Metabolic changes in Arabidopsis thaliana plants overexperssing chalcone synthase

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Chapter 4

Chalcone synthase protein expression in CHS transgenic *Arabidopsis*

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Abstract

Chalcone synthase is the enzyme responsible for the production of chalcones, which are precusors for a large range of flavonoids and related compounds. In order to confirm the expression of CHS in the CHS transgenic *Arabidopsis*, five transformants were checked by immunoblot assay and two of them were examined by an enzyme activity assay. Western blot analysis showed that CHS was expressed in all transformants. High performance liquid chromatography analysis showed that the activity level of endogenous CHS in *Arabidopsis* wild type (WT) line was less than that of the transgenic *Arabidopsis* ACS 20 line, whereas CHS activity of transgenic line ACS 2 was similar to the WT line.

Key words: chalcone synthase, *Arabidopsis*, protein assay, HPLC, naringenin.

4.1. Introduction

In plants, chalcone synthase is expressed under stress condition such as pathogen attack, UV light or during early development stages [Dixon *et al.,* 1995, Estabrook *et al.,* 1991]. Chalcone synthase (EC 2.3.1.74), known as a type III polyketide synthase (PKS), is the key enzyme in the flavonoid biosynthesis and catalyses the reaction of one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA to yield 4,2',4',6'-tetrahydroxychalcone (naringenin chalcone) the precursor for a large number of flavonoids [Weisshaar, 1998]. Naringenin chalcone is converted to naringenin by chalcone isomerase but can also be converted non–enzymetically to narigenin [Hahlbrock *et al.,* 1979; Mol *et al.,* 1985; Sankawa *et al.,* 1997; Schröder *et al.,* 1997]

A major goal of plant biotechnology is the production of genetically engineered crops that express natural or foreign functional proteins at high levels. A previously cloned *Cannabis CHS* gene (~ 45kDa) was overexpressed in *E coli*, and showed a chalcone synthase activity [Raharjo *et al.,* 2004]. In previous work (**Chapter 3**) six *chs* transgenic lines of *Arabidopsis* were collected for molecular analysis and *chs*-mRNA were shown to express. However, an analysis on protein expression and activity level is still needed. We want to evaluate whether the heterologous CHS protein expressed in plants also acts as a typical CHS.

In this study we tested CHS expression in CHS transgenic *A. thaliana* (ACS). To confirm the presence of the heterologous CHS protein immunobloting was applied. To determine the activity of the protein in transgenic plants a funtional assay was used. This assay was performed by measuring the conversion of the precusors (malonyl-CoA and p-coumaroyl-CoA) into naringenin in the protein extract. The final product (naringenin) was measured by HPLC [Zuurbier *et al.,* 1993].

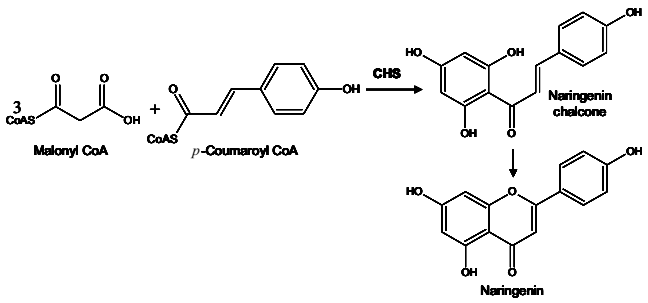

Figure 4.1. Reaction catalyzed by chalcone synthase (CHS)

4.2. Materials and methods

4.2.1. Plant material

Arabidopsis thaliana ecotype Columbia (Col.0) is the genetic background for all wild type and 6 transgenic ACS lines used. Seeds were surface sterilized by incubation for 1 min in 70% ethanol, 15 min in 2.5% sodium hypochlorite and rinsed with sterile water five times. Surface sterilized seeds were grown on plates containing half MS medium with 0.6% agar and supplemented with 20 mg/L hygromycin for selection of transgenic plants. Following stratification in 3 days at 4^{o}C and in dark environment, seeds were germinated at 23^{o}C and long day condition (16/8 h light/dark cycle) in a growth chamber. All the tissues were collected from five days old seedlings. Immediately after harvesting the material was frozen in liquid nitrogen and kept at −80 °C until used.

4.2.2. Chemicals

Tetramethylethylenediamine (TEMED), K_2HPO_4, sucrose, ascorbic acid, PMSF, $CaCl_2$, EDTA, SDS, polyvinylpolypyrrolidone (PVP), NaCl, $(NH_4)_2SO_4$, $(HOCH_2)_3CNH_2$ (Tris), ammonium persulfate (APS), malonyl-CoA were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Naringenin and *p*-Coumaryl-CoA were purchased from TransMIT (GmbH, Marburg, Germany).

4.2.3. Protein extraction

Enzyme was extracted as reported by Zuurbier *et al.,* [1995] with a slight modification. All steps were carried out at 0–4 °C. Frozen plant material (10 g) was ground using a pestle and mortar in the presence of 10% PVP (w/w). The frozen powder was mixed with extraction buffer (0.5 M K-Pi of pH 8, 0.4 M sucrose, 1 mM $CaCl_2$, 0.1% BSA (w/v), 0.2 M ascorbic acid, 50 mM EDTA, 50 mM cysteine, 10% DOWEX 1WX2 100 mesh, 10 μM leupeptin, 0.2 mM PMSF). After thawing, the homogenate was filtered using a Miracloth filter (Calbiochem, La Jolla, CA, USA) then centrifuged at 14 000 rpm for 20 min. The protein was then precipitated using a range from 30 to 70% $(NH_4)_2SO_4$. The 70% $(NH_4)_2SO_4$ pellet was collected and dissolved in 2.5 ml PD10 buffer (0.1 M K–Pi pH 6.8, 1.4 mM 2-mercaptoethanol, 40 mM ascorbic acid and 5% (w/v) trehalose, flushed with N_2 gas before use) and then desalted in the same buffer with the use of a PD10 column (Amersham Pharmacia Biotech, Uppsala, Sweden)

according to the manufacturer's instructions. The protein concentration was determined by the method of Peterson, [1977]. The protein sample was then frozen in liquid nitrogen and stored at −80 °C until used.

4.2.4. SDS-PAGE and Western blot

About 5 µg of protein extract was loaded on mini gels. The separation and stacking gel composition is as follows: Separating gel solution (30 ml) contains 16 ml of 28% acrylamide solution, 4.5 ml of 3 M Tris pH 8.9, 3 ml of 1% SDS, 6.45 ml of distilled water, 100 µl of 10% APS, and 30µl of TEMED. Stacking gel solution (5 ml) contains 0.9% acrylamide (28%), 2 ml of 0.5 M Tris-Cl pH 6.7, 0.5 ml of 1% SDS, 0.6 ml of distilled water, 40 µl of 10% APS, and 10 µl of TEMED. Electrophoresis was carried out at 100 V in running buffer (0.025 M Tris base, pH 8.3, 192 mM glycine, and 0.1% SDS). Samples were then transferred to a nylon membrane (0.2 micron Biotrans, ICN, Irvine, CA, USA) at 100 V for 30 minutes. Protein transfer was confirmed by Ponceau S staining. Upon destaining the blot, it was blocked with Blotto (5% nonfat dry milk in TTBS; 0.3 M NaCl, 20 mM Tris base, pH 7.4, 0.5 ml 100% Tween-20) for 1 hour at room temperature. Monoclonal CHS (aC-20) antibody (Santa Cruz Biotechnology, Inc. CA, USA) was diluted 1:200 in 3% bovine serum albumin and incubated with the blot for 1 hr at 37^{o}C. The blot was then washed for five minutes with three times changes of TTBS. Donkey anti-goat antibody conjugated to horseradish peroxidase was diluted 1:3000 in Blotto and incubated with the blot for 1 hr at room temperature. TTBS was again used to wash the blot three times, five minutes each. The blot was then placed in substrate for 10 minutes at room temperature.

4.2.5. Enzyme assay

One hundred µg protein extract (approximately 100 µl) was added to 25 µl malonyl-CoA 0.8 mM (20 nmol) and 25 µl *p*- Coumaroyl-CoA 0.4 mM (10 nmol). The mixture was then made up to 500 µl by adding assay buffer (0.5 M K–Pi of pH 6.8, 2.8 mM 2-mercaptoethanol and 2% BSA (w/v) were mixed. Incubation took place at 30 °C for 1 hr. At the end of the incubation period the mixture was extracted two times using 800 µl EtOAc by mixing using a vortex followed by centrifugation for 2 min. The EtOAc layer was then transferred to a new tube and evaporated using N_2 gas. The

residue was redissolved in 100 µl of HPLC mobile phase and then injected to the HPLC system.

4.2.6. HPLC analysis

The HPLC system consisted of a Waters 712 pump, a Waters 600E system controler, a Waters 717plus autosampler and Waters 991 photo diode array detector (Waters Corp. Milford, MA, USA). The column was a Hypersil C_{18} 240×4.6 mm separation column (Phenomenex, Torrance, CA, USA). The solvent system consisted of solvent A: H_2O containing 0.01% H_3PO_4 and solvent B: CH_3OH containing 0.01% H_3PO_4. The gradient profile was as follows: an isocratic step of 50% B for 1 min, then a linear gradient from 50%–100% of B for 10 min, followed by an isocratic step at 100% of B for 10 min. After this gradient, the eluent was returned to 50% of B for 5 min and was finally kept for 10 min before injection of the next sample. The flow rate was 0.8 ml/min. The chromatogram was monitored at 290 nm.

4.3. Results and discussion

4.3.1. Immunoblot assay

The immunolblot assay was performed to confirm the expression of CHS in CHS transgenic and control plants. Protein was purified from five ACS lines and wild type *Arabidopsis* and analysed first by SDS-PAGE gel (**Figure 4.2A**). The result showed that a protein of ca. 45 kDa accumulated more in transgenic plants. This size is the same as the size of heterogolous CHS. By using an antibody against CHS (*Arabidopsis*) in western blot analysis we confirmed that the band was CHS (**Figure 4.2B**).

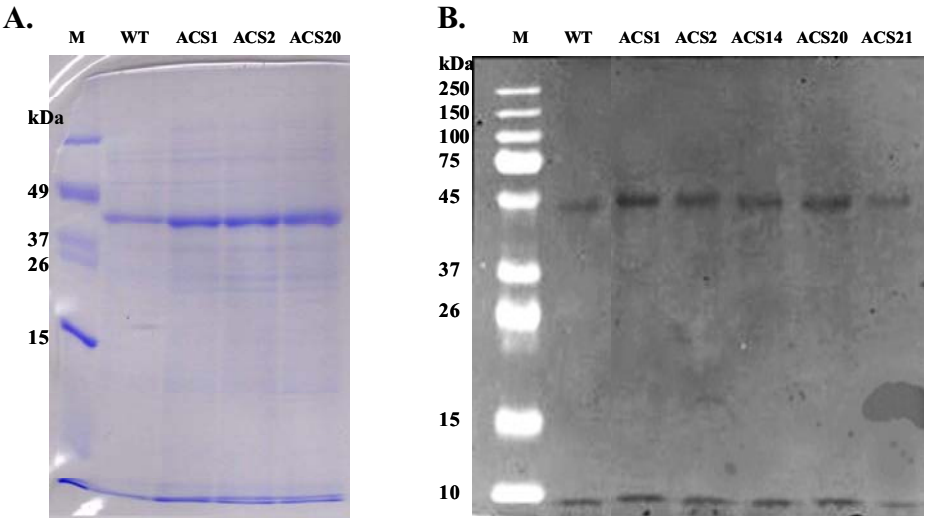

Figure 4.2. SDS PAGE (**A**) of and western blot (**B**) analysis of wild type (WT) and CHS transgenic plants (ACS)

The western blot showed that a wild type CHS band (~43kDa) appeared in the WT *Arabidopsis* lane with a molecular weight a bit lower than the CHS bands of ACS lanes (~45kDa) (**Figure 4.2B**). Also that band did not appear in all CHS transgenic plant lines. Generally, *CHS* is not expressed except under some circumstances such as in the development period or under environmental stress [**Chapter 2**]. In this experiment we used young seedlings for protein extraction so apparently the endogenous CHS *Arabidopsis* was expressed. That explains the appearance of a specific CHS *Arabidopsis* band (~43kDa) in WT lane. The endogenous *CHS* was not expressed in CHS transgenic lines because the induction of heterogolous CHS might inhibit endogenous CHS by co-suppression, or the high level of CHS in transgenic plants might inhibit endogenous CHS expression. This was also observed in CHS transgenic *Petunia* [Van der Krol *et al.,* 1990a]

4.3.2. Chalcone synthase activity assay

The positive result of the western blot confirmed the expression of CHS in the transgenic plant but an enzyme activity study is necessary to confirm activity. *p*-coumaroyl-CoA and malonyl-CoA were used as substrates for the protein extract to test the activity. Naringenin was expected as final product in case CHS activity is present.

A method was developed for measuring naringenin by HPLC. A C18 column and the mobile phase (CH_3OH-H_2O 0.01% phosphoric acid) with the gradient 30%-100% CH_3OH in 40 minutes was found to be suitable. In the HPLC system used, naringenin eluted at a retention time of 24.9 min as a relatively sharp peak with the maximum absorbance wavelength of naringenin at 290 nm. The HPLC elution profiles of the chalcone synthase assay using a protein extract from ACS 2, and ACS 20 five days old seedlings and WT are shown in **Fig. 4.3**. A peak appeared with the same retention time as naringenin reference compound and their UV spectra are the same. High activity was found in ACS 20 (**Figure 4.3C**) whereas CHS activity of transgenic line ACS 2 is similar to wild type. This result is in accordance with the results in **Chapter 3** where we showed that the mRNA expression level of ACS2 is much lower than ACS20 (~12 fold). To learn more about the channeling of substrates related to the flavonoids, a metabolomic study was made (**Chapter 6**)

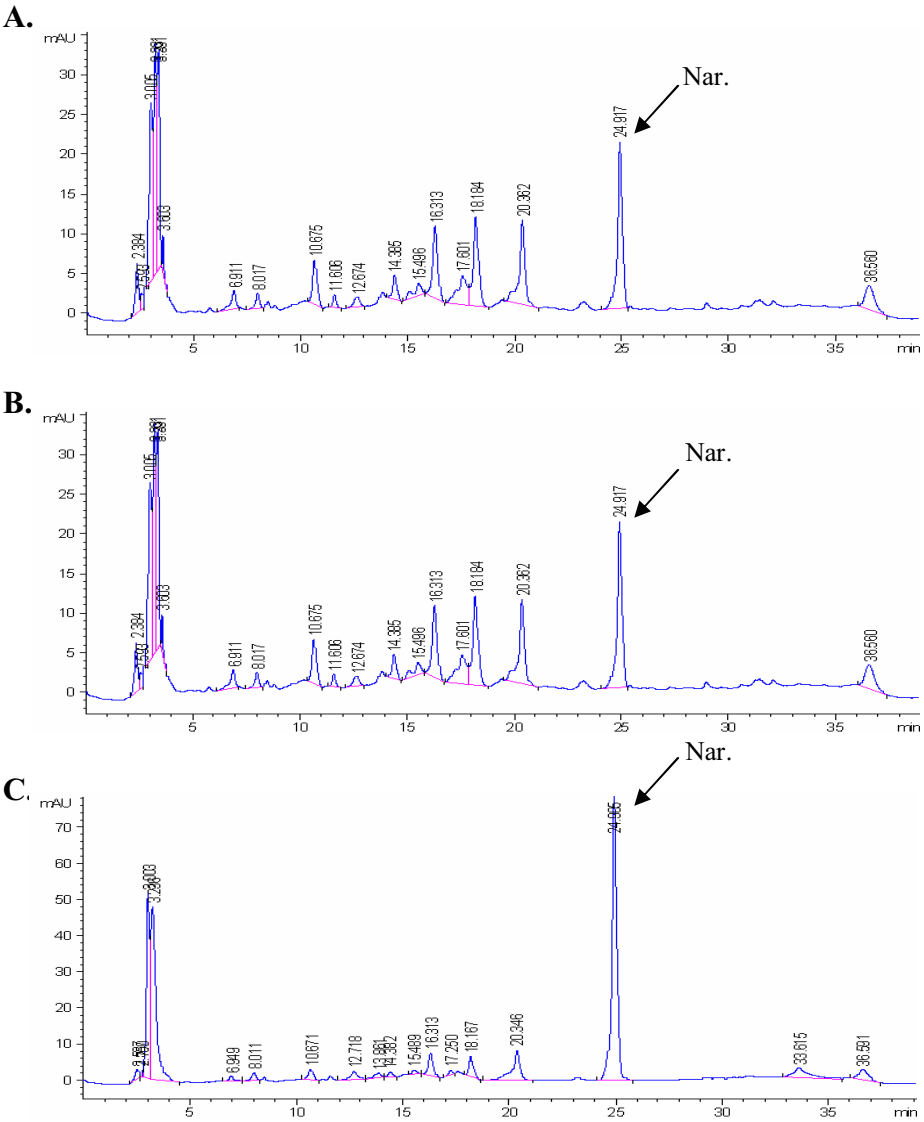

Figure 4.3. HPLC chromatograms of the EtOAc extract from the chalcone synthase activity assay with WT *Arabidopsis* (A), CHS transgenic *Arabidopsis* ACS2 (B) and ACS20 (C) protein extracts