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Metabolic changes in *Arabidopsis thaliana* plants overexpressing chalcone synthase

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Chapter 3

***Agrobacterium* -mediated transformation of *Arabidopsis thaliana* with *Cannabis sativa* cDNA encoding chalcone synthase**

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Abstract

The cDNA encoding chalcone synthase from *Cannabis sativa* was introduced into *Arabidopsis thaliana* Col. 0 via *Agrobacterium tumefaciens*-mediated transformation. This method involved the use of floral dip with disarmed *Agrobacterium* strain LBA4404 containing a plasmid in which the T-DNA region carries the CaMV 35S promoter driven *CHS* gene, as well as *hptII* encoding hygromycin phosphotransferase and the gene encoding the GFP protein. Twenty one transgenic *Arabidopsis* lines (ACS 1 - 21) were collected and six of them were subjected to molecular analysis. The results indicate that the exogenous gene was successfully integrated into the genome and expressed in *Arabidopsis thaliana* plants. All of the six transgenic lines contained multi copies of the *CHS* gene.

Key words: *Arabidopsis thaliana*, *Agrobacterium tumefaciens*, chalcone synthase, transformation.

3.1. Introduction

Chalcone synthases are a family of polyketide synthase enzymes (CHS) catalyzing the first reaction in the flavonoid pathway yielding chalcones, a class of organic compounds found mainly in plants as natural defense compounds and as biosynthetic intermediates. In plant, these compounds serve as antibacterial, antifungal and antitumor and anti-inflammatory activities. Chalcones are also intermediates in the biosynthesis of flavonoids, which are substances widespread in plants, with a wide array of biological activities.

Expression of the *CHS* gene has been well studied in a number of plant species. The expression can be quite differently regulated. E.g., in early developmental stages this enzyme is present in leaf tissue [Knogge *et al.*, 1986], while in adult *Petunia* plants CHS is limited to floral tissue [Koes *et al.*, 1986; Koes *et al.*, 1989]. Environmental stress, such as UV light, phytopathogens and elicitors, or wounding may lead to an induction of *CHS* gene expression [Koes *et al.*, 1989; Winkel, 2002]. *CHS* genes are involved in the biosynthesis of a number of different plant metabolites such as flavonoids, anthocyanins, isoflavonoids and prenylated phenolics. These compounds play important roles in the interaction of plants with the environment. Different substituted cinnamic acid derivatives are the pool from which the enzyme CHS taps the intermediates for the above-mentioned compounds. Moreover cinnamic acid derivatives are precursors for lignin, lignans, coumarins, chlorogenic acids and other esters of cinnamic acid. *CHS* is encoded by a gene family of between 4–8 members in many legume species, such as *Phaseolus vulgaris* [Ryder *et al.*, 1987], *Glycine max* [Estabrook *et al.*, 1991; Wingender *et al.*, 1989], *Medicago sativa* [Dalkin *et al.*, 1990, Junghans *et al.*, 1993], and *Pisum sativum* [An *et al.*, 1993; Harker *et al.*, 1990], whereas *Arabidopsis thaliana* contains only one *CHS* gene in its genome [Feinbaum *et al.*, 1988]. *Arabidopsis thaliana* has one of the smallest genomes among plants, and its genome is completely sequenced. Because of its rapid life cycle it is an important model plant for studying the function of genes. Because of those reasons, *Arabidopsis thaliana* was chosen as model to study *CHS* gene expression in the plant. The *Agrobacterium*-mediated transformation of *Arabidopsis* using the “floral dip” method is a routine protocol [Clough and Bent, 1998]. This method involves simply dipping a flower into a

solution containing *Agrobacterium tumefaciens* bearing the DNA of interest, thus avoiding the need for tissue culture or plant regeneration.

So far, most studies of CHS in plants considered only molecular aspects of gene expression, only few studies have been done on the effects of CHS on the plant metabolome and plant physiology [Koes *et al.*, 1989; Winkel, 2002, Le Gall *et al.*, 2005; reviewed in **Chapter 2**]. Previously, we cloned a polyketide synthase (~1.2Kb) from *Cannabis sativa* young leaves. By expression of the cDNA encoding CHS in *Escherichia coli* the gene product was shown to have CHS activity [Raharjo *et al.*, 2004]. In the present study, we investigated the effect of the overexpressed CHS on the biosynthesis pathways in *A. thaliana* plants.

3.2. Materials and Methods

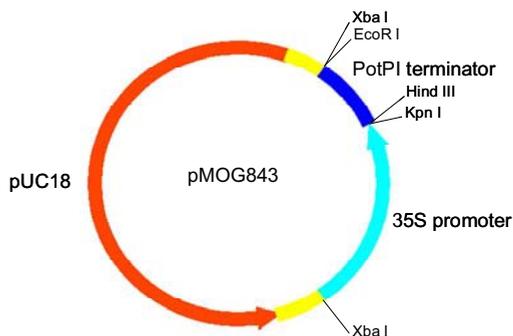
3.2.1. Plant materials

Arabidopsis thaliana ecotype Col-0 seeds were obtained from the section Plant Cell Physiology (IBL, Leiden University, The Netherlands) and were used throughout the study. Seeds were sown on a mixture of vermiculite, peat moss, and perlite 2:1:1 (by vol.). The pots were placed at 4°C for 4 days in the dark and transferred to a growth chamber at 21°C and long day conditions (16/8 h light/dark cycle). When the primary inflorescence reached 5 to 10 cm, plants were clipped to favor the growth of multiple secondary bolts.

For the molecular experiments, the samples (leaves of transgenic and non-transgenic plants) were collected, frozen immediately into liquid nitrogen and kept at -80°C.

3.2.2. Transformation vectors

A.



Transformation of *Arabidopsis* was according to the floral dip method [Clough and Bent, 1998] using *Agrobacterium tumefaciens* LBA4404 with minor modifications. Transgenic plants were selected on half MS medium containing 25mg/l hygromycin. Fluorescence of GFP protein in transgenic *Arabidopsis* was visualized by using an inverted Axiovert Zeiss 100 M microscope (Zeiss, Jena, Germany). After further selection of transgenic lines with a 3:1 segregation ratio, T₃ or T₄ homozygous lines were used for the phenotypic investigation.

3.2.4. Extraction of DNA

Approximately 100 mg of leaf tissue from transgenic and non-transgenic plants was ground to a fine powder under liquid nitrogen. DNA was isolated by using a DNAeasy Plant Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Each DNA sample was dissolved in 50 µL sterile ddH₂O, and 2 µL of DNA solution was used for each real-time PCR. DNA was quantified by spectrophotometric measurements.

3.2.5. Extraction of RNA and RT-PCR

Total RNA was extracted from the frozen samples by using the Plant RNeasy extraction kit (Qiagen, The Netherlands). To remove residual genomic DNA, the RNA was treated with an RNase-free DNaseI according to the manufacturer's instructions (Qiagen). The concentration of RNA was measured by spectrophotometer, and 5 µg of total RNA was separated on 1.2% formaldehyde agarose gel to check the concentration and to monitor integrity. RT-PCR was employed to detect the expression of *chs* in the transgenic *Arabidopsis* plants. A 500 ng sample of total RNA was used in the RT-PCR reaction.

3.2.6. Northern blot analysis

Total RNA (30 µg) was used for each experiment. Denatured RNA was subjected to electrophoresis through a 1.2% agarose/formaldehyde gel in MOPS buffer [Sambrook *et al.*, 1989] and then transferred onto a nylon membrane as described by Sambrook *et al.*. The RNA-labelled probes were synthesized using digested pCAMBIA-CHS, T7 (or T3) polymerase (Gibco-BRL) and ³²P UTP using the Riboprobe Gemini II core system kit (Promega).

RNA quantification was achieved by ethidium bromide staining. This experiment was repeated with different RNA extracts with the same RNA quantity and similar patterns were obtained for each analysis.

3.2.7. Quantitative real-time PCR and calculation methods

Quantitative real-time PCR was performed on a Chromo4 Real-Time PCR Detector system (Bio-Rad laboratories). Samples were amplified in a 50 µl reaction containing 1× SYBR Green Master Mix (Eurogentec, Maastricht, The Netherlands) and 300 nM of each primer. The thermal profile consisted of 1 cycle at 95°C for 5 min followed by 40 cycles at 95°C for 0.5 min, at 58°C for 0.5 min and 72°C for 1 min.

Changes in gene expression and copy number of the transgene as a relative fold difference between transgenic samples and control ones were calculated using the comparative C_t ($2^{-\Delta\Delta C_t}$) method [Livak *et al.* 2001; Winer *et al.* 1999; Ingham *et al.*, 2001; Schmittgen *et al.*, 2000]. *Actin3* gene was used as a reference gene for normalization. To exclude the DNA genomic contamination in the total RNA samples, the intron actin was used as a reference matrix.

Final copy number was calculated according to the following equation.

Copy number = $2^{-\Delta\Delta C_t}$ where, $\Delta\Delta C_t = \Delta C_t$ (unknown sample) – ΔC_t (reference).

In the copy number of transgenes experiment, the reference C_t is the C_t of 4-Hydroxyphenylpyruvate Dioxygenase gene (4HPPD) from *Arabidopsis*, because it has only a single copy in the *Arabidopsis* genome [Garcia *et al.*, 1999].

The PCR primer sets for real-time PCR are shown in **Table 3.1**.

Table 3.1. PCR primer sets

Primer name	Sequence
CHSR	5' CGCGGATCCGGTACCGTGGAGGAATTTC 3'
CHSF	5' CGCGGATCCCTAAATAGCCACACTGTGAAGG 3'
qCHSR	5' CTATTGGTGATCCTGAAGTAGTAATCC 3'
qCHSF	5' ACCGTGGAGGAATTCGCAAGG 3'
4HPPDR	5' TCATCCCCTAAATGTTTGGCTTC 3'
4HPPDF	5' GTGTCTATCGTTAGCTTCTACAGC 3'
ACTINR	5' CAGCGATACATGAGAACATAGTGG 3'
ACTINF	5' CCTCATGCCATCCTCCTGCT 3'
ACTINF-uni	5' AGTGGTCGTACAACCGGTATTGT 3'
ACTINR-7	5' GAGGAAGAGCATACCCCTCGTA 3'
ACTINF-7	5' GTTGTACATGTGTAAGACTACTGATCATG 3'

3.2.8. Reagents

Oligonucleotide primers were purchased from Isogen Benelux (IJsselstein, The Netherlands). Nucleoside triphosphates were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Invitrogen (Breda, The Netherlands) provided restriction endonucleases. All PCR and ligation reagents were purchased from Promega (Leiden, The Netherlands). Miniprep, plant genomic extraction, RT-PCR, and PCR product purification kits were purchased from Qiagen (Venlo, The Netherlands). Bacterial and plant growth media components were all purchased from Gibco-BRL (Breda, The Netherlands), Sigma-Aldrich (Zwijndrecht, The Netherlands).

3.3. Results and discussions

3.3.1. Transformation

The binary vector suitable for *A. tumefaciens*-mediated transformation was prepared with full-length *Cannabis sativa chs*-cDNA [Raharjo, 2004]. This binary vector named *chs*-pCAMBIA contains the *chs* coding region under the control of the constitutive CaMV-35S promoter. The construct also contains the hygromycin phosphotransferase (*HPT*) gene and the green fluorescent protein (*GFP*) reporter gene. *Arabidopsis* flowers were inoculated with a suspension of hypovirulent *A. tumefaciens* when numerous immature floral buds and only a few siliques were present. This method is simple and a high rate of transformed plants can be obtained. The transformation was successful and twenty one transgenic *Arabidopsis* lines were established and named ACS1- ACS21. Amongst these six transgenic lines (ACS1, ACS2, ACS3, ACS14, ACS20, and ACS21) were selected randomly for further molecular analysis.

3.3.2. Transgene expression experiments

ACSs were selected in half MS containing Hygromycin (25 mg/ml). Expression of GFP protein in ACSs can be detected in 5 days old seedlings (**Figure 3.2**). **Figure 3.2A** shows an ACS plantlet with high expression of GFP protein; GFP protein is present in all plant tissues. **Figure 3.2B** shows a plantlet with low expression of GFP, in which the GFP protein is only visible in the trichomes.

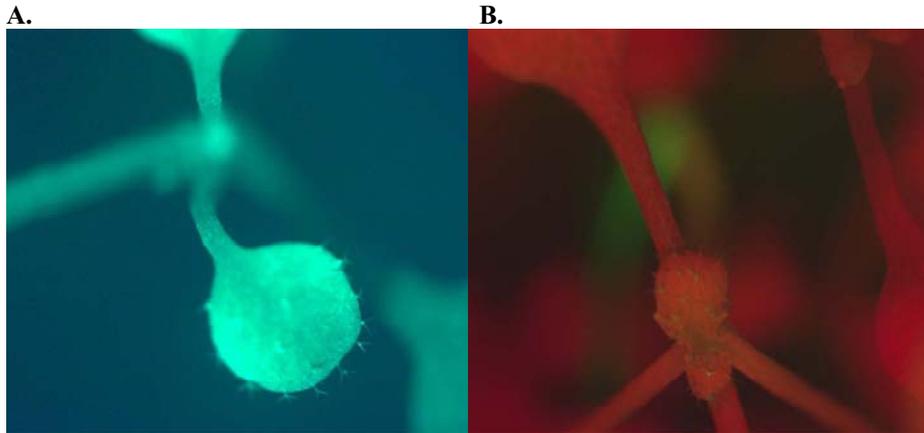


Figure 3.2. A. Transformed *Arabidopsis* with high GFP expression, B. Transformed *Arabidopsis* with low GFP expression

Common genetic transformation methods such as *Agrobacterium*–mediated transformation frequently result in multiple transgene copies at the same or different integration sites [Kohli *et al.*, 1998; Srivastava *et al.*, 1999, De Neve *et al.*, 1997; De Buck *et al.*, 1999; Tzfira *et al.*, 2006]. In transformed plants, the first step to be done is to estimate how many copies of the transgene have been integrated in the plant genome because this may influence the level of transgene expression and the ease of stabilizing expression in following generations. This can be measured by Southern blot analysis, but in this study we used Real-time PCR to estimate the gene copy number in our transgenic plants. This method has shown to be a reliable tool for such analyses [Li *et al.*, 2004, Yuan *et al.*, 2007, Mitrecic *et al.*, 2005].

In the real-time PCR assay, DNA samples from a CHS transgenic plant were serially diluted 2-fold to obtain a standard curve. Standard curves for the endogenous *Actin3* gene the *CHS* transgene and reference gene (*4HPPD*) were produced by using Opticon Monitor Continuous Fluorescence Detector software (**Figure 3.3**). The correlation (R) between C_t value and \log_{DNA} concentration was 0.99 for the *Actin* gene, *CHS* transgene and reference gene. The DNA concentration was linear with respect to gene copy number. The results confirm the linear relationship between C_t value and \log_{DNA} concentrations, thus making the C_t value a reliable way to quantify DNA amount to

estimate gene copy number as both genes amplify with approximately equal efficiencies and always constant regardless of DNA concentration.

Table 3.2. Estimated CHS copy number from real-time PCR

Transgenic line	Estimated Copy Number by Real-Time PCR
ACS 1	7-8
ACS 2	5
ACS 14	3-4
ACS 20	7
ACS 21	5

All transgenic plant lines showed multicopies of the transgene. Multiple transgene copies may cause a higher expression of mRNA or even cause transgene silencing [Flavell, 1994; Iyer *et al.*, 2000; Vaucheret *et al.*, 1998] so we used northern blot analysis to detect the expression level of the cannabis *CHS* transgene in all six transgenic plant lines. The results showed that the steady-state level of *GFP*-mRNA was slightly induced in ACS1, 3 and 20 and strongly induced in ACS2, 14 and 21 but unfortunately we were not able to detect *chs* mRNA on the northern blots (results not shown). Apparently, the steady-state levels of *chs*-mRNA expression are low in the transgenic *Arabidopsis* lines. Therefore, we used RT-PCR and real-time PCR to detect and quantify levels of *chs*-mRNA expression in transgenic *Arabidopsis*.

The RT-PCR result is presented in **Figure 3.4**. It shows that *chs*-mRNA is present in all ACS lines (**Figure 3.4A**) and no genomic DNA contamination was detected in RNA samples (**Figure 3.4B**). Thus only expression of the gene is measured. The *chs*-mRNA expression levels were quantified and can be seen in **Figures 3.5**. The expression levels are very low in transgenic line 2 whereas transgenic line 1 and lines 20 have high expression levels (**Figures 3.5**).

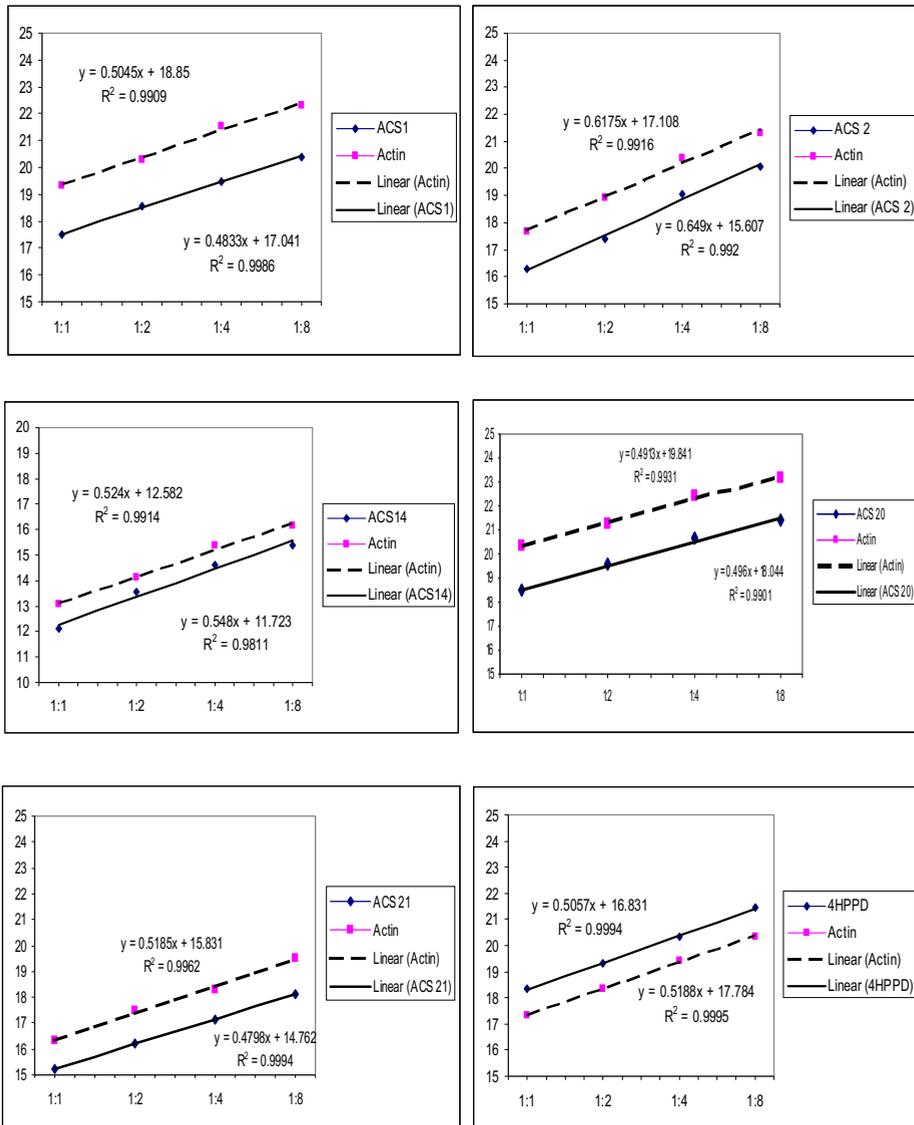


Figure 3.3. Efficiency of duplex real-time PCR for detection and quantitation of *Actin* and *CHS* DNA from a transgenic plant or a nontransgenic plant was diluted serially 2-folds.

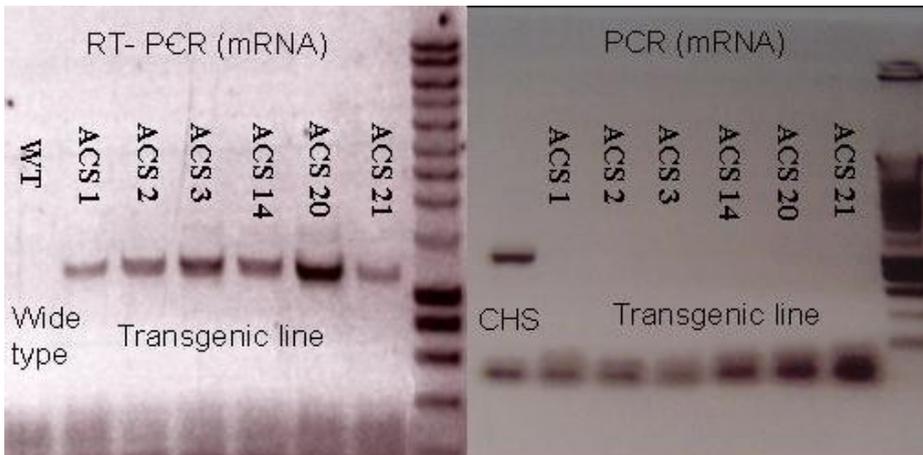


Figure 3.4. A. Qualitative analysis of *CHS* gene expression by RT-PCR, B. Analysis of genomic DNA contamination in mRNA samples by PCR

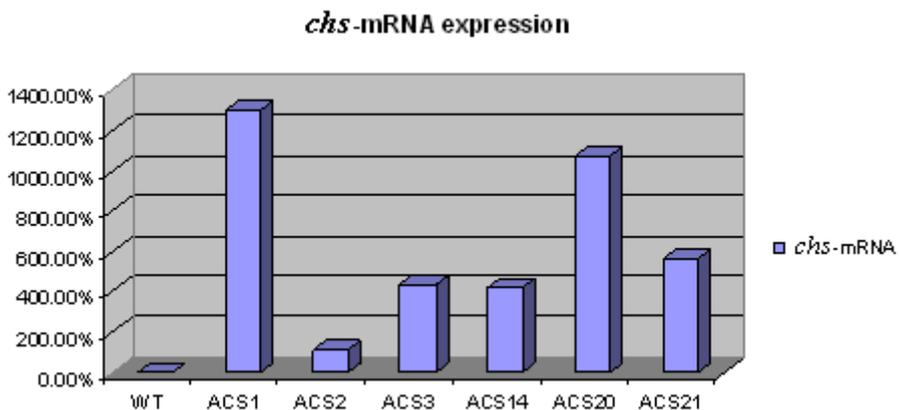


Figure 3.5. *chs*-mRNA expression levels optimized by Real-time PCR

3.4. Conclusions

Among 21 *CHS* transgenic *Arabidopsis* lines, 6 lines (ACS 1, ACS 2, ACS 3, ACS 14, ACS 20, and ACS 21) were analysed for their transcriptional and genomic levels. We found that *chs*-mRNA was expressed in all 6 transgenic lines and all contain multicopies of *CHS*. The metabolic changes due to the transformation will be studied in these lines.