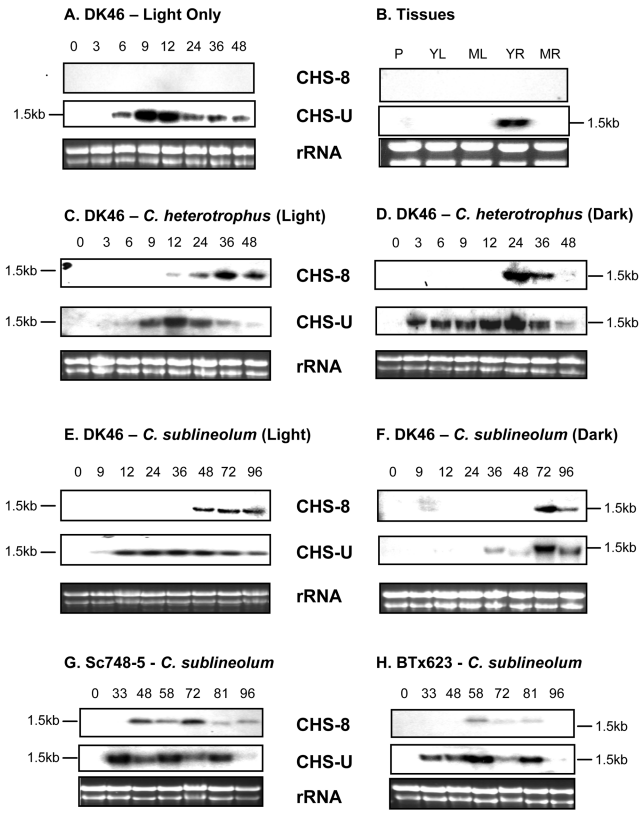

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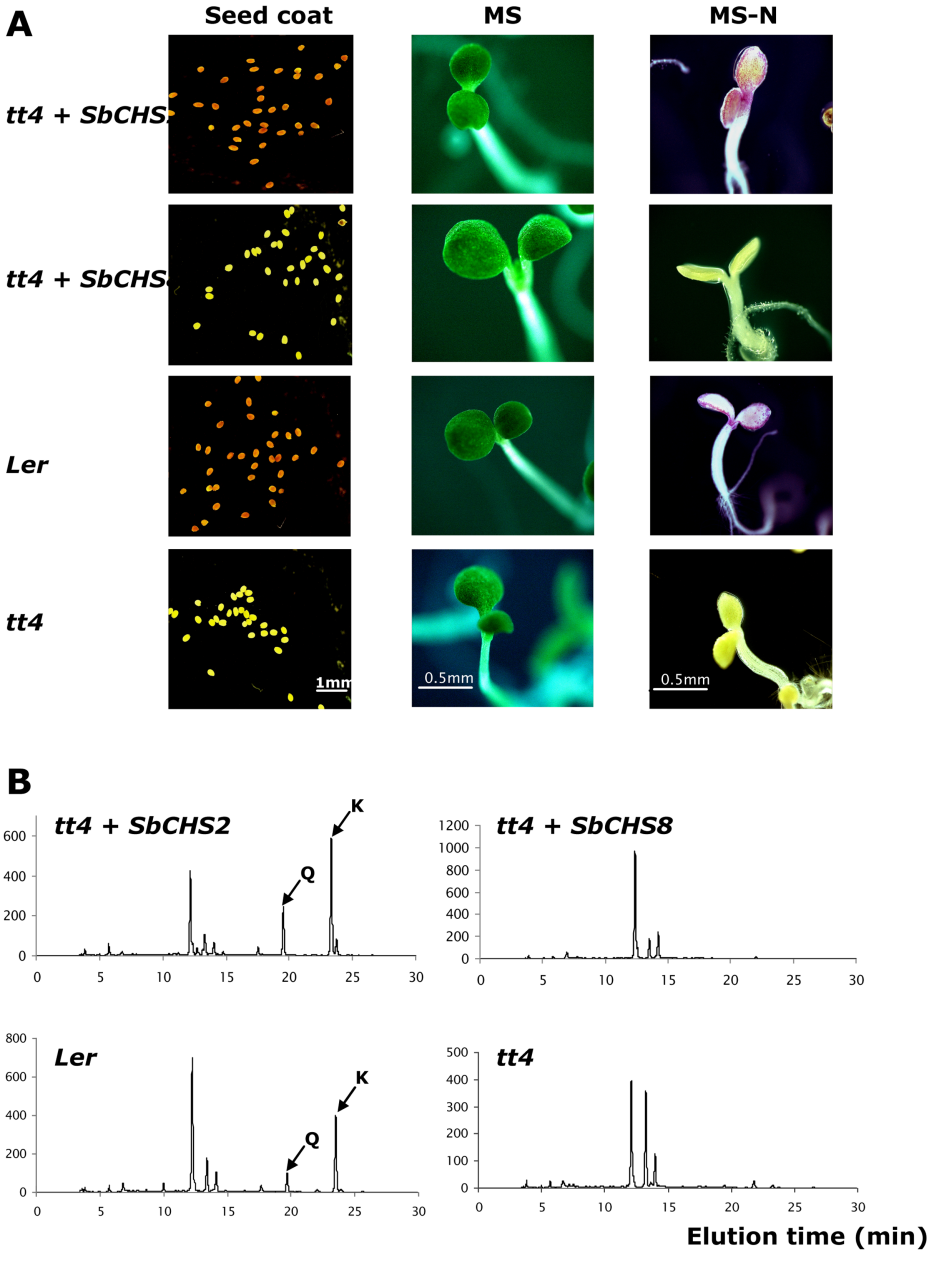

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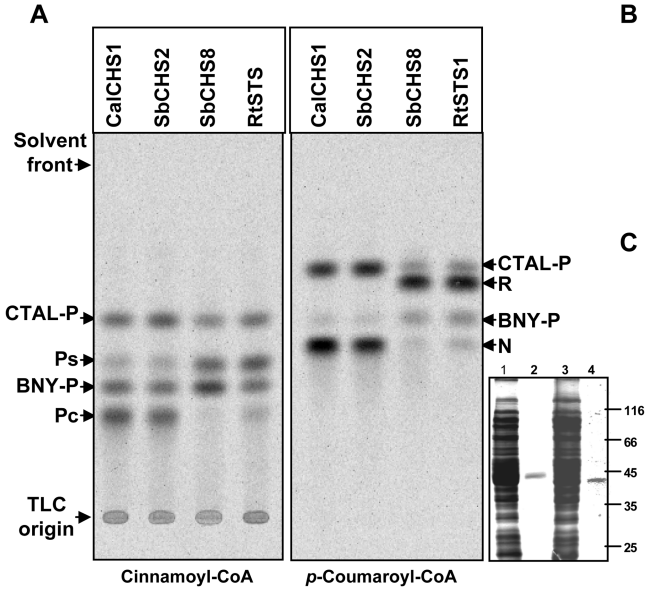

B

	Flavanone: Stilbene ratio			
	CalCHS1	SbCHS2	SbCHS8	RtSTS1
Cinnamoyl-CoA (Pc: Ps)	100: 1.8	100: 2.5	5.5: 100	26: 100
p-Coumaroyl-CoA (N: R)	100: 0	100:0	2.0: 100	8.7: 100

C

Compound	RT (min)	CE (eV)	SRM
Pc	15.53	-25	m/z 257 $[M+H]^+$ → m/z 131
		-25	m/z 257 $[M+H]^+$ → m/z 153
N	12.83	-25	m/z 273 $[M+H]^+$ → m/z 147
		-25	m/z 273 $[M+H]^+$ → m/z 153
Ps	14.44	+25	m/z 211 $[M-H]^-$ → m/z 169
R	11.42	+25	m/z 227 $[M-H]^-$ → m/z 185
		+25	m/z 227 $[M-H]^-$ → m/z 143

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