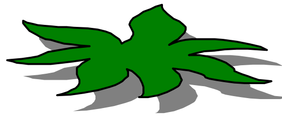
Metabolic changes in *Arabidopsis thaliana* plants overexpressing chalcone synthase



Thi Thanh Hien Dao

Metabolic changes in *Arabidopsis thaliana* plants overexpressing chalcone synthase

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List of Abbreviations

ACS	*Arabidopsis* chalcone synthase
BSA	bovine serum albumin
CHS	chalcone synthase
COSY	correlated spectroscopy
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
EtOAc	ethyl acetate
GABA	γ-amino butyric acid
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
PCA	principal component analysis
PLS-DA	partial least square-discriminant analysis
PMSF	phenylmethanesulphonylfluoride
PVP	polyvinylpolypyrrolidone
SDS	sodium dodecyl sulfate
TTBS	Tween® / Tris-buffered salt solution
UV	ultra violet

Chapter 1

General Introduction

T.T.H. Dao

Crop plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavors, fragrances, food, and pesticides [Balandrin and Klocke, 1988]. An in-depth understanding of the plant's metabolism is helpful for the improvement of their growth and yield [Carrari *et al.,* 2003]. As we know, green plants produce simple sugars by combining CO_2 and H_2O with energy from the sun by photosynthesis. Plants use sugars to make primary compounds such as starch, pectin, cellulose, fat, amino acids, proteins and nucleic acids for nutrition and construction of the plant structure. They also produce compounds which seem to have no explicit use for the plants and these are usually termed secondary metabolites. Secondary metabolites are defined as compounds that play a role in the interaction of the cell/organism with its environment to ensure the survival of the organism in its ecosystem [Verpoorte, 2000]. There are many secondary metabolites present in plants and they are classed in groups such as alkaloids, terpenoids, flavonoids, essential oils, phenolics and others.

Metabolic engineering of plants promises to create new opportunities in agriculture, environmental applications, production of chemicals, and even medicine. Metabolic engineering is referred to as the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones, with the use of recombinant DNA technology [Stephanopoulos, 1999]. It is generally referred to as "*the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly*" [Lessard, 1996]. Plant biotechnology and transgenic plants are based on the latest technologies and current research on the engineering, synthesis, utilization, and control of primary and secondary plant metabolism. In terms of DNA techniques, several approaches have been used for

the modification of the host cell to achieve the desired goal. These include: inhibiting gene expression or inhibiting encoded enzyme itself to eliminate a competitive pathway or a toxic product; amplification of a gene or group of genes to improve the synthesis of existing products [Shimada *et al.*, 1998; Cameron *et al.*, 1998] the expression of a heterologous enzyme(s) to extend the substrate range [Panke *et al.*, 1998], to produce novel products [Stassi *et al.*, 1998], to provide pathways for the degradation of toxic compounds [Keasling *et al.*, 1998; Xu *et al.*, 1996], or to design a more environmentally resistant plant [Smirnoff, 1998].

Among the secondary metabolite groups in plants, flavonoids are the most common group of polyphenolic plant secondary metabolites. In plants, flavonoids play an important role in biological processes. Besides their function as pigments in flowers and fruits to attract pollinators and seed dispersers, flavonoids are involved in UV-scavenging, fertility and disease resistance [Winkel, 2001]. Since flavonoids are present in a wide range of fruits and vegetables, flavonoids form an integral part of the human diet. Currently there is broad interest in the effects of dietary polyphenols on human health. In addition to the potent antioxidant activity of many of these compounds *in vitro*, an inverse correlation between the intake of certain polyphenols and the risk of cardiovascular diseases, cancer and other age related diseases has been observed in epidemiological studies [Harborne *et al.,* 2000]. Enhancing flavonoid biosynthesis in chosen crops may provide new raw materials that have the potential to be used in food designed for specific benefits to human health.

Characterization of flavonoid biosynthesis at the genetic level has been done in *Arabidopsis,* maize, snapdragon, parsley and petunia [Christie *et al.,* 1996; Feldbrügge *et al.,* 1997; Wade *et al.,* 2001; Koes *et al.,*1989; Junghans *et al.,*1993]. Since *Arabidopsis thaliana* was the first plant to have its entire nuclear genome sequenced, it has become the most important model system for plant biology. *Arabidopsis* is particularly useful in the characterization of the flavonoid biosynthetic pathway due to the relative simplicity of the genetics for the pathway's enzymes and with exception of flavonol synthase, all the major enzymes of the flavonoid biosynthesis pathway in *Arabidopsis* are encoded by single-copy genes [Winkel, 2001]. In all plants, the precursor of the first flavonoid molecule is naringenin chalcone. Naringenin chalcone is synthesized by the first enzyme of the flavonoid biosynthesis, chalcone synthase (CHS).

Modifications by specific suites of downstream enzymes this intermediate goes into a variety of end products.

Many analytical methods e.g. gas chromatography (GC)/mass spectrometry (MS), high-performance liquid chromatography (HPLC)/MS, capillary electrophoresis (CE)/MS, and nuclear magnetic resonance spectroscopy (NMR) have been used for identification of metabolites in crude plant extracts. There is no single technique that allows a comprehensive detection of all metabolites but in principle ^{1}H-NMR can detect any metabolite containing hydrogen. Thus the ^{1}H-NMR spectra of biological fluids or tissue extracts are a rich source of qualitative and quantitative information on the compounds present, covering compounds of all chemical classes. NMR is therefore considered as an important technique that can contribute to metabolic profiling of an organism.

Furthermore, integration of metabolomic data with other -omic data is performed to identify the gene/protein functions and eventually leading to metabolic and cellular simulation *in silico*. For this purpose, data processing and analysis methods have to be applied. For example multivariate data analysis such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) are carried out for data mining and can be used for identification of biomarkers for the response of the plants to certain forms of stress, comparison of plants for identifying resistance related compounds, chemotaxonomy and for quality control of food and botanicals.

Many studies related to CHS or using CHS mutant plants have been published [Mol *et al.,* 1983; Saslowsky *et al.,* 2001; Le Gall *et al.,* 2005] but so far most studies are restricted to the molecular level and information on metabolic changes is still lacking. Introduction of *CHS* in *Arabidopsis thaliana* would be the way to study the effect of overexpression of this gene on the metabolome of the plants and the flavonoid biosynthesis pathway.

Aim of thesis

The aim of the present study was to investigate the effect of chalcone synthase (*CHS*) overexpresion in *Arabidopsis thaliana* on primary and secondary metabolism.

Outline of the thesis

This thesis starts with a review of the function of CHS in plants and especially in plant resistance (**Chapter 2**). **Chapter 3** deals with the work on *Agrobacterium*-mediated transformation of heterologous chalcone synthase in *Arabidopsis thaliana* Col. 0. The effect of overexpression of *CHS* on the transcriptional level is discribed in this chapter. The activity of the CHS enzyme in the transgenic plants is reported in **Chapter 4.** In **Chapter 5** metabolic profiling of *Arabidopsis thaliana* using nuclear magnetic resonance spectroscopy (NMR) is described. In this chapter the primary and secondary metabolites of *Arabidopsis thaliana* Col. 0 which can be detected by NMR are reported. **Chapter 6** reports the metabolic profiling of CHS transgenic *Arabidopsis.* Metabolomic changes upon UV-A/blue light treatment of *Arabidopsis thaliana* were investigated (**Chapter 7). Chapter 8** deals with the study of the effect of the non-pesticide chemical, Benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) on the *Arabidopsis* metabolome. Finally, the general summary and discussion of thesis are given in **Chapter 9**.

Chapter 2

Chalcone synthase and its funtions in plant resistance

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Abstract
Chalcone synthase (CHS, EC 2.3.1.74) is a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway. Besides being part of the plant developmental program the *CHS* gene expression is induced in plants under stress conditions such as UV light, bacterial or fungal infection. CHS expression causes accumulation of flavonoid and isoflavonoid phytoalexins and is involved in the salicylic acid defense pathway. This review will discuss CHS and its function in plant resistance.

Keywords: Chalcone synthase, flavonoids, plant resistance.

2.1. Introduction

During their life cycle, plants respond actively to stress by producing phytoalexins and other stress metabolites. Such stress can result from injuries caused by the attack of insects and microbes or by mechanical wounding, and can induce many distinctive biochemical changes. These include the production of protective compounds either at the site of injury, or systemically in distant unwounded tissues [Kuhn, 1988; Bowles, 1990; Ryan, 1990]. In plants, phenylalanine is derived from the precursor chorismate and leads to the flavonoid, phenylpropanoid and stilbenoid biosynthesis pathways. All are interesting in connection with plant defense but in this review we will focus on the flavonoid biosynthesis pathway and its key enzyme chalcone synthase (CHS).

CHS is a member of the plant polyketide synthase superfamily, which also includes stilbene synthase (STS), acridone synthase, pyrone synthase, bibenzyl synthase, and *p*-coumaroyltriacetic acid synthase [Sanchez *et al.,* 2008]. Chalcone synthases, the most well known representatives of this family, provide the starting materials for a diverse set of metabolites (flavonoids) which have different and important roles in flowering plants, such as providing floral pigments, antibiotics, UV protectants and insect repellents [Hahlbrock and Scheel, 1989]. Flavonoids also have benefits for human health, as they exhibit amongst others cancer chemopreventive [Jang *et al*., 1997], antimitotic [Edwards *et al.,* 1990], estrogenic [Gehm *et al*., 1997] antimalarial [Li *et al.,* 1995] antioxidant [Jang *et al*., 1997] and antiasthmatic [Zwaagstra *et al.,* 1997] activities.

Flavonoids are synthesized via the phenylpropanoid and polyketide pathway, which starts with the condensation of one molecule of CoA-ester of cinnamic acid or derivatives such as coumaric or ferulic acid, and three molecules of malonyl-CoA, yielding a naringenin chalcone as major product. This reaction is carried out by the enzyme chalcone synthase (CHS). The chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyzes the stereospecific 3ß-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of glucosides is catalyzed by UDP glucose-flavonoid 3-*O*-

glucosyl transferase (UFGT), which stabilizes the anthocyanidins by 3-*O*-glucosylation [Harborne and Grayer, 1994; Bohm, 1998]. An overview of the flavonoid pathway is presented in **Fig. 2.1**. Flavonoids play an important role in plant defense, and CHS as the gatekeeper of flavonoid biosynthesis plays an important role in regulating the pathway. In fact *CHS* gene expression is influenced by many stress and environmental factors such as UV, wounding or pathogen attack [Dixon and Paiva 1995; Gläßgen *et al.,* 1998; Ito *et al.,* 1997].

In this review we will evaluate the present understanding about CHS and its regulation in plant resistance.

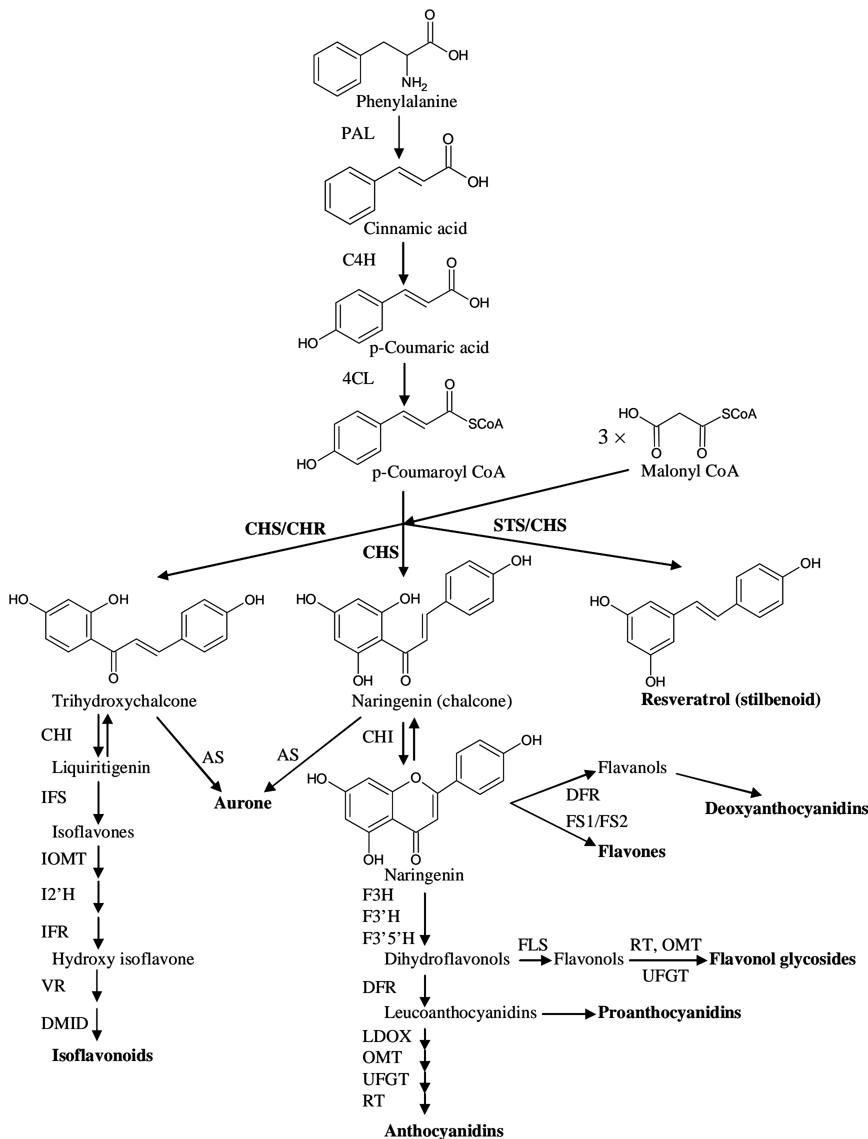


Figure 2.1. Flavonoid biosynthetic pathway

ANS, anthocyanidin synthase; AS, aureusidin synthase; C4H, cinnamate-4-hydroxylase; CHR, chalcone reductase; DFR, dihydroflavonol 4-reductase; DMID, 7,2′-dihydroxy, 4′-methoxyisoflavanol dehydratase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′ hydroxylase; F3′5′H, flavonoid 3′5′ hydroxylase; FS1/FS2, flavone synthase; I2′H, isoflavone 2′-hydroxylase; IFR, isoflavone reductase; IFS, isoflavone synthase; IOMT, isoflavone *O*-methyltransferase; LCR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; OMT, *O*-methyltransferase; PAL, phenylalanine ammonia-lyase; RT, rhamnosyl transferase; UFGT, UDP flavonoid glucosyl transferase; VR, vestitone reductase; STS, stilbene synthase; FLS, flavanol synthase.[Winkel, 1999; KEGG pathways]

2.2. Chalcone synthase

2.2.1. Structure of chalcone synthase

The chalcone synthase (CHS) enzyme - known as a type III polyketide synthase enzyme (PKS) is structurally and mechanistically the simplest PKS [Schröder *et al.,* 1997; Sanchez *et al.,* 2008]. These enzymes function as homodimeric iterative PKS (monomer size of 42–45 kDa) with two independent active sites that catalyze a series of decarboxylation, condensation, and cyclization reactions [Tropf *et al*., 1995]. The three dimensional structure of alfalfa CHS2 was studied intensively by Ferrer *et al.,* (1999). X-ray crystallography revealed that each alfalfa CHS2 monomer consists of two structural domains. The conserved architecture of the upper domain maintains the three-dimensional structure of the catalytic residues of the enzyme (Cys164, His303, and Asn336) was defined as the catalytic machinery of CHS. The lower domain of CHS has a large active site providing space for the tetraketide required for chalcone formation (i.e., naringenin and resveratrol) from one p-coumaroyl-CoA and three malonyl-CoA [Jez *et al.,* 2001a,b]. Cys164 serves as the nucleophile for polyketide formation and is not essential for malonyl-CoA decarboxylation. His303 plays a role in chalcone formation and malonyl-CoA decarboxylation, as histidine is able to abstract a proton from Cys164 to form the reactive thiolate necessary for initiation of the polyketide elongation. Asn336 works as the second component of the decarboxylation machinery. Phe215 was proposed to interact with acyl-CoA substrates through van der Waals interactions.

The crystal structure further revealed three interconnected cavities that intersect with the four catalytic residues and form the active site architecture of the CHS: a CoA-binding tunnel, a coumaroyl-binding pocket and a cyclisation pocket. This division of the active site into discrete pockets provides a structural basis for the ability of the CHSs to orchestrate the multiple reactions of chalcone synthesis.

2.2.2. Mechanism of chalcone synthase

Production of chalcone requires the condensation of one molecule of *p*-coumaroyl-CoA and three malonyl-CoA molecules which is catalyzed by CHS. It starts with the transfer of a coumaroyl moiety from a *p*-coumaroyl-CoA starter molecule to an active site

cysteine (Cys164) [Lanz *et al.,* 1991]. Next, a series of condensation reactions of three acetate units derived from three malonyl-CoA molecules, each proceeding through an acetyl-CoA carbanion derived from malonyl-CoA decarboxylation, extends the polyketide intermediate. Following generation of the thioester-linked tetraketide, a regiospecific intramolecular Claisen condensation forms a new ring system to yield chalcone. In plants, chalcone isomerase (CHI) will convert the chalcone to (2*S*)-5,7,4'-trihydroxyflavanone (naringenin); however, spontaneous ring closure *in vitro* results in mixed enantiomers of naringenin [Hahlbrock *et al.,* 1970*;* Jez *et al.,* 2000]. *In vivo* chalcone can convert to narigenin without need of CHI. Four amino acids (Cys164, Phe215, His303, and Asn336) situated at the intersection of the CoA-binding tunnel and the active site cavity play an essential and distinct role during malonyl-CoA decarboxylation and chalcone formation. The general reaction mechanism of CHS is presented in **Figure 2.2.**

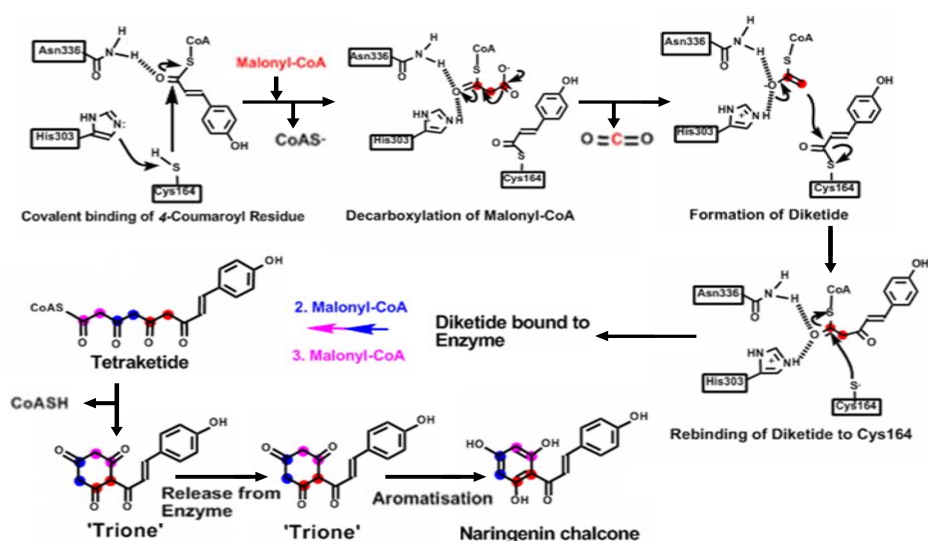


Figure 2.2. Reaction Mechanism of Chalcone Synthase (CHS)

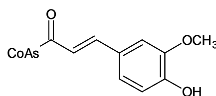
In CHS, three amino acids play key roles in the catalytic functions of type III PKS: Cys164: active site, covalent binding site of starter residues and intermediates, His303 and Asn336: stabilization/activation of both starter (e.g. 4-coumarate) and extender units (malonyl-/acetyl-residues) [Ferrer *et al.,* 1999, Bomati *et al.,* 2005, modified by Schröder 2008].

Several other cyclization reactions are possible besides the one yielding a chalcone. In addition to the starter molecule *p*-coumaroyl-CoA, *in vivo* alfalfa CHS accepts other CoA-linked thioesters as alternate starter molecules to generate corresponding chalcones, tetraketide lactone, and triketide lactone products (**Figure 2.3**). The substrates can be feruloyl-CoA, hexanoyl-CoA, phenylacetyl-CoA, benzoyl-CoA, butyryl-CoA, isobutyryl-CoA and isovaleryl-CoA. With the starter substrates p-coumaroyl-CoA and malonyl-CoA, CHS catalyzes an intramolecular Claisen condensation yielding the chalcone naringenin. Alfalfa CHS2 and parsley CHS [Hrazdina, 1976], accept feruloyl-CoA as a starter molecule and produce the tetraketide lactone (2b) and methylpyrone as the major products with the triketide lactone (2c) generated as a minor product. With hexanoyl-CoA, alfalfa CHS2 yields the tetraketide lactone (3b) as the major product, triketide lactone (3c) and methylpyrone are minor products [Jez *et al.,* 2001a]. Parsley CHS accepts butyryl-CoA and hexanoyl-CoA as substrates *in vitro*, which yield, respectively, the chalcone analogues, phlorobutyrophenone (5b) and phlorocaprophenone (4b) at pH 6.5 [Schuez *et al.,* 1983]. *Medicago sativa* CHS2 accepts phenylacetyl-CoA as a starter molecule yielding a phlorobenzyl ketone (4a), the chalcone-like product, accounts for less than 10% and others like tetraketide lactone (4b), triketide lactone (4c), and methylpyrone comprise the other products. The overall product distribution with phenylacetyl-CoA is similar to *Scutellaria baicalensis* CHS [Morita *et al.,* 2000]. With benzoyl-CoA as the starter molecule, alfalfa CHS2 generates phlorobenzophenone (5a) and methylpyrone as the major product, and tetraketide lactone (5b) and triketide lactone (5c) as minor products [Jez *et al.,* 2001a]. The recombinant hop CHS1 expressed in *E. coli* showed activity with isobutyryl-CoA and isovaleryl-CoA substrates, which produced as main products phloroisobutyrylphenone (6b) and phloroisovalerophenone (7b) [Zuurbier *et al.,* 1998; Novák *et al.,* 2006].

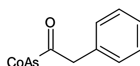
Table 2.1. Steady-State Kinetic Constants of *Medicago sativa* CHS2 with different starter substrates [Jez *et al.,* 2001a; Novak *et al.,* 2006]

	*k*cat (min^{-1})	*K*m (μM)
p-coumaroyl-CoA	5.14 ± 0.30	6.1 ± 1.3
Malonyl-CoA	4.58 ± 0.24	4.7 ± 1.1
feruloyl-CoA	1.04 ± 0.17	5.2 ± 0.9
Hexanoyl-CoA	2.52 ± 0.22	4.1 ± 1.2
phenylacetyl-CoA	2.17 ± 0.35	5.1 ± 0.7
benzoyl-CoA	1.73 ± 0.21	2.2 ± 0.2
Isobutyryl-CoA	-	14.9 ± 0.2
Isovaleryl-CoA	-	8.0 ± 0.2

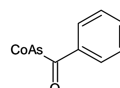
Starter molecules



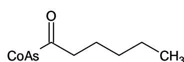
Feruloyl-CoA (1)
R1= [-CH=CH-C_6H_3(OH)(OCH_3)]



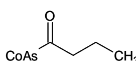
Phenylacetyl-CoA (2)
R2= [-CH_2 -C_6H_5]



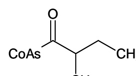
Benzoyl-CoA (3)
R3= [-C_6H_5]



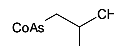
Hexanoyl-CoA (4)
R4= [-CH_2-CH_2-CH_2-CH_2-CH_3]



Butyryl-CoA (5)
R5= [-CH_2-CH2 –CH_3]

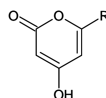


Isobutyryl-CoA (6)
R6= [-CH–(CH_3)$_2$]

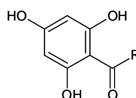


Isovaleryl-CoA (7)
R7= [-CH_2-CH–(CH_3)$_2$]

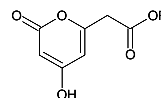
Products



Chalcones
1a: R=R1
2a: R=R2
3a: R=R3
4a: R=R4
5a: R=R5



Tetraketide lactone
1b: R=R1
2b: R=R2
3b: R=R3
4b: R=R4
5b: R=R5
6b: R=R6
7b: R=R7



Triketide lactone
1c: R=R1
2c: R=R2
3c: R=R3
4c: R=R4

Figure 2.3. Alternate starter molecules and their predicted reaction products catalyzed by CHS

The steady-state kinetic parameters of *Medicago sativa* CHS2 for *p*-coumaroyl-CoA, malonyl-CoA, feruloyl-CoA, hexanoyl-CoA, phenylacetyl-CoA and benzoyl-CoA have been determined, these are presented in **Table 2.1** [Jez *et al.,* 2001a; Novak *et al.,* 2006]

2.3. Control of CHS activity

In plants, CHS is activated by a wide range of environmental and developmental stimuli. Theoretically, there are many ways to regulate CHS activity *in vivo,* from metabolic control to the control of initiation of transcription of the *CHS* gene [Martin, 1993].

2.3.1. Metabolic control

There are many studies showing that CHS is inhibited noncompetitively by flavonoid pathway products like naringenin, chalcone naringenin and the other end products of CoA esters. For example, the parsley CHS is 50% inhibited by 100 μM naringenin and 10 μM CoA esters [Hinderer and Seitz, 1985; Kreuzaler and Hahlbrock, 1975], the flavonoids luteolin and apigenin are inhibitory to rye CHS *in vitro* [Peters *et al.,* 1988], whereas in carrot, among the range of flavonoids tested, only naringenin and chalcone narigenin can inhibit CHS at 100 μM [Hinderer and Seitz, 1985]. It seems that flavonoids accumulate in the cytosol to a level that blocks CHS activity to avoid toxic levels for the plant [Whitehead and Dixon, 1983], though there is no direct evidence that this inhibition happens *in vivo*.

2.3.2. Control of CHS *turnover*

In plants, CHS may always be present in the cells but is only activated under certain specific conditions. Studies on parsley cell cultures showed that the induction of CHS activity by UV light was the result of *de novo* synthesis and active enzyme subsequently decayed with a half-life of 6h, whereas inactive enzyme decayed more slowly with a half-life of 18h [Schröder and Schäfer, 1980]. Inactive CHS could be detected by CHS antibodies and the size of the protein was not changed. In another study about accumulation of CHS during UV induction, Chappell and Hahlbrock (1984) concluded that the accumulation of flavonoid end products is presumably determined by activity of the rate-limiting step(s) in flavonoid biosynthesis and may not precisely reflect the dynamics of CHS activity *in vivo.*

1.3.3. Control of CHS *through trans-gene*

The activity of CHS can be controlled by antisense or sense genes. The studies on expression of antisense genes in *Petunia* [e.g. Van der Krol *et al.,* 1988; Van der Meer *et al.,* 1993], tobacco [Wang *et al.*, 2006], *Gerbera hybrida* [Elomaa *et al.,* 1996] and *Arabidopsis* [Le Gall *et al.,* 2005] have shown that the presence of antisense *CHS* could inhibit the expression of the endogenous *CHS* in plants. In flowers of antisense *CHS* transgenic *Petunia,* the antisense construct was able to inhibit expression of the endogenous *CHS* genes to varying degrees, which is observed phenotypically as an inhibition of anthocyanin production to give completely acyanic or patterned flowers. In the cyanic sectors and flowers, transcripts of the endogenous *CHS* genes were under the detection limit, but the antisense transcripts were also barely detectable [Van der Krol, 1990b]. The antisense effect most likely involves homologous pairing between the transcripts of endogenous *CHS* genes and transcripts of the introduced antisense *CHS* gene to form double stranded RNA that is very rapidly degraded, thus inhibiting *CHS* transcript accumulation and hence CHS activity.

Introducing a heterologous *CHS* gene in sense orientation can inhibit CHS activity in transgenic plants. This phenomenon is called co-suppression since it involves the reduction of transcriptional level of both endogenous and introduced genes in tissues where the endogenous gene is normally expressed [Napoli *et al.,* 1990; Jorgensen 1995]. This is known as gene silencing in which the transgene triggered not only its own silencing but also the endogenous chalcone synthase gene [Hammond *et al.,* 2001]. But on the other hand the introduced *CHS* gene may be expressed to high levels in tissue where the endogenous *CHS* genes are not expressed, such as in leaves of Petunia [Van der Krol *et al.,* 1990a]. Some studies have shown that co-suppression correlates with DNA methylation of the silenced sequences, presumably leading to a blockade at the transcriptional level or/and failure of transcript to accumulate in the cytoplasm resulting in a lack of enzyme activity [Ingelbrecht *et al.,* 1994, Furner *et al.,* 1998; Amedeo *et al.,* 2000]. Nowadays, the molecular mechanism of co-suppression of gene expression is thought to be related to the RNAi mechanism [Hannon, 2002]

2.4. Control of *CHS* gene expression

In *Arabidopsis*, parsley, and snapdragon only a single copy of the *CHS* gene has been found. In most angiosperms CHS has been shown to be encoded by a multigene family, such as in petunia (violet 30) [Koes *et al.,* 1987], morning glories (Ipomoea) [Durbin *et al.,* 2000], Gerbera [Helariutta *et al.,* 1996], leguminous plants [Ryder *et al.,* 1987; Wingender *et al.,* 1989; Ito *et al.,* 1997], and *Cannabis sativa* [Sanchez *et al.*, 2008].

2.4.1. Regulation of CHS gene expression

Many studies have shown that the *CHS* gene is constitutively expressed in flowers, but also its expression can be induced by light/UV light and in response to phytopathogens, elecitors or wounding in different parts of the plant, resulting in enhanced production of flavonoids [Koes *et al.,* 1987; Ryder *et al.,* 1984; Bell *et al.,* 1986; Ryder *et al.,* 1987; Burbulis *et al.,* 1996]. *CHS* expression is also regulated by the circadian clock [Thain *et al.,* 2002].

The level of *CHS* gene expression is reflected by the level of the *CHS* transcripts in plant cells. In order for transcription to take place, the RNA polymerase II must attach to specific DNA sequences in the *CHS* promoter in the vicinity of the TATA box and must be activated by specific DNA-binding proteins (transcription factors) binding to response elements further upstream in the promoter. The *CHS* promoter was studied extensively in *Phaseolus vulgaris, Antirrhinum, Arabidopsis,* and parsley [Dixon *et al.,* 1994; Faktor *et al.,* 1997a, b; Feinbaum *et al.,* 1991; Lipphardt *et al.,* 1988].

The *CHS* promoter contains the nucleotide sequence CACGTG regulatory motif known as G-box, which has been found to be important in the response to light/UV light [Kaulen *et al.,* 1986; Staiger *et al.,* 1989; Dixon *et al.,* 1994; Schulz *et al.,* 1989]. Besides the G-box there are other domains in the *CHS* promoter involved in the light activation of *CHS* transcription. Those domains have been identified in the parsley *CHS* promoter as Box I, Box II, Box III, Box IV or three copies of H-box (CCTACC) in the *Phaseolus vulgaris CHS15* promoter. These boxes play a role as core promoter together with the G-box and all are required for light inducibility [Lawton *et al.,* 1990; Weisshaar *et al.,* 1991; Block *et al.,* 1990].

The environmental and developmental control of CHS transcription has been investigated for the *CHS15* bean gene (**Figure 2.4**) [Dixon *et al.,* 1994; Harrison *et al.*

1991]. The sequence elements required for transcriptional activation of the *CHS15* gene in response to fungal elicitors and glutathione are contained in a 130 bp region of the promoter [Dron *et al.,* 1988; Choudary *et al.,* 1990; Harrison *et al.,* 1991]. This region contains a G-box and H-box III. There is a silencer element located between positions -326 and -173 of the *CHS15* promoter [Dron *et al.*, 1988]. No *trans*-acting factors were found that could bind to *cis* elements in this region but the region reduced expression of *CHS* [Harrison *et al.* 1991]. An enhancer element was found in the *Antirrhinum CHS* promoter. It is located in the region between -564 and -647 and increased *CHS* gene expression in roots, stems, leaves, and seeds but not in petal tissue [Fritze *et al.,* 1991].
The *Petunia CHSA* promoter was studied by van der Meer *et al.* (1990, 1993) to understand the role of the promoter in tissue-specific *CHS* expression. The studies showed that the promoter sequence between +1 and -67 confers flower specific *CHS* gene expression. Another study on the *Antirrhinum CHS* promoter has shown that the sequences between +1 and -39 allow *CHS* expression in root and stems, whereas sequences between -39 and -197 are required for expression in petals and seeds [Fritze *et al.,* 1991].
The regulators of *CHS* in plants are controlled by some specific loci. In maize, there are four loci, *cl, r, vp,* and *clf,* involved in the regulation of *CHS* expression [Dooner, 1983]. Multiple regulatory loci for *CHS* expression have also been described for the petunia regulatory mutant Red Star. The phenotype of this mutant of red and white sectors in the flower petals is thought to depend on at least four regulatory genes, all of which regulate *CHS* expression in *trans* [Mol *et al.,* 1983]. In the *CHS* gene family of *Phaseolus vulgaris,* the regulation is via the *a* and *a2* loci though they regulate different *CHS* members in different ways. The *CHS* genes might have different combinations of *cis* elements that determine their response to the products of these regulatory loci. The expression of *CHSl* in flower tissue has an absolute requirement for the products of both the *a* and *a2* loci, whereas, in root tissue, the products of these loci are not required. It is possible that the *CHSl* gene interacts with one or more factors present in roots, which are absent in flowers, that can substitute for the products of the *a* and *a2* loci. *CHS3* expression in flower tissue is more complicated: it requires the product of the *a2* locus, but has a lower level of expression in *a* mutants compared with wild type. This suggests that *CHS3* interacts with both the *a2* and *a* locus products, but, unlike the *CHSl* gene, it

may also interact with other products, allowing transcription at a low level in *a* mutants. *CHS2* is expressed in roots but not in petal tissue, suggesting that it may not be able to interact with the products of *a* and *a2* loci in petal tissue [Harker *et al.,* 1990].

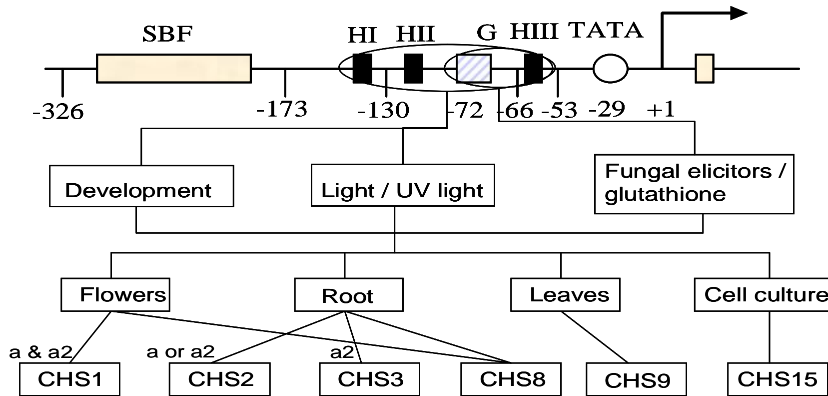


Figure 2.4. Bean *CHS15* promoter and regulators

SBF: silencer binding factor, H: H-Box (CCTACC), G: G-Box (CACGTG), a/a2 regulation loci

1.4.2. Transcription factors involved in of CHS gene expression

Trans-acting factors of bean *CHS15* that bind to two short sequences centered on the G-box and H-box also make major contributions to the *in vivo* transcription of the promoter [Arias *et al.,* 1993, Yu *et al.,* 1993]. *Trans* activation required both a MYB-binding site and a G-box like element [Sablowski *et al.,* 1994]. MYB305, one of the MYB-like proteins that have been implicated in the transcriptional control of tissue-specific *CHS* gene expression, is also recognized by a *cis* element of the light-regulatory unit 1 (LRUI) of *CHS* in parsley [Feldbrügge *et al.,* 1997]. G-box/H-box binding factor 1(G/HBF-1), a basic leucine zipper (bZIP) protein, that binds to both the G-box and the adjacent H-box in the proximal region of the *CHS15* bean promoter, is rapidly phosphorylated in elicited soybean cells, this happen also to the *CHS15, CHS7,* and *CHS1* promoter [Dröge *et al.*, 1997; Yoshida *et al.,* 2008]. Protein and mRNA levels of G/HBF-l do not change during the induction of *CHS* genes following pathogen attack [Yoshida *et al.,* 2008] but *CHS* gene expression is strongly stimulated following phosphorylation responding to fungal elicitor treatment *in vitro* [Dröge *et al.,* 1997].

2.5. CHS localization and dynamics

The CHS protein in buckwheat (*Fagopyrum esculentum*) hypocotyls is located in the cytosol and associates with the cytoplasmic face of the rough endoplasmic reticulum (rER), but not with nuclei, plastids, mitochondria, Golgi, or tonoplasts [Hrazdina, 1992]. Saslowsky *et al.,* [2001] examined the subcellular location of CHS and CHI in *Arabidopsis* roots. High levels of both enzymes were found in the epidermal and cortex cells of the elongation zone and the root tip, consistent with the accumulation of flavonoid endproducts at these sites. Co-localization of CHS and CHI was observed at the endoplasmic reticulum and tonoplast in these cells.

However, there is evidence that flavonoids located in the nucleus may be synthesized *in situ* [Saslowsky et al., 2001]. Several recent reports describe the accumulation of flavonoids in the nucleus in such diverse species as *Arabidopsis thaliana*, *Brassica napus*, *Flaveria chloraefolia*, *Picea abies, Tsuga Canadensis,* and *Taxus baccata* [Hutzler *et al.,* 1998; Kuras *et al.,* 1999; Buer *et al.,* 2004; Grandmaison *et al.,* 1996; Feucht *et al.,* 2004; Peer *et al.,* 2001]. For the enzymes of the flavonoid pathway, several mechanisms may be involved. In the cytoplasm, flavonoid enzyme complexes are believed to assemble at the ER and in electron dense particles through the association of operationally-soluble enzymes such as CHS and CHI with the membrane-bound P450 hydroxylase, flavonoid 3'-hydroxylase [Saslowsky *et al.*, 2001; Hrazdina *et al.*, 1985]. CHS possesses sequences resembling a classic nuclear localization signal (NLS). This signal is located on the surface, on the opposite side of the protein from the dimerization interface and could function to direct CHS, and perhaps associated enzymes into the nucleus. The localization of end products such as flavonol sulfate esters and flavan-3-ols to the nucleus suggests that additional flavonoid enzymes are also present in the nucleus [Grandmaison *et al.*, 1996; Feucht *et al.*, 2004].

There is an immuno gold-labeling study in grape berry showing that CHS was localized in rough endoplasmic reticulum (ER) and cytoplasm of the skin cells, while few gold particles were found on the cell wall. Besides, two novel sites of CHS were observed within cells of developing grape berry, one is in the plastids which remain unchanged throughout all stages of berry development. At the ripening stage of grape berry, CHS is present in the vacuole and in the vacuole membrane (tonoplast) [Tian *et al.,* 2008]. It is

suggested that in grape berries, the synthesis of flavonoids in the ripening stage may occur in the vacuole.

2.6. CHS activity in plant resistance

In nature plants are exposed to a variety of biotic and abiotic stresses. Viruses, bacteria, fungi, nematodes and other pests attacking plants are biotic stresses, while light, temperature, wounding, drought, etc. are abiotic stresses. During stress conditions a plant is expressing a number of genes as part of its defense. Among these genes, CHS is quite commonly induced in different plant species under different forms of stress like UV, wounding, herbivory and microbial pathogens resulting in the production of compounds that have e.g. antimicrobial activity (phytoalexins), insecticidal activity, and antioxidant activity or quench UV light directly or indirectly. The current knowledge about regulation of CHS in plant pathogen resistance is presented in **Table 2.2**.

2.6.1. Phytoalexins

Phytoalexins are antimicrobial metabolites produced by plants in response to microbial attack (or biotic and abiotic elicitors) [Dixon *et al.*, 1986]. Phytoalexins come from many different metabolite classes such as flavonoids, stilbenoids, sesquiterpenoids, steroids and alkaloids. CHS can help the plant to produce more flavonoids, isoflavonoid-type phytoalexins and other related metabolites to protect it against stress. Accumulation of flavonoids and isoflavonoids in response to pathogen attack is seen in many plant species, and their importance as antimicrobial phytoalexins is well established [Matthews *et al.,* 1989; Van Etten *et al.,* 1976]. Flavonoid phytoalexins have been described in legumes, cereals, sorghum, rice, *Cephalocereus senilis, Beta vulgaris* [Hipskind *et al.,* 1990; Kodama *et al.,* 1992; Pare *et al.,* 1992; Johnson *et al.,* 1976]. Some isoflavonoids were increased in *Lupin luteus* after infection with *Fusarium oxysporum* such as genistein, wighteone and luteon [Morkunas *et al.,* 2005]. The isoflavones, daidzein, genistein and glycitein, in soybean were strongly increased after infection by *Sclerotinia sclerotiorum* [Wegulo *et al.,* 2005]. Stilbenes are known as the phytoalexins in peanut [Ingham, 1976] and grapes [Langcake and Pryce, 1977a, b]. There is also evidence that stilbene synthase (STS) has developed from CHS several times in the evolution [Tropf *et al.*, 1994].

2.6.2. Phytoanticipins

Van Etten *et al.,* [1995] defined phytoanticipins as low molecular weight, antimicrobial compounds that are constitutively expressed in plants without the need for infection with fungal pathogens or are produced after infection solely from preexisting constituents. The distinction between phytoalexins and phytoanticipins is not always clear as some compounds may be phytoalexins in one species and phytoanticipins in another species. Phytoanticipins also are classed into several chemical groups such as flavonoids, terpenoids, steroids, glucosinolates, and alkaloids.

The flavonoid epicatechin plays an important role as phytoanticipin in avocado fruits [Guetsky *et al.,* 2005,] and antimicrobial isoflavones desmodianones A, B and C have been isolated from *Desmodium canum* [Monache *et al.,* 1996]. Anthocyanins as products of the flavonoid metabolism are, for example responsible for the red to purple and blue colors of many fruits, vegetables, flowers, and cereal grains. In plants they serve as attractants for pollination and seed dispersal, give constitutive protection against the harmful effects of UV irradiation, and as phytoanticipins provide antiviral and antimicrobial activities in plants [Wrolstad, 2000]. Genotypes of *Ipomoea purpurea* with nonfunctional copies of chalcone synthase (*CHS*) received greater herbivore damage and twice the intensity of infection by the fungal pathogen *Rhizoctonia solani* than the wild type [Zufall and Rausher, 2001].

2.6.3. Light protection

Phenolic compounds like flavonoids strongly absorb UV light and thus are able to protect plants from DNA damage caused by UV. Anthocyanins belong to a class of flavonoids that accumulate in leaves and stems as plant sunscreen in response to light intensity [Leyva *et al.,* 1995]. Expression of *CHS* genes is known to be regulated by light through a photoreceptor-mediated mechanism [Koes *et al.,* 1989]. In several cases, it was found that the photoregulated production of flavonoids is at least in part due to the transcriptional induction of *CHS* [Chappell and Hahlbock, 1984; Feinbaum and Ausubel, 1988; van Tunen *et al.,* 1988; Taylor and Briggs, 1990). Examination of *CHS* expression in parsley cell culture suggested that a UV-B light receptor, a blue light receptor and phytochrome may all play a role in light-induced *CHS* expression [Bruns *et al.,* 1986; Ohl *et al.,* 1989).

High intensity light and UV-A were found to regulate expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants [Feinbaum *et al.,* 1991]. High-intensity light treatment of *A. thaliana* plants for 24 h caused a 50-fold increase in CHS enzyme activity and an accumulation of visibly detectable levels of anthocyanin pigments in the vegetative structures of these plants [Feinbaum *et al.,* 1988]. The expression of *CHS* genes was increased with time during a 24 h exposure to UV-A on swollen hypocotyls of the red turnip 'Tsuda' and induced anthocyanin accumulation [Zhou *et al.,* 2007]. The flavonoids accumulate in epidermal cells of the leaves and it is specifically in these cells that *CHS* gene expression is induced by light stimuli [Schmelzer *et al.,* 1988]. However, in mustard the expression of two *CHS* genes is induced coordinately in seedlings grown in a dark environment for 36-42 hours, though this induction is enhanced by supplying red or far red light [Ehmann *et al.,* 1991].

2.6.4. Auxin and jasmonic acid signaling

In plant increase of CHS activity causes a high accumulation flavonoid level that inhibit polar auxin transport [Jacobs and Rubery, 1988; Faulkner and Rubery, 1992; Brown *et al.,* 2001]. Inhibitors of auxin transport could increase the resistance of tomato plants to *Fusarium oxysporum* [Davis *et al.,* 1954]. Also other research showed that CHS is expressed in the nodule primordium and later primarily in uninfected cells of the nodule apex in *Rhizobium* infected legumes. This may explain the induction of nodule on infected legume roots, higher accumulation of flavonoids blocks auxin transport, causing a local accumulation of auxin, a growth hormone, which caused the induction of nodule growth and development [Estabrook and Sengupta, 1991; Yang *et al.,* 1992].
Jasmonic acid and its esters, such as methyl jasmonate (MeJA) are a group of plant hormones having a signaling role in insect and disease resistance [Xu *et al.,* 1994]. They could activate *CHS* in soybean and parsley cell cultures [Creelman *et al.,* 1992] and *Picea glauca* [Richard *et al.,* 2000]. It is thought that volatile jasmonates are released from wounded tissue; thus elicitating plants to activate *CHS* which cause a production of phytoalexins in advance to resist an infection.

Table 2: Chalcone synthase expression in plant under stress conditions

No.	Host		Pathogen	Metabolites	References
1	*Petroselinum crispum*	Parsley	UV	Flavonoids	Schmelzer *et al.,* 1988 Schulze-Lefert *et al.,*1989
2	*Phaseolus vulgaris* cells	French bean	*Colletotrichum lindemuthianu*		Ryder *et al.,* 1984
3	*Arabidopsis* cells		UV-B and UV-A/Blue Light		Christie *et al.,*1996
4	*Arabidopsis thaliana*		Low temperature	Anthocyanins	Leyva *et al.,*1995
			UV-B, UV-A, and Blue Light		Fuglevand *et al.,* 1996 Hartmann *et al.,* 1998 Wade *et al.,* 2001
			High-intensity light	Anthocyanins	Feinbaum *et al.,*1988
			Salicylic Acid, Ethylene Methyl jasmonate *Alternaria brassicicola*		Schenk *et al.,* 2000
			Pseudomonas syringae	Phenolic compounds, lignin, camalexin	Soylu, 2006
5	*Petunia hybrida*		UV		Koes *et al.,*1989
			Low temperature	Anthocyanin	Shvarts *et al.,*1997
6	*Petroselinum hortense cells*		UV		Kreuzaler *et al.,*1983
7	*Secale cereale*	Rye	UV		Haussuehl *et al.,*1996
8	*Pinus sylvestris*	Scots pine	UV-B	Phenolic compounds, flavonoids, catechin	Schnitzler *et al.,*1996
9	*Picea abies*	Norway spruce	*Ceratocystis polonica*		Nagy *et al.,*2004
			Ophiostoma polonicum and wounding	Catechin	Brignolas *et al.,*1995
10	*Lycopersicon esculentum*	Tomato	Nitrogen	Flavonoids	Bongue and Phillips, 2009

No.	Host		Pathogen	Metabolites	References
11	*Hordeum vulgare*	Barley	*Blumeria graminis* *Erysiphe graminis* UV		Christensen *et al.*,1998a,b
12	*Medicago truncatula* *Medicago sativa*	Alfalfa	*Glomus versiforme*	Isoflavonoids	Harrison *et al.*,1993
13	*Antirrhinum majus*	Snapdragon	*Erwinia chrysanthemi* *Rhizobium meliloti* $CuCI_2$ Wounding *Phoma medicaginis*		Junghans *et al.*,1993
14	*Glycine max*	Soybean	*Colletotrichum lindemuthianum*		Dalkin *et al.*,1990
			UV		Lipphardt *et al.*,1988 Staiger *et al.*,1989
			Pseudomonas syringae pv *glycinea* *Phytophthora megasperma* f. sp. *glycinea*		Dhawale *et al.*,1989
15	*Picea glauca*	White Spruce	Wounding, JA, MeJA		Richard *et al.*,2000
16	*Daucus carota*	Carrot cell	UV, *Pythium aphanidermatum*	Anthocyanins	Gläßgen *et al.*,1998
17	*Brassica rapa*	Turnip	UV	Anthocyanins	Zhou *et al.*, 2007
18	*Sorghum bicolor*	Sorghum mesocotyl, juvenile sorghum tissues,	*Colletotrichum graminicola* *Helminthosporium maydis*	3-deoxyanthocyanidins, apigeninidin luteolinidin .	Lue *et al.*, 1989 Nicholson *et al.*, 1987

2.7. Conclusion

CHS is known as the key entry enzyme commited to the production of the polyketide phenylpropanoids in plants. The product of CHS activity, naringenin, is the starter of a large variety of secondary metabolites such as flavonoids, isoflavonoids, anthocyanins, and phloroglucinols. These multi-functional compounds serve diverse functions in different plant species, e.g. as pigments, phytoalexins, UV protectants, signal molecules in plant-microbe interactions, antioxidants, and pollinator attractants or feeding deterrents. In other words these unique plant compounds play a major role in the interaction of plants with their environment. Besides that, many flavonoids are active principles of medicinal plants and exhibit pharmacological effects [De Bruyne *et al.,* 1999; Kong *et al.,* 2003: Marles *et al.,* 2003; Yilmaz and Toledo, 2004].

With the advent of reverse-genetic tools and molecular cloning, one may develop CHS transgenic plants and eventually thus open new avenues to better understand the flavonoid biosynthesis pathways and their functions in plant resistance. Eventually this may lead to breeding or engineering of plants with an improved resistance or better consumer quality, e.g. healthier food.

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 deverivates are precusors 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 *CH*S 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

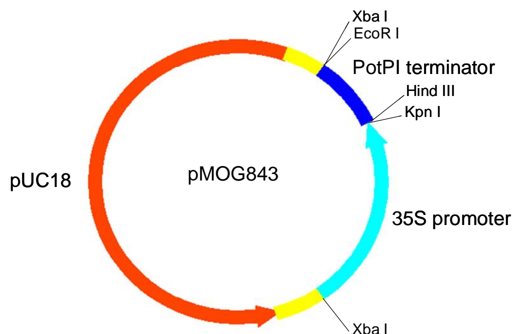
3.2.1. Plant materials

Arabidopsis thaliana ecotype Col-0 seeds were obtained from the section Plant Cell Physiology (IBL, Leiden Universiy, 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.



B.

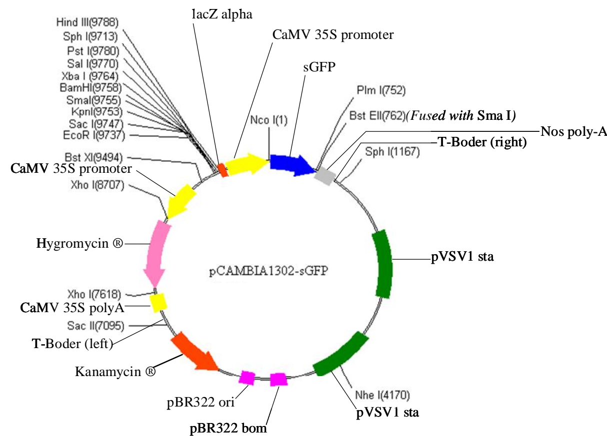


Figure 3.1. A. Subcloning vector pMOG843, **B.** Transformation vector pCAMBIA 1302-sGFP. *chs* cDNA transgene was subcloned in pMOG843 in position between *Hind*III and *Kpn*I restriction site then *CHS* containing the PotPI and 35S promoter was constructed in the polylinker site of pCAMBIA1302-sGFP

3.2.3 Vector construction and plant transformation

To generate *chs* overexpression constructs, the coding region of *chs* cDNA [Raharjo *et al.* 2004] was obtained by PCR using primers containing restriction sites *Kpn*I and *Hind*III, respectively, and was ligated into the pGEM-T easy vector (Promega). The vector was then digested using a *Kpn*I/*Hind*III double digestion, and the resulting DNA was subcloned into the pMOG843B (**Fig. 3.1A**) behind the 35S promoter. Subsequently, the XbaI/EcoRI digested 35S:CHS:PotPI terminator fragment was cloned into the pCAMBIA1302-sGFP (**Fig. 3.1B**) and transformed into *Agrobacterium* LBA4404. Plasmid vector pCAMBIA1302-sGFP also contains *hptII* encoding hygromycin phosphotransferase and a gene encoding the GFP protein, which permits easy detection of transformed plantlets. All DNA manipulations were according to standard procedures [Sambrook *et al.*, 1989], and the *chs* coding region and the junction sequences were confirmed by DNA sequencing.

The PCR conditions were following: one µl *chs* plasmid DNA was used as template for PCR using CHSR and CHSF primers (**Table 3.1**), PCR was performed with a Perkin Elmer DNA Thermal Cycler 480 with the following parameters: 30 sec at 95°C, 1 min at 50°C, 1 min at 72°C, 30 cycles. The final step was an extension at 72°C for 10 min.

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, T_3 or T_4 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 ddH_2O, 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 ^{32}P 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 ($2^{-\Delta\Delta Ct}$) 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 = $2^{-\Delta\Delta Ct}$ where, $\Delta\Delta Ct = \Delta Ct$ (unknown sample) – ΔCt (reference).

In the copy number of transgenes experiment, the reference Ct is the Ct 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’ ACCGTGGAGGAATTTCGCAAGG 3’
4HPPDR	5’ TCATCCCACTAAATGTTTGGCTTC 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.

A. **B.**

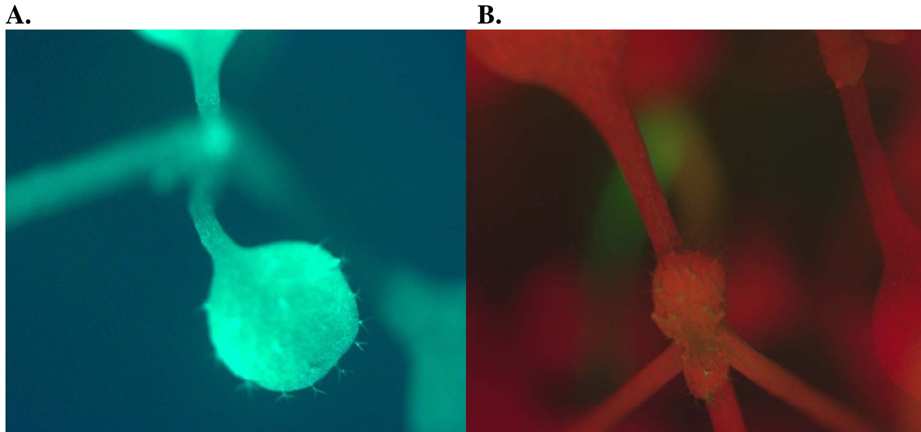


Figure 3.2. A. Transformed *Arabidopsis* with high GFP expression, B. Transformed *Arabidopsis* with low GFP expression

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 Flourescence 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 Ct 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**).

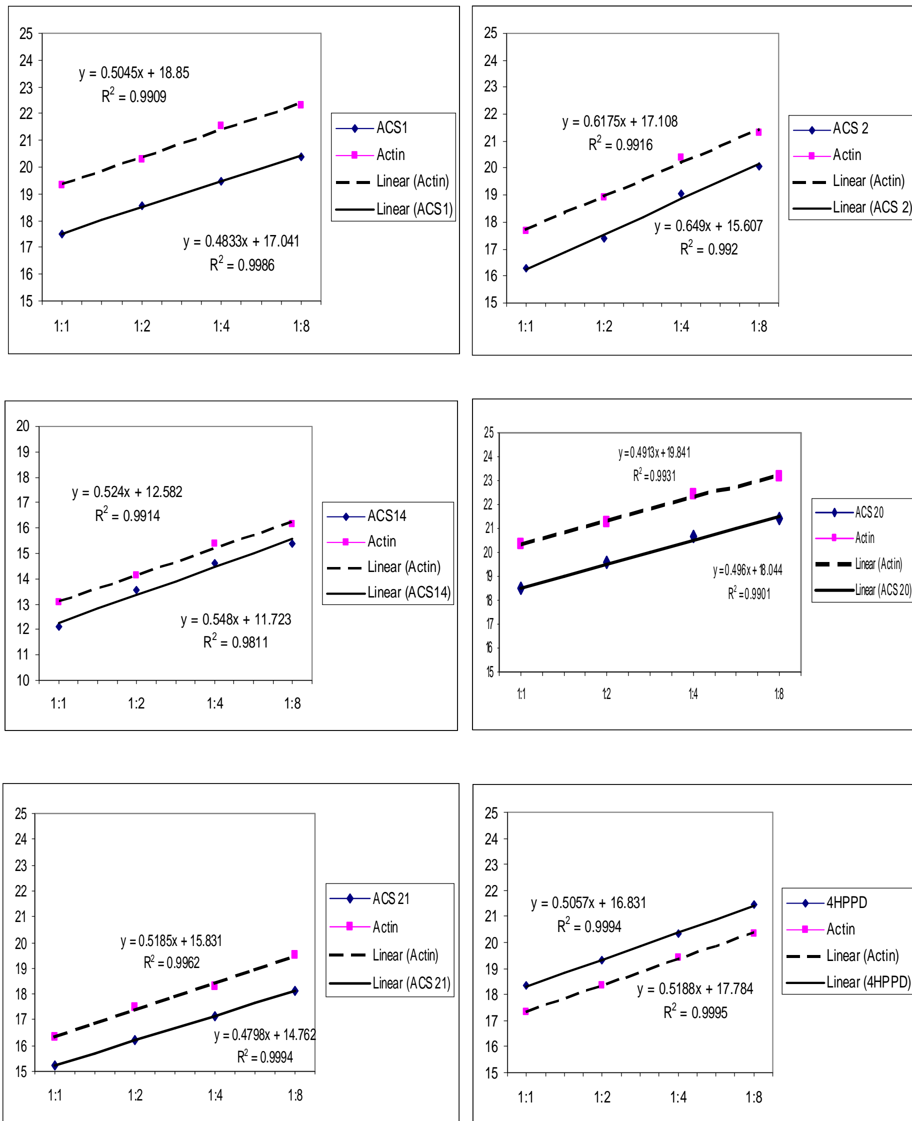


Figure 3.3. Efficiency of duplex real-time PCR for detection and quantitation of *Actin* and *CHS* DNA from a transgenic plant or a nontransgenic plant was diluted serially 2-folds.

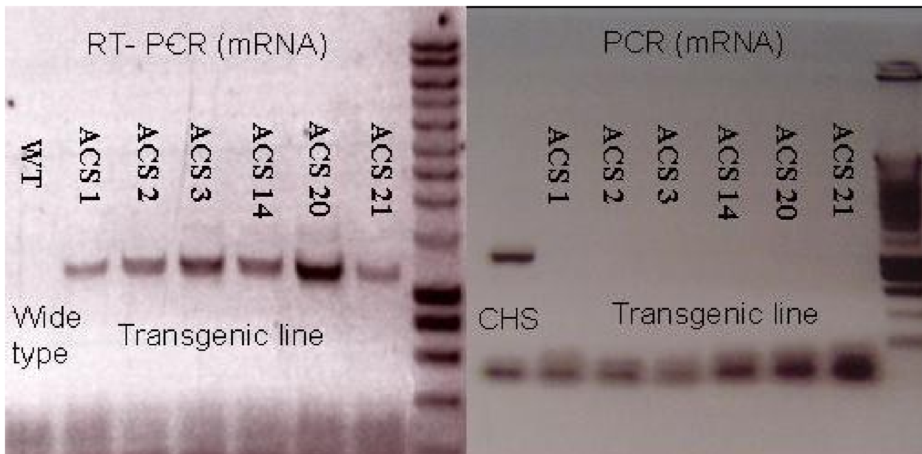


Figure 3.4. A. Qualitative analysis of *CHS* gene expression by RT-PCR, **B**. Analysis of genomic DNA contamination in mRNA samples by PCR

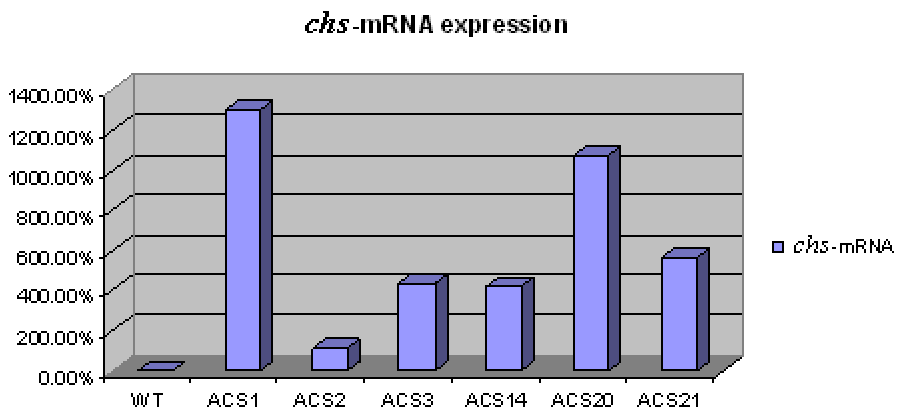


Figure 3.5. *chs*-mRNA expression levels optimized by Real-time PCR

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.

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].

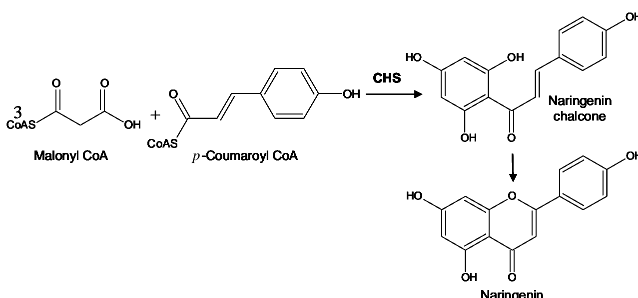

Figure 4.1. Reaction catalyzed by chalcone synthase (CHS)

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^oC and in dark environment, seeds were germinated at 23^oC 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°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**).

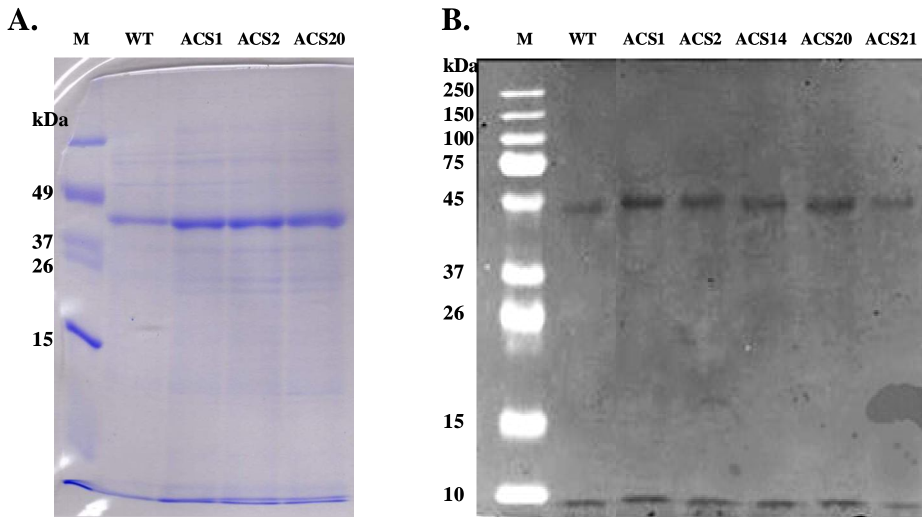

Figure 4.2. SDS PAGE (**A**) of and western blot (**B**) analysis of wild type (WT) and CHS transgenic plants (ACS)

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**)

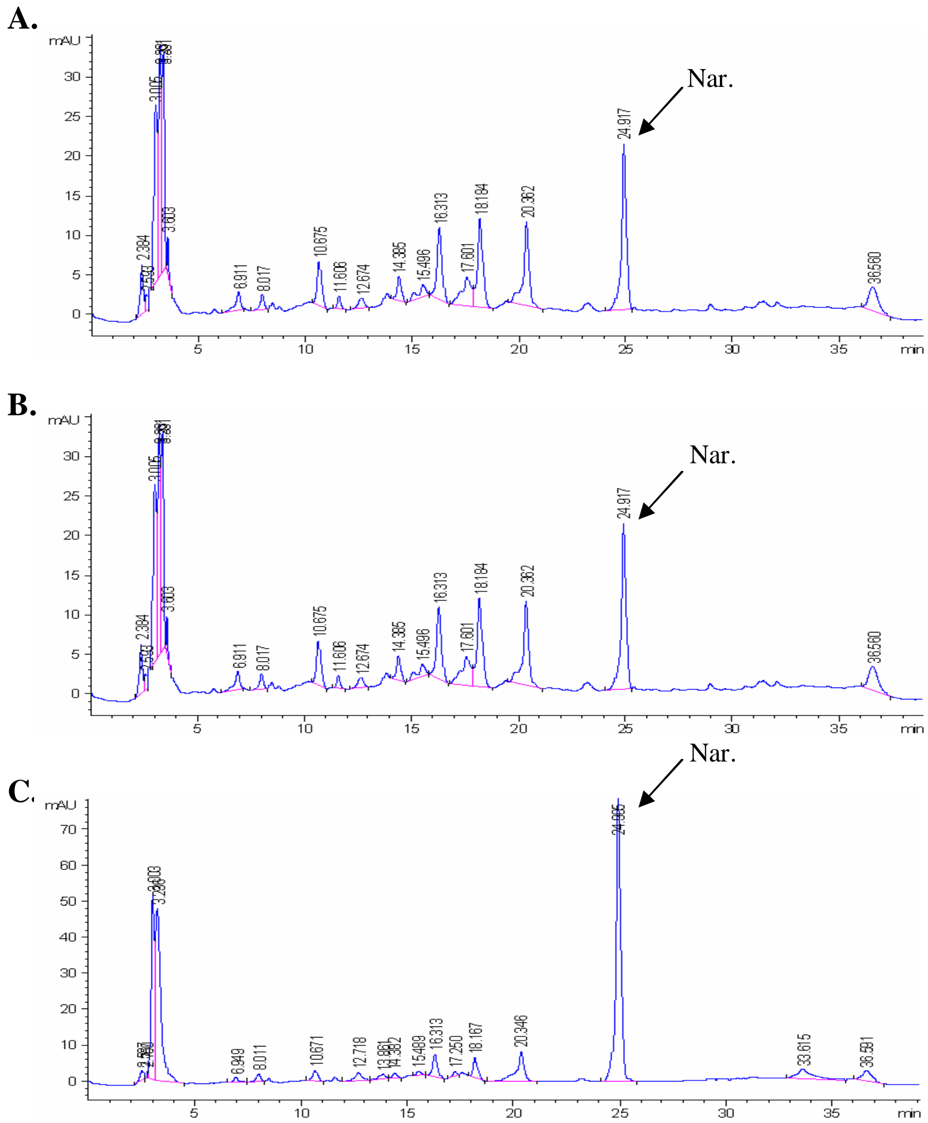

Figure 4.3. HPLC chromatograms of the EtOAc extract from the chalcone synthase activity assay with WT *Arabidopsis* (A), CHS transgenic *Arabidopsis* ACS2 (B) and ACS20 (C) protein extracts

Chapter 5

Identification of metabolites in *Arabidopsis thaliana*

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Abstract

Identification of *Arabidopsis thaliana* Col.0 metabolites by use of NMR spectroscopy is described in this chapter. Among the different extraction solvent tested, MeOD was the best solvent to extract phenolic compounds from *Arabidopsis*. By column chromatography using Sephadex LH-20 and prep HPLC, several flavonoids were isolated from the methanol extract of *Arabidopsis*, and their structures were identified by LC-MS and NMR spectroscopy as kaempferol 3-*O*-glucopyranoside-7-*O*-rhamnopyranoside, kaempferol 3-*O*-rhamnosyl (1–2) glucoside-7-*O*-rhamnopyranoside, kaempferol 3,7-*O*- dirhamnopyranoside and quercetine 3-*O*-rhamnopyranoside. Twenty four major metabolites of *Arabidopsis thaliana* Col.0 including amino acids, organic acids, sugars, phenylpropanoids, and flavonoids were identified and their NMR cherecteristics are also summerized in this study.

Keywords: NMR, metabolites, phenolics, flavonoids, extraction method, *Arabidopsis thaliana*

5.1. Introduction

Arabidopsis thaliana has become an extremely popular model system for studying plant biology. The biosynthesis of plant secondary metabolites represents a complex cellular network involving the transcription, translation and post-translational modification of many gene products. Analysis of whole plant metabolomes is a difficult task due to the huge number and great diversity of primary and secondary metabolites present in plant tissues [Dixon and Strack, 2003; Sumner *et al.,* 2003; Stobiecki and Kachlicki, 2005]. A good profiling method should be simple and detect as many of the metabolites as possible in a single extract of the material. In addition, the method should be reproducible to archive the data for future datamining.

The chromatography and spectroscopic technologies as HPLC-UV, GC/MS, LC/MS and NMR employed in plant metabolomics have been extensively reviewed [Fan, 1996; Fiehn *et al.,* 2000; Wagner *et al.,* 2003]. NMR is a tool to analyze the metabolome with a lot of advantages. Sample preparation for NMR measurement is usually simple and rapid, measurement times are short and readily automated and advanced data analysis methods are available. The 1D and 2D-NMR spectra of complex mixtures may provide sufficient information for the structures of unknown components to be elucidated, either from the NMR spectrum of the mixture itself, or after some purification. Another advantage of NMR is the linearity of quantitative responses on increasing metabolite concentrations, irrespective of the chemical compound class. Large signals in NMR can directly be interpreted as high level concentrations, whereas in MS, quantitative responses strongly rely on the ionization potential of each metabolite. Therefore, quantitation in MS is limited to relative abundances of a given metabolite between samples, or requires calibration curves if absolute comparison of different metabolites is needed. NMR either generates a metabolite profile, in which the NMR signals are assigned to specific metabolites, or a metabolite fingerprint, in which the analysis is based on the distribution of intensity in the NMR spectrum rather than the assignment of the signals [Krishnan et *al.,* 2005]. However, NMR has some limitations such as low sensitivity in comparison with MS. But new technology in NMR equipment such as higher-field spectrometers and use of cryogenically cooled probes improved the NMR sensitivity many folds.

Large numbers of metabolites of *Arabidopsis* have been identified. Understanding a significant part of *Arabidopsis* biology requires methods allowing the sensitive detection and quantification as well as the identification of secondary metabolites. Applying such techniques to various genetic backgrounds and to different environmental and developmental conditions then would help elucidate the function of such compounds and of the genes involved in their biosynthesis. Metabolic profiling of Arabidopsis and other plants have been developed in recent years. Most commonly used are gas chromatographies (GC)-mass spectrometry (MS)-based approaches. Several hundred of metabolites can be robustly and reliably detected but most of them are primary metabolites such as sugars, amino acids, organic acids [Roessner *et al.,* 2000; Fiehn *et al.,* 2000; Wagner *et al.,* 2003]. Beisdes that, liquid chromatography (LC)-MS based metabolomic has been used for profiling of metabolites [Roessner *et al.,* 2000]. Every analytical procedure is necessarily limited as to what type of compounds can be separated and detected. GC-MS is predominantly applied to very polar or unpolar substances though requiring derivatization to obtain volatile derivatives, whereas the main application of LC-MS is more related to compounds of medium polarity. About 300 metabolites were detected in *A. thaliana* leaf extracts and about half of them were identified by using GC-MS [Fiehn *et al.,* 2000], LC-MS and NMR [Hendrawati *et al.,* 2006; Le Gall *et al.,* 2005; Von Roepenack *et al.,* 2004].

Phenolic compounds are ubiquitous constituents of higher plants found in a wide range of commonly consumed plant foods such as fruits, vegetables, cereals and legumes, and in beverages of plant origin, such as wine, tea and coffee [Cheynier, 2005; Manach *et al.,* 2004]. These compounds are secondary metabolites of plants that are generally involved in defense against ultraviolet radiation or often attack by pathogens. They constitute an important class of plant secondary metabolites and are mostly present as glycosidic conjugates. The major flavonoid compounds in *A. thaliana* are the kaempferol glycosides flavonols [Rohde *et al.,* 2004; Veit and Pauli, 1999], but quercetin glycosides can also accumulate after exposure to UV radiation [Graham, 1998]. Another group of flavonoids present in *A. thaliana* are the anthocyanins, the major red, purple and blue pigments of plants best known from flowers and fruits. The major anthocyanin in *A. thaliana* has a cyanidin core with four attached sugars [Bloor and Abrahams, 2002]. Some flavonoids from green tissues of *A. thaliana* have been

fully structurally characterized with various physicochemical methods. Kaempferol 3-*O*-*β*-[*β*-D-glucosyl(1–6)D-glucoside]-7-*O*-*α*-L-rhamnoside, kaempferol 3-*O*-*β*-D-glucoside-7-*O*-*α*-L-rhamnoside, kaempferol 3-O-*α*-L-rhamnoside]-7-*O*-*α*-L-rhamnoside, kaempferol 3-*O*-*β*-[*α*-L-rhamnosyl (1–2)D-glucoside]-7-*O*-*α*-L-rhamnoside were identified [Veit and Pauli, 1999; Bloor and Abrahams, 2002]. Most studies on flavonoid characterization have been done by analytical procedures using the isolated flavonoids. In further experiments in this thesis a profiling of metabolites in crude extract will be applied so an identification of *Arabidopsis* flavonoids in plant crude extracts needs to be developed. So far the flavonoids of *Arabidopsis* are not available commercially so isolation and identification of flavonoids in *Arabidopsis thaliana* Col. 0 were done in this study in order to have reference compounds for further analysis.

Profiling applications of NMR in plant tissues have usually focused on the identification of particular metabolites, and so the extraction techniques need to be considered for optimal extraction recovery of the compounds of interest. The aim of this study is the application of NMR to identify *Arabidopsis thaliana* Col. 0 metabolites, focusing on phenolic compounds in plant crude extracts because we studied the effect of CHS expression, a key enzyme in flavonoid biosynthesis pathway (**Chapter 2**), on the *Arabidopsis* metabolome. Thus a suitable extraction method for this purpose was developed in this study.

5.2. Methods and Materials

5.2.1. Plant materials and extraction for flavonoid isolation

Arabidopsis thaliana above ground parts were used as a plant material for extraction. 500 ml of CH_3OH was added to 256 mg of dried and ground leaves and ultrasonicated for 30 minutes and then vacuum filtered. The procedure was repeated for 3 times and all the supernatants were pooled and dried using a rotary evaporator. The dried extract was redissolved in 100 ml of deionized water and partitioned with different solvents like *n*-hexane, chloroform, and *n*-butanol. All the fractions were separately dried by rotary evaporator and stored at 4 °C until further use.

5.2.3. Sample Fractionation

The *n*-butanol extract (1.2 g) was selected for fractionation as high flavonoids content was expected in this fraction. Sephadex column LH-20 (145 cm length x 16 mm diameter) was used for sample fractionation with 100% CH_3OH as a mobile phase. Total 84 fractions were collected of 5 ml each. TLC indexing was performed for every fourth fraction and observed under 254 nm and 366 nm. The solvent system for TLC indexing was composed of ethyl acetate, formic acid, acetic acid, and water, in the ratio of 100:11:11:27 (v/v). The fractions that showed a similar pattern under UV were pooled and seven combined fractions (from A to G) were obtained. Fraction A contained fractions from 1-19, B from 20-30, C from 31-34, D from 35-38, E from 39-48, F from 49-71, and G from 72-84. ^{1}H-NMR analyses were performed for all the pooled fractions and on the basis of flavanoids signals, fraction C, D, E, and F were selected for further purification by HPLC. Sixty sub-fractions (C1-4, D1-4, E1-4, and F1-4) were collected and analysed with H-NMR. The results show that flavonoids are mainly in sub-fraction F2 and F4. F4 sub-fraction contained more than one flavonoid so we applied one more HPLC step to fractionate F4 and four fractions (F4.1, 4.2, 4.3, 4.4) were collected each mainly containing a single compound.

5.2.4. HPLC analysis

The selected fractions were further separated using an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) equipped with a UV detector operating at 254 nm. A semi-preparative reversed phase column (Phenomenex Luna 5µ C18; 250 x 10 mm, 5µ) was used for separations, with a solvent gradient of 0.1 % formic acid with water and 0.1 % formic acid with CH_3OH. The gradient starts from H_2O-CH_3OH in the ration of 60:40 for the first 30 minutes, then shifted towards 20:80 for two minutes. After this the columm was reequilibrated again for the next analysis by ruinning the initited solvent 60:40 for eight minutes. Total time for each run is fourty minutes with the flow rate of 2 ml/min.

5.2.2. Plant materials and extraction of crude extract for NMR measurements

Plants were ground in liquid nitrogen and pooled before subjected to freeze-drying. Twenty-five milligrams of freeze-dried material were transferred to a micro-centrifuge

tube before adding 600 µl of CH_3OH-d_4. The mixture was vortexed for 2 minutes and sonicated for 20 minutes, followed by centrifugation at 13,000 rpm for 5 minutes at room temperature. Five hundred microliters of the supernatant were then transferred into 2ml micro-centrifuge tubes and were added two hundred fifty microliters of KH_2PO_4 buffer, pH 6.0, containing 0.1% trimethyl silyl propionic acid sodium salt (w/v). The mixture was left for 30 minutes in 4°C and followed by centrifugation at 6000 rpm for 5 minutes at room temperature. Seven hundred microliters of the supernatant were then transferred into 5 mm NMR tubes for analysis.

5.2.5. NMR measurements

The dried sub-fractions were redissolved in 1.0 ml of 50% CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% TMSP (trimethyl silyl propionic acid sodium salt, w/v) and then 800 µl of the supernatant was transferred to a 5 mm NMR tube. ^{1}H-NMR and 2D *J*-resolved spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. CH_3OH-d_4 was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µs), and relaxation delay (RD) = 1.5 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D *J*-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. *J*-Resolved spectra tilted by 45°, was symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H–^{1}Hcorrelated spectroscopy (COSY) and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. Window

function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 s relaxation delay, 6361 Hz spectral width in F2 and 27,164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectrum except for 30,183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

5.3. Results and discussions

5.3.1. Optimization of extraction method

The aim of this thesis is to study the effect of overexpression of CHS, a key enzyme of the flavonoid biosynthesis pathway, on the metabolism in *Arabidopsis.* As this enzyme will result in the production of flavonoids and related compounds, the focus is on the phenolic compounds. This includes also the compounds from earlier part of the phenylpropanoid pathway which might be affected because of competitive for the same precusors. Thus an efficient extraction method with good yield and reproducibility which provides reliable metabolic profiling data on phenolic compounds by using NMR spectroscopy was investigated. Due to the large differences of metabolites e.g. in molecular weight and polarity, in general CH_3OH-H_2O is usually used as extraction solvent for metabolic profiling because it is medium polar, and it penetrates cell walls and membranes quite effectively. In order to optimize the extraction for metabolic profiling, different ratios of water were mixed with CH_3OH, from 0 % to 100 % following the gradient 0%, 25%, 50%, 75%, 100%. The solvent CH_3OH/H_2O ratio of 1/1(v/v) give both signals of primary and secondary metabolites in the ^{1}H-NMR spectra, whereas the solvent 100% CH_3OH preferably extracts the secondary metabolites, such as phenolic compounds. Multivariable data analysis of the various extracts (data not shown) only revealed a clear difference between wild type and CHS transgenic plants with the 100% CH_3OH extracts. Therefore CH_3OH-d_4 was chosen as extraction solvent. As CH_3OH-d_4 also extracts chlorophyll, D_2O was added (30% in total volume) to the primary crude CH_3OH-d_4 extract to precipitate chlorophyll before NMR analysis. The final supernatant was analyzed directly by NMR.

5.3.2. Isolation and characterization of Arabidopsis thaliana Col. 0 flavonoids

The flavonoid glycosides of *Arabidopsis thaliana* Col. 0 were isolated and structure elucidated by use of HPLC, NMR and LC/MS.

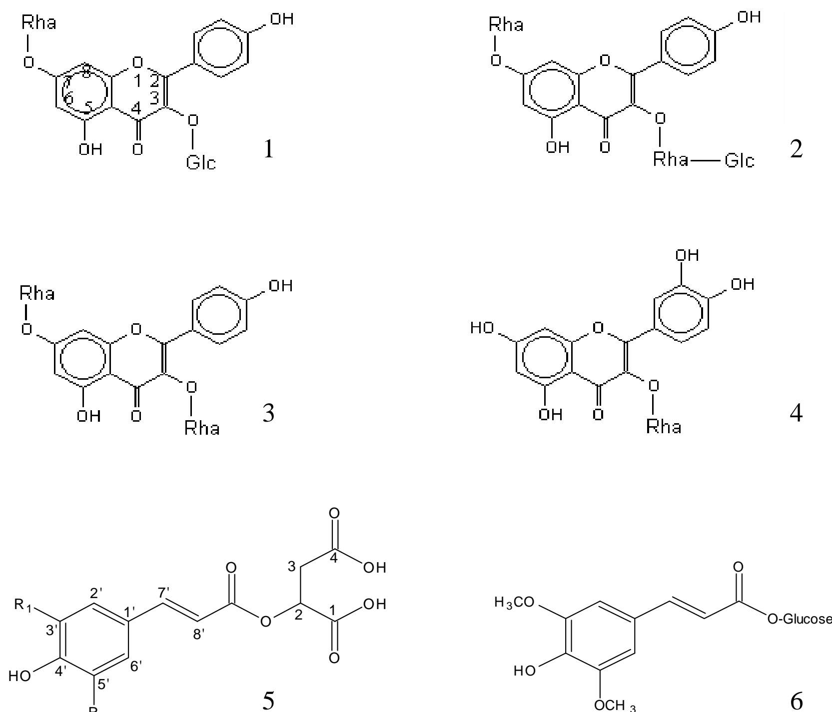
The NMR spectrum of the F2 sub-fraction shows two signals at δ 6.84 (d, J=2.0 Hz) δ 6.82 (d, J=2.0 Hz). Those are H-6, H-8 characteristic signals of a flavonoid glycoside. Another two signals at δ 6.99 (d, J=8.8 Hz) δ 8.11 (d, J=9 Hz) are charactistic signals of H-3'& 5', H-2' & 6' in kaempferol. The signals at δ 5.56 (d, J=1.5 Hz) and δ 1.25 (d, J=6.2 Hz) are characteristic signals of 7-*O*-rhamnose and the signals at δ 5.77 (d, J=7.8 Hz) and δ 0.95 (d, J=6.2 Hz) were identified as signals of a futher 3-*O*- (rhamnosyl (1–2) glucoside) [Kerhoas *et al.,* 2006]. This compound was assigned as kaempferol 3-*O*-[rhamnosyl (1–2) glucoside]-7-*O* -rhamnopyranoside which also fit with its $[M–H]^{-}$ signal on LC-ESI-MS is *m/z* 739.

The F4.1 fraction also showed the characteristic signals of a keampferol derivative at δ 6.52 (H-6, d, J=2.0 Hz) δ 6.82 (H-8, d, J=2.0 Hz) δ 7.0 (H-3'& 5', d, J=8.8 Hz) δ 8.09 (H-2' & 6', d, J=9 Hz). The signals at δ 5.56 (d, J=1.6 Hz) and δ 1.25 (d, J=6.2 Hz) are characteristic signals of 7-*O*-rhamnose and the signals at δ 5.77 (d, J=7.8 Hz) and δ 5.33 (d, J=7.7 Hz) were identified as signals of a 3-*O*- glucoside [Kerhoas *et al.,* 2006]. $[M–H]^{-}$ signal in LC-ESI-MS of F4.1 showed a *m/z* 577 which was confirmed that F4.1 is kaempferol (3-*O*-glucopyranoside-7-*O*-rhamnopyranoside) [Kerhoas *et al.,* 2006].

The NMR signals of the F4.3 fraction at δ 6.43 (H-6, d, J=2.0 Hz) δ 6.81 (H-8, d, J=2.0 Hz) δ 7.83 (H-2'& 6', d, J=9.0 Hz) δ 7.04 (H-3'&5', d, J=9.0 Hz) are in accordance with a kaempferol glycoside moiety. The signals at δ 5.56 (d, J=1.6 Hz) and δ 1.25 (d, J=6.2 Hz) are characteristic signals of a 7-*O*-rhamnose and the signals at δ 0.94 (d, J=6.0 Hz) were identified as signals of 3-*O*-rhamnoside [Kerhoas *et al.,* 2006]. Therefore F4.3 were assigned as kaempferol (3,7-*O*-dirhamnopyranoside) and fits with *m/z* 593 [Kerhoas *et al.,* 2006].

The NMR spectrum of the F4.4 fraction shows typical the quercetin derivative NMR signals at δ 7.72 (H-6, d, J=2.0 Hz) δ 6.97 (H-8, d, J=2.0 Hz) δ 6.89 (H-5', d, J=8.0 Hz) δ 7.27 (H-6', dd, J=8.0, 2.0 Hz) δ 7.32 (H-2', d, J=2.1 Hz). The sugar attached to quercetin was identified as rhamnose with the NMR signal at δ 0.94 (d, J=6.0 Hz). This compound also was confirmed as quercetin 3-*O*-rhamnopyranoside (**Fig. 5.1**) with a $[M–H]^{-}$ signal is *m/z* 477.

Based on the above mentioned information we could thus identify three kaempferol glycosides and one quercetin glycoside in *Arabidopsis thaliana* Col.0 leaves.



1) Kaempferol 3-*O*-glucopyranoside-7-*O*-rhamnopyranoside.
(2) Kaempferol 3-*O*-rhamnosyl (1–2) glucoside-7-*O*- rhamnopyranoside.
(3) Kaempferol 3,7-*O*- dirhamnopyranoside.
(4) Quercetine 3-*O*-rhamnopyranoside.
(5) Phenylpropanoids: $R_1 = OCH_3$, $R_2 = OH$, hydroxyferuloyl malate
$R_1 = OH$, $R_2 = H$, caffeoyl malate
$R_1 = H$, $R_2 = H$, coumaroyl malate
$R_1 = OCH_3$, $R_2 = OCH_3$, sinapoyl malate
(6) Synapoyl glucose

Figure 5.1. Chemical structures of flavonoids and phenylpropanoids in *A. thaliana* Col.0

5.3.3. NMR analysis of Arabidopsis in methanol crude extract

Metabolic profiling of *Arabidopsis* CH_3OH-d_4 crude extracts by using NMR 600MHz will be applied in the next experiments (**Chapter 6, 7, 8**). Identification of compounds is based on NMR spectra as described in this chapter.

Figure 5.2 shows the ^{1}H-NMR spectrum of the *Arabidopsis* Col.0. The combined information gathered from ^{1}H-NMR, COSY, J-resolved and HMBC spectra and the use of a library of ^{1}H-NMR spectra of reference compounds allowed an almost complete assignment. Sugars, organic acids and amino acids signals are present in the high field region of the NMR spectra, between 0.5 to 6.0 ppm (**Figure 5.2 b**). In the amino acid region (*δ* 0.8–*δ* 4.0) the main identified signals were alanine δ 1.48 (H-3, d, J=7.0 Hz), glutamic acid δ 2.07 (H-2, m) δ 2.41 (H-3, m), glutamine δ 2.12 (H-2, m) δ 2.48 (H-3, m) , leucine δ 0.96 (H-5, d, J=8.0 Hz), threonine δ 1.32 (H-5, d, J=6.6 Hz), valine δ 1.03 (H-5, d, J=7.8 Hz), aspartic acid δ 2.67 (m) and asparagine δ 2.8 (m). The organic acid regions of the NMR spectrum only show signals of formic acid δ 8.5 (s) and malic acid δ 4.32 (H-2, dd, J=4.0 Hz, 11 Hz) because the other organic acids have very poor solubility in CH_3OH. Hence only formic acid and malic acid can be detected in the CH_3OH extract. The signals of the terminal CH_3 of choline was identified at *δ* 3.23 (s). For sugars, the anomeric proton of *β*-glucose at δ 4.57 (H-1, d, J=8.0 Hz), α-glucose at *δ* 5.18 (H-1, d, J=3.7 Hz), sucrose at *δ* 5.4 (H-1, d, J=4.0 Hz), rhamnose at δ 5.62 (H-1, d, J=8.0 Hz), and fructose at δ 4.17 (H-1, d, J=9.0 Hz) were assigned.

Signals of four flavonoids present in the low field region (6.8-8.2 ppm) have been analyzed (see above), Quercetine derivatives are present as minor compounds in the crude extract but difficult to detect in the NMR spectrum. Moreover, in the aromatic region, the presence of five major doublets with the same coupling constants (d, J=16.0 Hz) in the range of δ 6.31–δ 6.50 indicate the presence of the *trans* olefinic protons H-8’ of phenylpropanoids (**Figure 5.3**) [Liang *et al.,* 2006]. This also was confirmed by the correlation of H-8′ of the phenylpropanoids with the H-7′ (d, J=16.0 Hz) protons at δ 7.54–δ 7.59 in the COSY spectrum (**Figure 5.4**). Five *trans*-phenylpropanoids were elucidated by two dimensional NMR. Those are *trans*-caffeoyl malate (H-8’, δ 6.32; H-7’, δ 7.66), *trans*-5-hydroxyferuloyl malate (H-8’, δ 6.34; H-7’, δ 7.66), *trans*-coumaroyl malate (H-8’, δ 6.37, H-7’, δ 7.66), sinapoyl malate (H-2 & 6, δ 6.99 s; H-8, δ 6.48 d, J=16 Hz; H-7 δ 7.66 d, J=16 Hz), and sinapoyl glucose (H-2 & 6, δ 6.97 s; H-8 δ 6.49 d, J=16 Hz; H-7 δ 7.77 d, J=16 Hz) [Liang *et al.,* 2006] (**Figure 5.3**). The *cis*-form of those phenylpropanoids are present only at very low concentration in crude extract so we could not identify them.

The ^{1}H chemical shifts (*δ*) and coupling constants (Hz) of the indentified *Arabidopsis thaliana* Col.0 metabolites are presented in **Table 5.1**.

Table 5.1. [1]H chemical shifts (*δ*) and coupling constants (Hz) of *Arabidopsis thaliana* Col. 0 metabolites identified by references and using 1D and 2D NMR spectra (CH_3OH-d_4–KH_2PO_4 in D_2O, pH 6.0)

Compounds	Chemical shifts (ppm) and coupling constants (Hz)
Amino/organic acids	
Threonine	δ 1.32 (H-5, d, J=6.6 Hz)
Alanine	δ 1.48 (H-3, d, J= 7.0 Hz)
Glutamine	δ 2.12 (H-2, m) δ 2.48 (H-3, m)
Glutamic acid	δ 2.07 (H-2, m) δ 2.41 (H-3, m)
Valine	δ 1.03 (H-5, d, J=7.8 Hz)
Leucine	δ 0.96 (H-5, d, J=8.0 Hz)
Asparagine	δ 2.8 (m), 2.97(m)
Aspartic acid	δ 2.67 (m)
Malic acid	δ 4.32 (H2, dd, J=4.0 Hz, 11 Hz) δ 2.80 (H3, dd, J=8.8 Hz, 16.0 Hz) δ 2.96 (H2, dd, J=3.6 Hz, 16.0 Hz)
Formic acid	δ 8.5 (s)
Sugars	
β-glucose	δ 4.57 (H-1, d, J=8.0 Hz)
α-glucose	δ 5.18 (H-1, d, J=3.7 Hz)
Rhamnose	δ 5.62 (H-1, d, J=8.0 Hz)
Fuctose	δ 4.17 (H-1, d, J=9.0 Hz)
Sucrose	δ 5.40 (H-1, d, J=4.0 Hz)
Phenylpropanoids/Flavonoids	
Kaempferol 3-*O*-glucopyranoside-7-rhamnopyranoside	δ 6.52 (H-6, d, J=2.0 Hz) δ 6.82 (H-8, d, J=2.0 Hz) δ 7.0 (H-3'& 5', d, J=8.8 Hz) δ 8.09 (H-2' & 6', d, J=9 Hz)
Kaempferol 3,7-*O*-dirhamnopyranoside	δ 6.43 (H-6, d, J=2.0 Hz) δ 6.81 (H-8, d, J=2.0 Hz) δ 7.83(H-2'& 6', d, J=9.0 Hz) δ 7.04 (H-5', d, J=9.0 Hz)
Kaempferol 3-*O* -rhamnosyl (1–2) glucoside-7-*O*-rhamnopyranoside	δ 6.84 (H-6, d, J=2.0 Hz) δ 6.82 (H-8, d, J=2.0 Hz) δ 6.99 (H-3'& 5', d, J=8.8 Hz) δ 8.11 (H-2' & 6', d, J=9 Hz)
Quercetine 3-*O*-rhamnoside	δ 7.72 (H-6, d, J=2.0 Hz) δ 6.97 (H-8, d, J=2.0 Hz) δ 6.89 (H-5', d, J=8.0 Hz) δ 7.27 (H-6', dd, J=8.0, 2.0 Hz) δ 7.32 (H-2', d, J=2.1 Hz)
trans-5-hydroxyferuoyl malate	δ 6.34 (H-8', d 16 Hz) δ 7.54 (H-7', d, J=16 Hz)
trans-feruoyl malate	δ 6.42 (H-8', d 16 Hz) δ 7.66 (H-7', d, J=16 Hz)
trans-caffeoyl malate	δ 6.32 (H-8', d 16 Hz) δ 7.55 (H-7', d, J=16 Hz)
trans-coumaroyl malate	δ 6.37 (H-8', d 16 Hz) δ 7.59 (H-7', d, J=16 Hz)
trans-sinapoyl malate	δ 6.99 (H-2 & 6, s) δ 6.48 (H-8, d, J=16 Hz) δ 7.66 (H-7, d, J=16 Hz)
trans-sinapoyl glucoside	δ 6.97(H-2 & 6, s), δ 6.49 (H-8,d, J=16 Hz), δ 7.77(H-7, d, J=16 Hz)
Other compounds	
Choline	δ 3.23 (s)
Inositol	δ 4.1 (H-2, dd, J=2.0 Hz, 13Hz) δ 3.62(H-4 and 6, dd, J=8.8 Hz, 16.2 Hz) δ 3.46 (H-1 and 3, dd, J=6.5 Hz, 13.9 Hz)

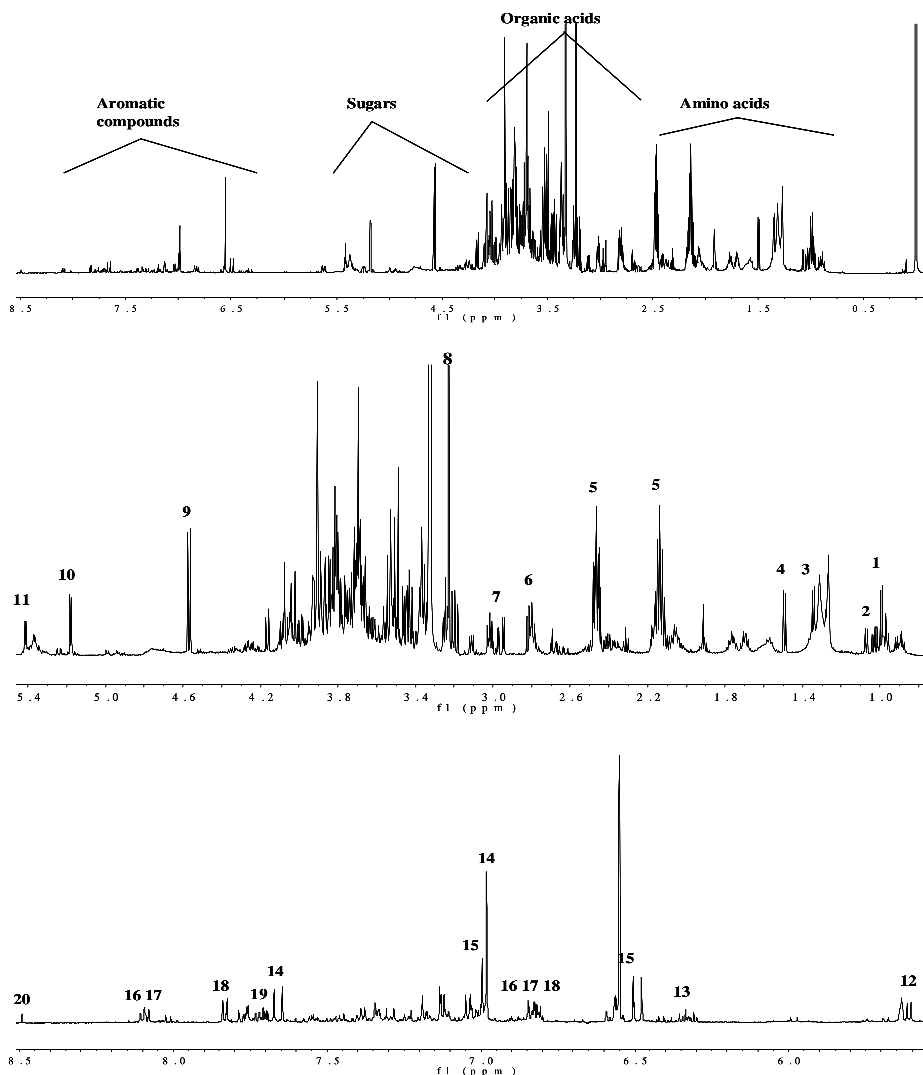


Figure 5.2. ^{1}H-NMR spectra of *A. thaliana* Col. 0 (a), extended high field region 0.0-5.5 ppm (b), extended low field region 5.6-8.2 ppm (c). 1. Leucine., 2. Valine, 3. Threonine, 4. Alanine, 5. Glutamine, 6. Asparagine, 7. Malic acid, 8. Choline, 9. β-glucose, 10. α-glucose, 11. Sucrose, 12. Rhamnose, 13. Phenylpropanoids (*trans*-feruoyl malate, *trans*-caffeoyl malate, *trans*-coumaroyl malate, *trans*-5-hydroxyferuoyl malate) 14. *trans*-sinapoyl malate, 15. *trans*-sinapoyl glucose, 16. Kaempferol 3-*O*-glucopyranoside-7-rhamnopyranoside, 17. Kaempferol 3-*O*-rhamnosyl (1–2) glucoside-7-*O*- rhamnopyranoside, 18. Kaempferol 3,7-*O*- dirhamnopyranoside, 19. Quercetin derivatives, 20. Formic acid.

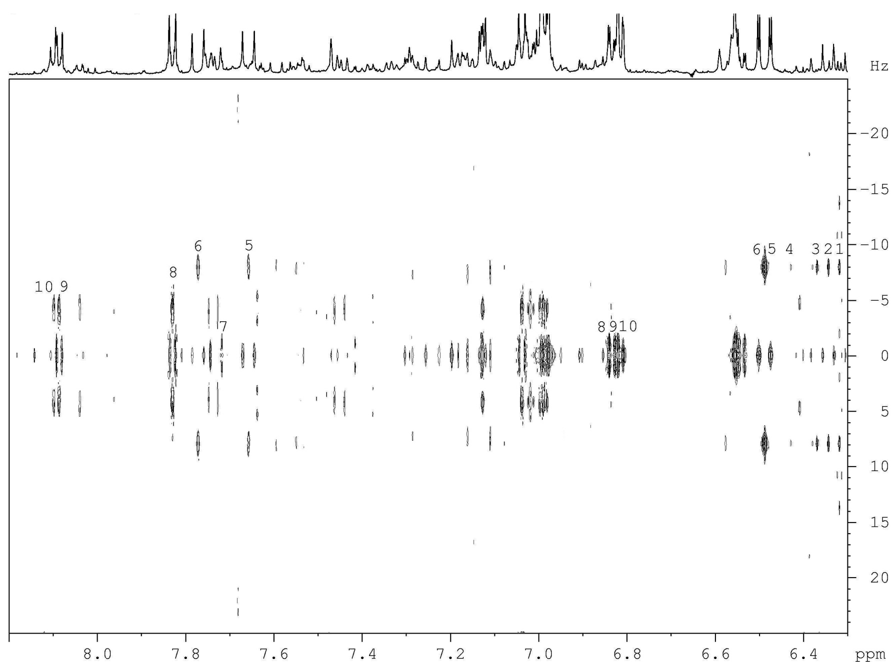


Figure 5.3. 2D NMR J-resolved spectra of *A. thaliana* Col. 0 in aromatic region from 6.2 – 8.2 ppm. 1. *trans*-caffeoyl malate, 2 *trans*-5-hydroxyferuloyl malate., 3. *trans*-coumaroyl malate, 4. *trans*-feruloyl malate, 5. *trans*-sinapoyl malate,,6. *trans*-sinapoyl glucose, 7. Quercetin derivatives., 8. Kaempferol 3,7-*O*- dirhamnopyranoside, 9. Kaempferol 3-*O*-rhamnosyl (1–2) glucoside-7-*O*- rhamnopyranoside, 10. Kaempferol 3-*O*-glucopyranoside-7-rhamnopyranoside.

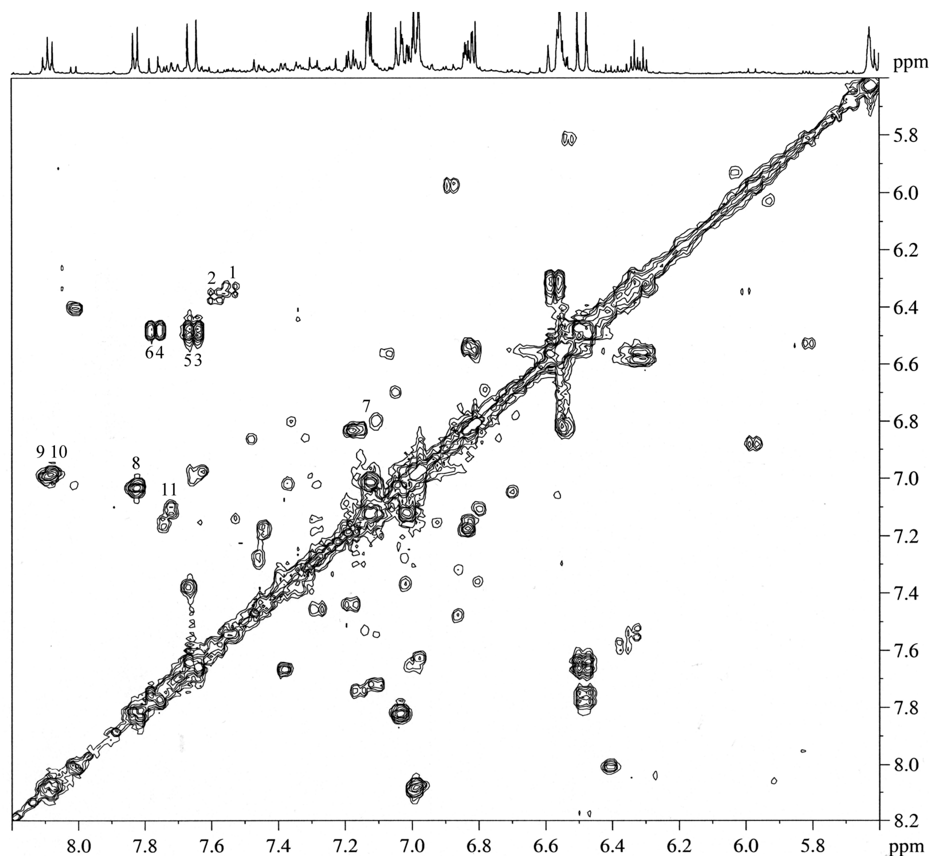


Figure 5.4. 2D NMR Cosy spectra of *A. thaliana* Col. 0 in aromatic region from 5.7 – 8.2 ppm. 1. *trans*-caffeoyl malate, 2 *trans*-5-hydroxyferuloyl malate., 3. *trans*-coumaroyl malate, 4. *trans*-feruloyl malate, 5. *trans*-sinapoyl malate,,6. *trans*-sinapoyl glucose, 7. Quercetin derivatives., 8. Kaempferol 3,7-*O*- dirhamnopyranoside, 9. Kaempferol 3-*O*-rhamnosyl (1–2) glucoside-7-*O*-rhamnopyranoside, 10. Kaempferol 3-*O*-glucopyranoside-7-rhamnopyranoside.

5.4. Conclusion

Three keampferol glycosides and one quercetin glycoside were isolated and identified in this study. Twenty six metabolites of *A. thaliana* Col.0 in methanol crude extract were identified and listed **Table 5.1.** These results are now used as reference for next studies.

Chapter 6

Metabolic profiling of *Arabidopsis thaliana* transformed with a heterologous *chs* cDNA from *Cannabis sativa*

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Abstract

Nuclear Magnetic Resonance (NMR) and Multivariate Data Analysis (PDA) are important analytical tools for macroscopic profiling of metabolomes. This study describes the use of this approach to measure the metabolome of transgenic *Arabidopsis thaliana* plants with a high expression level of a heterologous chalcone synthase gene. Five transgenic *Arabidopsis* lines were analyzed in this study. The Partial least square-Discriminant Analysis (PLS-DA) showed a very good separation between the transgenic plants and controls. This analysis indicated that the level of sugars, flavonoids and phenylpropanoids are higher in the CHS transgenic plants than in control plants. These results show that chalcone synthase overexpression affects both plant secondary metabolism and primary metabolism.

Keywords: chalcone synthase, flavonoids, phenylpropanoids, *Arabidopsis*, NMR, multivariate data analysis.

6.1. Introduction

We can look at what genes are being expressed, and what proteins are present, but what are the end products in the form of cellular functions? Metabolomics attempts to answer this question. Linking functional metabolomic information to mRNA and protein expression data makes it possible to visualize the functional genomic repertoire of an organism. Metabolomics is becoming a widely used technology to evaluate global metabolite levels. In the context of functional genomics, the non-targeted profiling of metabolites in biological samples is now regarded as a viable counterpart to protein and transcript profiling technologies.

Metabolomics is the study of all the metabolites of a biological sample. Several analytical tools such as gas chromatography (GC), liquid chromatography (LC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) have been used to profile the metabolome. None of these is able to give a comprehensive view of the complete metabolome. Issues as dynamic range, polarity, volatility, and stability are in fact limiting factors in obtaining a complete picture of all metabolites. A combination of the various methods should be used to have the most comprehensive view on the metabolome. However, with each single analytical method a wealth of information about the metabolome of an organism can already be obtained under certain conditions.

Nuclear magnetic resonance spectroscopy (NMR) has been considered as a powerful platform in metabolomics because of its ease of sample preparation, short time of analysis and as the only method that allows direct absolute quantitation of all metabolites. Also with ^{1}H-NMR analysis we can detect and do structure elucidation of various metabolites in the sample. However, most metabolites have many signals in their ^{1}H-NMR spectra, thus overlapping may cause a problem in identifying individual metabolites. 2D NMR or chromatographic separation can be used to overcome this problem.

Transgenic or mutant plants in combination with metabolomics provide an excellent means to look at changes in metabolic networks through the specific perturbation of a gene of interest. Plant extracts are very complex in composition and, if many samples are examined, it is difficult to make a meaningful comparison of large numbers of spectra or chromatograms ‘by eye.’ Thus multivariate statistical methods can be

extremely useful, as they are able to compress data into a more easily managable form, allowing the visualiation of the relation between samples.

In this study we used NMR spectroscopy and multivariate data analysis to monitor metabolome changes in transgenic CHS overexpressing *Arabidopsis thaliana* Col 0. Various metabolites were detected and their NMR signals were assigned. Finally, the differences between metabolites levels in transgenic plants and controls were determined.

The first committed step in the biosynthesis of flavonoids is catalysed by the enzyme chalcone synthase (CHS), resulting in a yellow coloured chalcone. In the majority of plants chalcones are not end-products, but intermediates in the pathway proceeding with several further enzymatic steps to other classes of flavonoids, such as flavanones, dihydroflavonols and finally to the anthocyanins, the major water-soluble pigments in flowers and fruits. Other flavonoid classes (i.e. isoflavones, aurones, flavones, pro-anthocyanidins (PA) and flavonols) represent side branches of the flavonoid pathway and are derived from intermediates in the anthocyanin formation. Little is known about the effect of CHS in the total plant metabolomic network, e.g. leading to lignan- and phenylpropanoid derivatives.

Prior to the present study, we transformed a *chs*-cDNA gene from *Cannabis sativa* into *A. thaliana.* The introduction of the first gene in the flavonoid biosynthesis pathway may alter directly or indirectly the level of other metabolites present in *A. thaliana*. This study aimed at the identification of metabolomic pathways that are affected by the overexpression of the *CHS* gene in this plant. ^{1}H-NMR based metabolomics coupled with multivariate data analysis was applied to distinguish between control and CHS transgenic *A. thaliana* plants (ACS).

6.2. Materials and methods

6.2.1. Growth of plant materials

Arabidopsis thaliana ecotype Columbia (Col.0) seeds were obtained from the section Plant Cell Physiology (IBL, Leiden Universiy, The Netherlands) and 5 transgenic ACS lines (ACS 1, 2, 14, 20, 21) were generated as described in **Chapter 3**. 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 a plate containing half MS medium with 0.6% agar supplemented with 20 mg/L hygromycin for selection of transgenic plants. After keeping 3 days at 4°C in the dark, the seeds were germinated at 23°C under long day condition (16/8 h light/dark cycle) in a growth chamber. All the plant tissues were harvested when the seedlings are 10-days old. Immediately after harvesting, the material was frozen in liquid nitrogen and kept at −80° C until used.

6.2.2. Solvents and chemicals

D_2O (99%) and CH_3OH-d_4 (99.8%) were obtained from Cambridge Isotope Laboratories Inc (Miami, FL, USA). NaOD was purchased from Cortec (Paris, France). Potassium dihydrogen phosphate and trimethylsilane propionic acid sodium salt (TSP) were purchased from Merck (Darmstadt, Germany). As buffering agent 1.232 g KH_2PO_4 and 10 mg TSP (internal standard) were added to 100 g D_2O. Finally, the pH of the solution was adjusted to 6.0 using 1 M NaOD.

6.2.3. Extraction of plant materials

Plants were ground in liquid nitrogen and pooled before subjected to freeze-drying. Twenty-five milligrams of freeze-dried material were transferred to a micro-centrifuge tube before adding 600 μl of CH_3OH-d_4. The mixture was vortexed for 2 min and sonicated for 20 min, followed by centrifugation at 13,000 rpm for 5 min at room temperature. Five hundred microliters of the supernatant were then transferred into 2 ml micro-centrifuge tubes and 250 microliters of KH_2PO_4 buffer, pH 6.0, containing 0.1% trimethyl silyl propionic acid sodium salt (w/v) were added. The mixture was left for 30 min in 4°C and followed by centrifugation at 6000 rpm for 5 min at room temperature. Seven hundred microliters of the supernatant were then transferred into 5 mm NMR tubes for analysis.

6.2.4. NMR measurement

^{1}H-NMR, 2D J-resolved, ^{1}H-^{1}H correlated spectroscopy (COSY), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded at 25 °C on a 600 MHz Bruker AV 600 spectrometer equipped with cryo-probe operating at a proton NMR frequency of 600.13 MHz. CH_3OH-d_4 was used as the internal lock. Each ^{1}H-NMR

spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 30° (10.8 μsec), and relaxation delay (RD) = 1.5 sec. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zerofilled to 32 K points. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, using Topspin (version 2.1, Bruker).

6.2.5. Data analysis

The spectral intensities were reduced to integrated regions of an equal width of 0.04 ppm (buckets or bins) corresponding to the region of δ 0.3 - δ 10.0. The regions of δ 4.8-δ 4.9 and δ 3.28- 3.40 were excluded from the analysis because of the residual signal of water and CH_3OH. Principal Compnent Analysis (PCA), Partial least square-Discriminant Analysis (PLS-DA) and Hierarchical Clustering Analysis (HCA) were performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden).

6.3. Results and discussion

Seedlings of Arabidopsis Col. 0 wild type (WT), transgenic plants with empty vector (E) and 5 CHS transgenic plant lines (ACS1, 2, 14, 20, 21) were subjected to metabolomic analysis by ^{1}H-NMR and 2D-NMR spectroscopy. For the assignments of metabolites in the ^{1}H-NMR spectra is referred to **Chapter 5.** ^{1}H-NMR spectra of ACS 2, ACS 20 and WT are shown in **Figure 6.1**. In the aromatic area, three kaempferol glycosides, quercetin rhamnose, 4 malate conjugated cinamic acid derivatives (hydroxyferulic, caffeic, coumaric, and sinapoyl), sinapoyl glucoside and formic acid were found. Visually in ^{1}H-NMR spectra we can see that all the identified phenolic compounds are significantly higher in ACS 20 compared to ACS 2 which has the same level of phenolic compounds as WT. This is in accordance with the results reported in **Chapter 4** showing that ACS 2 has similar CHS activity as WT.

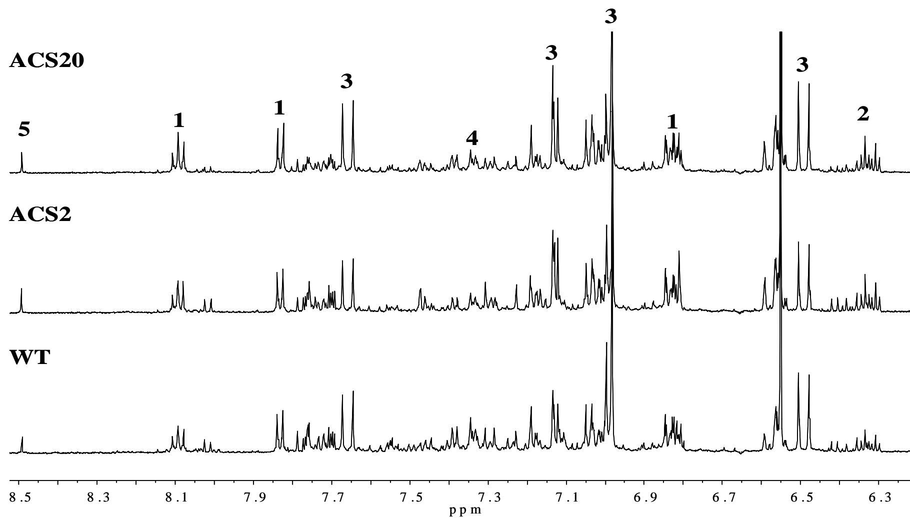

Figure 6.1. Aromatic regional ^{1}H-NMR spectra of Arabidopsis Col.0 (WT) and CHS transgenic Arabidopsis (ACS2, ACS20). 1. Keampferol glycosides (Kaempferol 3,7-*O*- dirhamnopyranoside, Kaempferol 3-*O*-rhamnosyl (1–2) glucoside-7-*O*- rhamnopyranoside, Kaempferol 3-*O*-glucopyranoside-7-rhamnopyranoside), 2. *trans*-phenylpropanoids (caffeoyl malate, coumaroyl malate, hydroxyferuoyl malate), 3. *trans*-synapoyl, 4. Quercetin derevative, 5. Formic acid.

In order to distinguish the samples based on the ^{1}H-NMR spectra, multivariate data analysis was applied. Firstly, principal component analysis (PCA) was used to reduce the data set from the ^{1}H-NMR spectra to fewer components enabling to group samples or to do pattern recognition. To remove the biological variation of the samples of CHS transgenic plants and non CHS transgenic plants, a supervised multivariate data analysis, termed Partial Least Square-Discriminant Analysis (PLS-DA), was employed. After the PLS-DA analysis, Hierachical Clustering Analysis (HCA) based on PLS-DA was performed. The results show that E samples are very close to WT and all the samples of transgenic line ACS 2 and two samples of transgenic line ACS 21 are separated from other transgenic lines (**Figure 6.2**). This result fits with the genetic data of the transgenic plants which are reported in **Chapter 3**. The transgenic ACS 2 and ACS 21 have 5 copies of the *CHS* transgene. Since the E group shows similar metabolites profiles as WT group, it was not further studied.

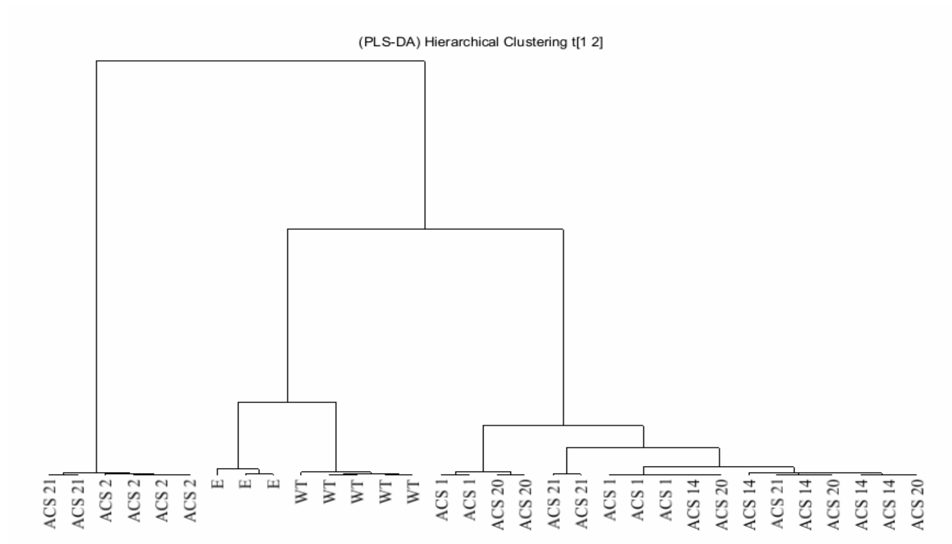

Figure 6.2. Hierarchical Clustering Analysis (HCA) based on PLS-DA of transgenic and control *Arabidopsis* plants. E: transgenic Arabidopsis with empty vector, WT: wild type *A. thaliana* Col.0; ACS 1….ACS 21: CHS transgenic *Arabidopsis* plants

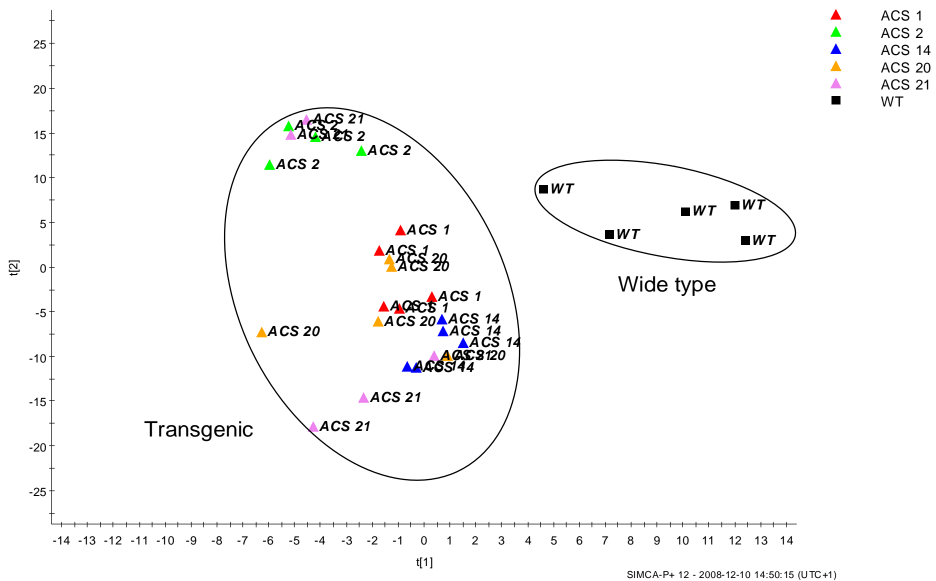

Figure 6.3. The PLS-DA score scatter plot of CHS transgenic (ACS 1-21) and wild type (WT) *Arabidopsis* Col. 0 plants.

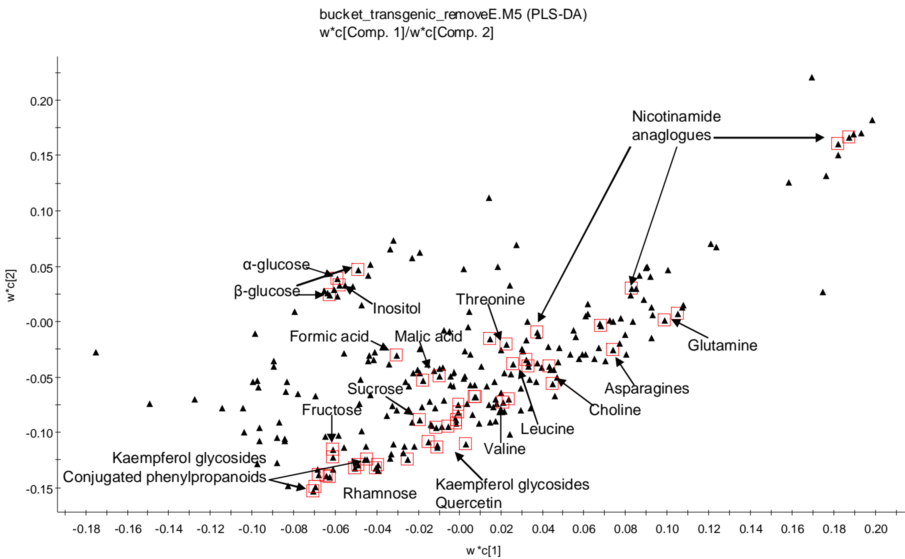

Figure 6.4. The PLS-DA loading scatter plot of transgenic (ACS 1-21) and wild type (WT) *Arabidopsis* Col. 0

When PLS-DA was applied, all the transgenic lines and WT plants could be clearly observed as separate clusters in the PLS-DA score scatter plot in PC 2 (**Figure 6.3**). Most of the transgenic plants are located on the negative side of the PLS PC 2 axis while all WT plants are on the positive side. To find out which metabolites contribute to the discrimination between the transgenic plants, a PLS-DA loading scatter plot (w*c) was analysed as shown in **Figure 6.4**. Positive values of w*c[1] are seen for the variable associated with WT plants whereas negative values are associated with transgenic plants. Examination of the loading plot shows that the metabolites strongly contributing to the separation of the WT plants were amino acids such as threonine (δ 1.32), alanine (δ 1.48), leucine (δ 0.96), asparagine (δ 2.8 and 2.97) and glutamine (δ 2.12 and 2.48). For the transgenic plants, the responsible loading plots correspond to the signals of sugars (α-glucose δ 5.18, β-glucose δ 4.58, rhamnose δ 5.62, sucrose δ 5.4), flavonoids (kaempferol 3-*O*-glucopyranoside 7-*O*-rhamnopyranoside δ 8.09, kaempferol 3,7-*O*-dirhamnopyranoside δ 7.83, kaempferol 3-O-[rhamnosyl (1–2) D-glucoside]-7-O-rhamnopyranoside δ 8.11, quercetine moiety δ 7.72), phenylpropanoids (*trans*-sinapoyl malate δ 6.48 and 7.66, *trans*-sinapoyl glucose δ 6.49 and 7.77, *trans*-5-hydroxyferuoyl malate, *trans*-caffeoyl malate, *trans*-coumaroyl malate δ 6.32 - 6.37 and 7.54 - 7.59).

These results indicate that sugars, flavonoids and phenylpropanoids are accumulated at higher levels in the transgenic plants than in WT plants, which contain higher levels of amino acid and nicotinamide analogues. It suggests that the presence of the heterogonous *CHS* gene in the transgenic plants influences the flavonoid pathway directly and indirectly, resulting in upregulation of sugar and phenylpropanoid metabolism. A simplified biosynthetic network including the compounds discussed in this chapter is shown in **Figure 6.4**. The phenylpropanoid pathways and secondary metabolic pathways are presented in **Figure 6.5** and **Figure 6.6.** Our results showed that the heterologous *CHS* gene may influence the expression of other genes so a micro array analysis could help to obtain a more complete picture of the effect of expression the *CHS* transgene on plant metabolism.

In plants, sugars have dual functions as nutrition and as important signal molecules [Rolland *et al.,* 2002]. There are several studies indicating that sugars upregulate the flavonoid and anthocyanin biosynthesis pathway strongly. The *CHS* gene derived from petunia (*Petunia hybrida*) petals expressed in transgenic *Arabidopsis* leaves was induced by sugars and *Arabidopsis* grown on a sucrose containing medium showed high levels of anthocyanins [Tsukaya *et al.,* 1991; Ohto *et al.,* 2001]. Here we also found a correlation between high expression of *CHS* and sugars, though now the effect seems inversed, the CHS expression causing increase of sugars.

The increase of flavonoid and phenylpropanoid levels in the transgenic plants suggests that CHS transgenic plants could be a way to achieve crop plants with a higher level of these phenolic compounds. They play an important role in plant development and a plant's interaction with the environment [Rasmussen and Dixon, 1999]. It is reported that flavonoids have antimicrobial activity, and can protect plants from herbivory. Their function as UV protectant in plants is well-known. Moreover important medicinal and nutritional values are described for flavonoids such as antioxidant activity, anti-inflammatory activity and anti-tumor activity [Harborne and William, 2000; Dixon, 2000]

A comparision of metabolites in wild type *Arabidopsis* and 5 CHS transgenic plants (ACS 1, 2, 14, 20, 21) based on the loading scatter plot are presented in **Table 6.1.**

Table 6.1. The metabolites in wild type *Arabidopsis* and transgenics

Compounds	WT	ACS1	ACS2	ACS14	ACS20	ACS21
Amino/organic acids						
Threonine	+	–	–	–	–	–
Alanine	+	–	–	–	–	–
Glutamine	+	–	–	–	–	–
Valine	+	–	–	–	–	–
Leucine	+	–	–	–	–	–
Asparagine	+	–	–	–	–	–
Formic acid	–	+	+	+	+	+
Sugars						
β-glucose	–	+	+	+	+	+
α-glucose	–	+	+	+	+	+
Rhamnose	–	+	+	+	+	+
Fuctose	–	+	+	+	+	+
Sucrose	–	+	+	+	+	+
Phenylpropanoids/Flavonoids						
Kaemferol 3-*O*-glucopyranoside-7-rhamnopyranoside	–	+ +	+	+	+ +	+
Kaemferol 3,7-*O*-dirhamnopyranoside	–	+ +	+	+	+ +	+
kaempferol 3-O- rhamnosyl (1–2) D-glucoside]-7-O-rhamnoside	–	+ +	+	+	+ +	+
Quercetine direvatives	–	+ +	+	+	+ +	+
trans-5-hydroxyferuoyl malate	–	+	+	+	+	+
trans-caffeoyl malate	–	++	+	+	++	+
trans-coumaroyl malate	–	++	+	+	++	+
trans-sinapoyl glucoside	–	+ +	+	+	+ +	+
trans-sinapoyl malate	–	+ +	+	+	+ +	+
Other compounds						
Choline	+	–	–	–	–	–
Nicotinamide analogue	+	–	–	–	–	–
Inositol	–	+	+	+	+	+

Note: – : lower, + : higher, ++: much higher

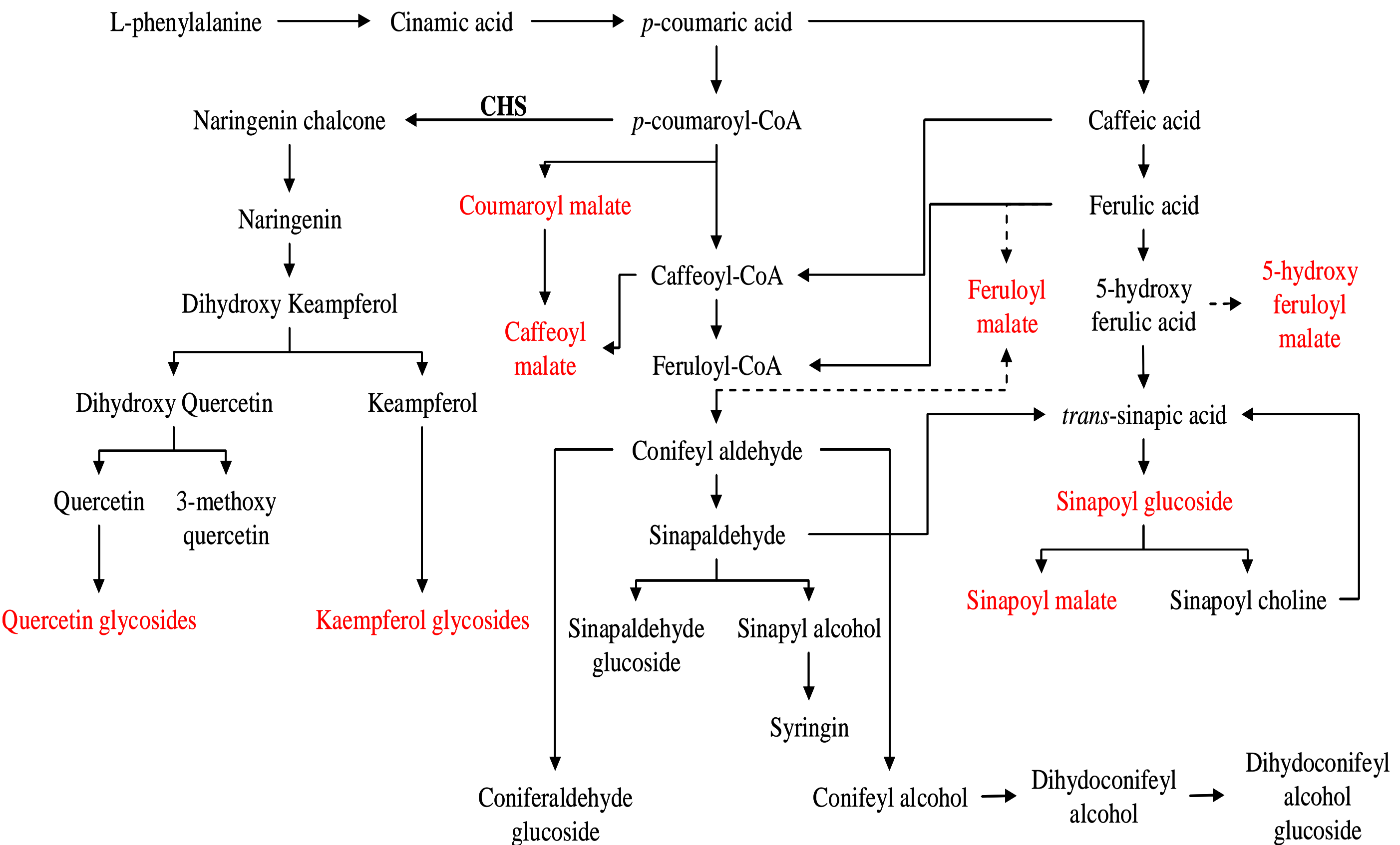

Figure 6.5. Phenylpropanoids biosynthesis pathways in *Arabidopsis* (Reference: *AraCyc* pathway; Sullivan, 2009)

The compounds with red letters are increased in transgenic CHS plants. ——▶ known pathway; - - - -▶ unknown pathway.

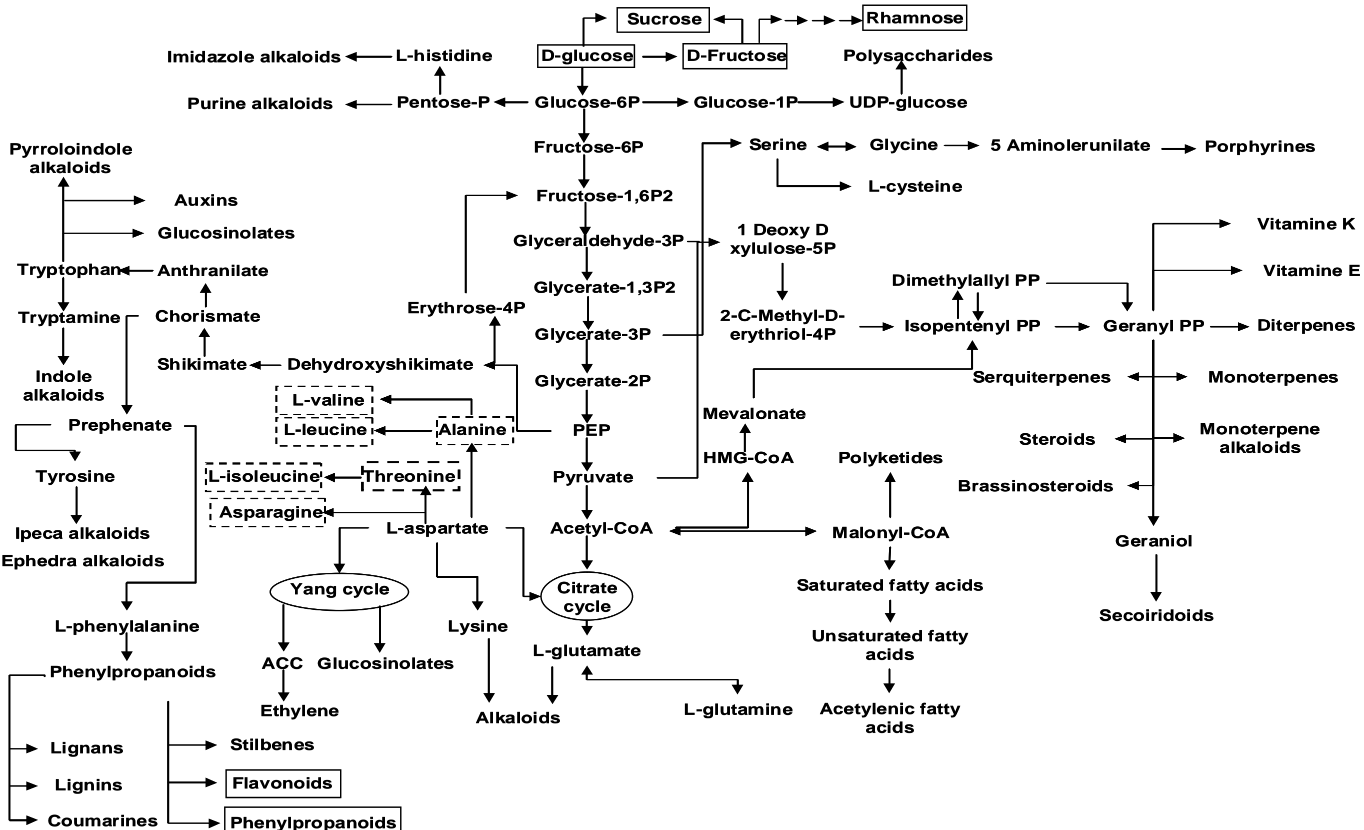

Figure 6.6. Secondary metabolomic biosynthesis pathways in plants (Reference: Kegg pathway)

The compounds in [solid box] are increased and in [dashed box] are decreased in transgenic CHS plants

6.4. Conclusion

This study shows that a specific extraction method focusing on phenolic compounds such as flavonoids and phenylpropanoids and using NMR-based metabolomics coupled with multivariate data analysis is able to visualize metabolome changes in CHS transgenic plants. This analytical method allows identification of a broad range of primary and secondary metabolites in crude samples without any purification steps. The introduction of the heterogonous *CHS* gene in *A. thaliana* has influence on the whole plant metabolism, and not only on the pathway where the transgenic protein ic expressed. The results also show a potential future for CHS transgenic plants, as modifying the flavonoid biosynthesis pathway could improve nutritional or medicinal value of a plant.

Chapter 7

^{1}H-NMR analysis of metabolic changes in *Arabidopsis thaliana* and CHS transgenic plants upon treatment with UV-A/blue light

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Abstracts

The metabolic response of *Arabidopsis thaliana* Col. 0 and CHS transgenic plants upon treatment with UV-A/blue light were investigated using high resolution ^{1}H-NMR spectroscopy combined with multivariate data analysis. The investigation of the score and loading plots of partial least square (PLS) and partial least square-discriminant analysis (PLS-DA) showed a high accumulation of flavonoids, phenylpropanoids, glucose, fructose, rhamnose, and organic acids in *A. thaliana* Col. 0 whereas no significant change was obtained in CHS transgenic plants after treatments with UV-A/blue light. The control transgenic plants in fact had already similar levels of flavonoids and phenylpropanoids as the UV-A/blue light treated wild type *Arabidopsis*.

Keywords: UV-A/blue light, chalcone synthase, flavonoid, phenylpropanoid, *Arabidopsis*, metabolome.

7.1. Introduction

Plants detect and respond to a wide range of endogenous and environmental signals that control their metabolism and development. The synthesis of secondary metabolites in response to stresses has been implicated as a major defense response of higher plants [Bell, 1981]. UV radiation from the sun induces various responses in higher plants. While the greatest portion of UV-B (280–320 nm) is absorbed by the ozone layer, UV-A (320–400 nm) penetrates the atmosphere to reach the earth surface. DNA is especially sensitive to UV-B, resulting in the formation of pyrimidine dimers [Taylor *et al.*, 1997; Frohnmeyer and Staiger, 2003]. Low UV-B stimulates distinct other responses in plants, such as the accumulation of UV-absorbing pigments and expression of stress response-related genes [Hahlbrock and Scheel, 1989; Mackerness *et al.*, 2001; Brosché and Strid, 2003; Frohnmeyer and Staiger, 2003]. UV and blue light regulate the expression of various plant genes. In several species, UV-B, UV-A, and blue light stimulate the transcription of genes encoding the key phenylpropanoid and flavonoid biosynthesis enzymes like phenylalanine ammonia–lyase (PAL) and chalcone synthase (CHS) [Fuglevand *et al.,* 1996, Chappell and Hahlbrock 1984; Kubasek *et al.,* 1992; Jenkins 1997; Schäfer *et al.,* 1997]. That causes an induction of flavonoid accumulation such as kaempferol derivatives and sinapoyls in *Arabidopsis* [Hahlbrock 1981; Beggs *et al.,* 1985; Li *et al.,* 1993; Lois, 1994]. UV-B and UV-A/blue light act through separate but synergistic pathways in inducing CHS expression in *Arabidopsis* [Fuglevand *et al.,* 1996]. UV-B, UV-A and blue light each stimulate *CHS* expression up to about 10-fold in mature *Arabidopsis* leaf tissue and the combination of light treatments give even a much larger increase [Fuglevand *et al*., 1996].

Plants are thought to produce natural sunscreens, which selectively absorb photons in the UV-B and UV-A range, and flavonoid pigments are generally regarded as UV-absorbing agents. The accumulation of UV-absorbing compounds (including flavonoids such as flavones, isoflavonoids and anthocyanins) in the vacuoles of the epidermal layer provide selective attenuation of UV-B radiation [Hrazdina *et al.*, 1982, Schmelzer *et al.*, 1988, Tevini *et al.*, 1991, Strid and Porra, 1992]. UV light induces anthocyanins in for example, the swollen hypocotyls of turnip, apple fruit, Gros Colman' grapes (*Vitis vinifera* L.), petals of *Rosa hybrida*, *Arabidopsis* [Zhou *et al.,* 2007; Arakawa, 1988;

Kataoka *et al.,* 2003; Nakamura *et al.,* 1980], and camalexin in *Arabidopsis* [Mert *et al.,* 2003].

There are 3 UV/blue light photoreceptors in *Arabidopsis* identified: cryptochromes 1 and 2 (cry1 and cry2), and the phototropism photoreceptor phototropin (nph1) [Lin, 2000]. Plant nph1 is tightly associated with the plasma membrane [Reymond *et al.,* 1992] and cry1 and cry2 are nuclear proteins. Cry1 is present largely in the cytosol of light-grown plants and mediates the UV-A/blue light induction of several genes involved in flavonoid biosynthesis and anthocyanin accumulation in *Arabidopsis* [Kuhn *et al.,* 1984, Kreuzaler *et al.,* 1983]. Cry2 has a minor role in this response and it is constitutively imported to the nucleus regardless of light treatment [Wade *et al.*, 2001; Ahmad *et al.*, 1998, Lin et al., 2003]. The blue-light increases the cytoplasmic calcium concentration strongly [Baum *et al.,* 1999]. UV-B and UV-A /blue light induction of *CHS* expression involves calcium and these responses are inhibited by the calcium channel blockers nifedipine and ruthenium red [Christie and Jenkins, 1996; Frohnmeyer *et al.*, 1997]. Calcium flux of the cytosol involves Ca^{2+}–ATPases activity and some Ca^{2+}–ATPases are activated by calmodulin [Bush, 1995; Askerlund and Sommarin, 1996] and may cause a calmodulin-stimulated Ca^{2+}–ATPase which is involved in UV-B signal transduction [Long and Jenkins, 1998]. A scheme of the affects of UV-blue light in plant cells is shown in **Figure 7.1**.

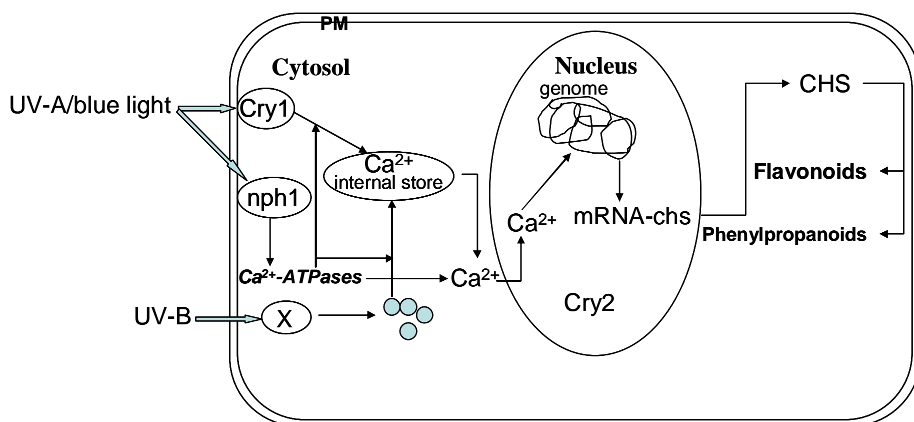

Figure 7.1. Model showing effect of UV/blue light on *Arabidopsis* cell and *CHS* expression. PM: Plasma membrane, X: UVB receptor (unknown)

So far flavonoid production has been regarded as the only major metabolic response of plants to UV-A/blue light irradiation. But flavonoids are part of the metabolic network and changes in this part of the network may affect the overall metabolic network in a cell. This study will use NMR-based metabolomics with multivariate data analysis to examine the whole of metabolic changes in Arabidopsis after UV-A/blue light stress. This allows the study of the total of metabolic networks in more detail and to understand more about the relationship between individual genes and metabolic processes.

7.2. Materials and methods

7.2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col.0) is the genetic background and 6 transgenic ACS lines were used. Seeds were surface sterilized by submersion for 1 min in 70% ethanol, 15 min in 2.5% sodium hypochlorite followed by five rinses with sterile water. 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. After 3 days at 4°C in the dark the seeds were germinated at 23°C and long day condition (16/8 h light/dark cycle) in a growth chamber. Plants were routinely grown in a long day growth conditions under white light for 14 days before transfer to different light qualities. Plants were separated in two sets. One set as control was kept growing under white light. The other set was transferred to a UV-A/blue light chamber. Samples were collected after 6 hours, 24 hours and 48 hours after UV-A/blue light irradiation. Immediately after harvesting the material was frozen in liquid nitrogen and kept at −80 °C until used.

7.2.2. Light sources

Illumination was performed in controlled environment rooms at 23°C. White light was provided by warm-white fluorescent tubes (Osram, Munich. Germany). UV-A light was provided by F35WIBI-26 blacklight-blue fluorescent tubes (GTE Sylvania, Shipley, UK), which emit light between 350 and 400 nm with a λ_{max} at 370 nm. Blue light was provided by 40W TI2 blue fluorescent tubes (GTE Sylvania) with λ_{max} at 430 nm,

covered with a UV226 filter (Lee Filters, Andover, UK) to remove wavelengths <400 nm

7.2.3. Extraction of plant materials

Plants were ground in liquid nitrogen and pooled before subjected to freeze-drying. Twenty-five milligrams of freeze-dried material were transferred to a micro-centrifuge tube before adding 600 µl of CH_3OH-d_4. The mixture was vortexed for 2 minutes and sonicated for 20 min, followed by centrifugation at 13,000 rpm for 5 minutes at room temperature. Five hundred microliters of the supernatant were then transferred into 2 ml micro-centrifuge tubes and 250 µl of KH_2PO_4 buffer, pH 6.0, containing 0.1% trimethyl silyl propionic acid sodium salt (w/v) were added. The mixture was left for 30 minutes in 4°C, and then centrifuged at 6000 rpm for 5 minutes at room temperature. Seven hundred µl of the supernatant were then transferred into 5 mm NMR tubes for analysis.

7.2.4. Solvents and chemicals

D_2O (99%) and CH_3OH-d_4 (99.8%) were obtained from Cambridge Isotope Laboratories Inc (Miami, FL, USA). NaOD was purchased from Cortec (Paris, France). Potassium dihydrogen phosphate and trimethylsilane propionic acid sodium salt (TSP) were purchased from Merck (Darmstadt, Germany). KH_2PO_4 was added to D_2O as a buffering agent. The pH of the D_2O was adjusted to 6.0 using a 1 M-NaOD solution.

7.2.5. NMR spectra measurement

^{1}H-NMR, 2D J-resolved spectra were recorded at 25 ^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany). ^{1}H-^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Kalsruhe, Germany). All the NMR parameters were the same to those of our previous reports (Jahangir *et al.*, 2008; Abdel-Farid *et al.*, 2007).

7.2.6. Data analysis

Spectral intensities of ^{1}H-NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4- δ 10.0. The regions of δ 4.8-δ 4.9 and δ 3.28-δ 3.40 were excluded from the analysis because of

the residual signal of water and CH_3OH. Principal component analysis (PCA) and partial least square regression analysis (PLS) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden).

7.3. Results

In order to exclude the separation in analysis due to the effect of the transgene and only observe the effect of UV-A/blue light, the sample data set was split into two groups: wild type and transgenic *Arabidopsis.* The results are presented below.

7.3.1. Metabolomic profiling of UV-A/blue light treated Arabidopsis thaliana Col. 0 by NMR spectroscopy

To reveal the change of metabolites after a UV-A/blue light treatment in *A. thaliana* Col. 0 the multivariate analysis partial least square (PLS) method was applied, that method can apply a time point (6, 24, 48 hrs) variable in the data analysis. Thus we can see the metabolite changes based on UV-A/blue light treatment as well as due to growth. A clear separation was obtained in the PLS score plot (**Figure 7.2**). There is no metabolomic change after 6 hrs of UV-A/blue light treatment, most differentiation was observed after 24 hrs UV-A/blue light treatment. However, during the developmental period the plant metabolome always changes and thus also affects the results of UV-A/blue light treated plants. To solve this problem the supervised method PLS-DA was applied. For PLS-DA, the data were divided into two classes: controls and UV-A/blue light treated plants. The score plot (**Figure 7.3**) of PLS-DA shows a distinct separation between the UV-A/blue light treated plants and controls. Most separation was displayed in component 1 (PC 1). The UV-A/blue light treated group (2) stays on the negative side of PLS-DA PC 1, whereas the control group is on the positive side. To interprete which metabolites correspond to each group in the PLS-DA diagram the loading plot was investigated. The loading plot of PC 1 (**Figure 7.4**) clearly shows that phenylpropanoids, flavonoids, glucose, fructose and organic acids are a higher in the UV-A/blue light treated group (**Figure 7.4 A, B, C**) and non-treated group has higher amount of sucrose and amino acids (**Figure 7.4 A, B**).

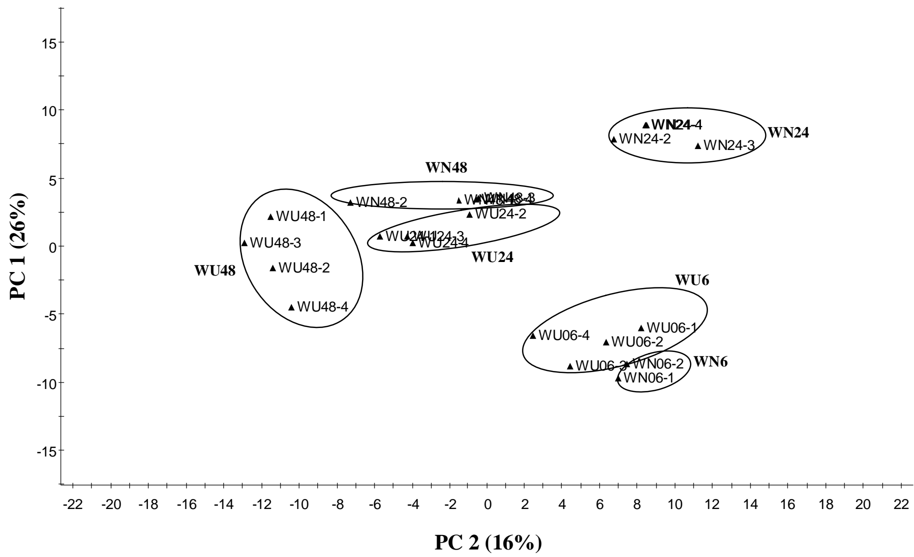

Figure 7.2. PLS score scater plot of UV-A/blue light treated *Arabidopsis* in 6 hr (WU6), 24 hr (WU24), 48 hr (WU48) and controls (WN6, WN24, WN48).

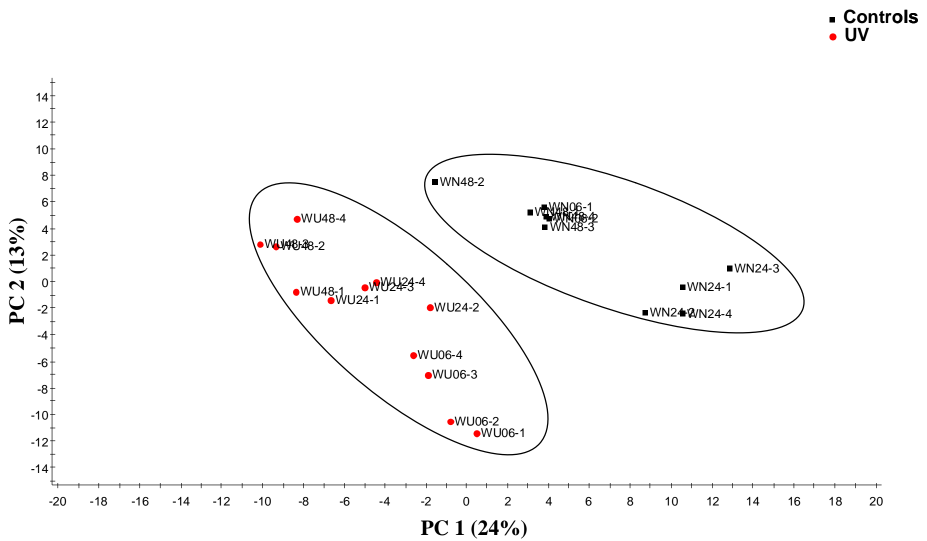

Figure 7.3. PLS-DA score scatter plot of UV-A/blue light treated *Arabidopsis* Col.0 in 6 hr (WU4), 24 hr (WU24), 48 hr (WU48) and controls (WN6, WN24, WN48)

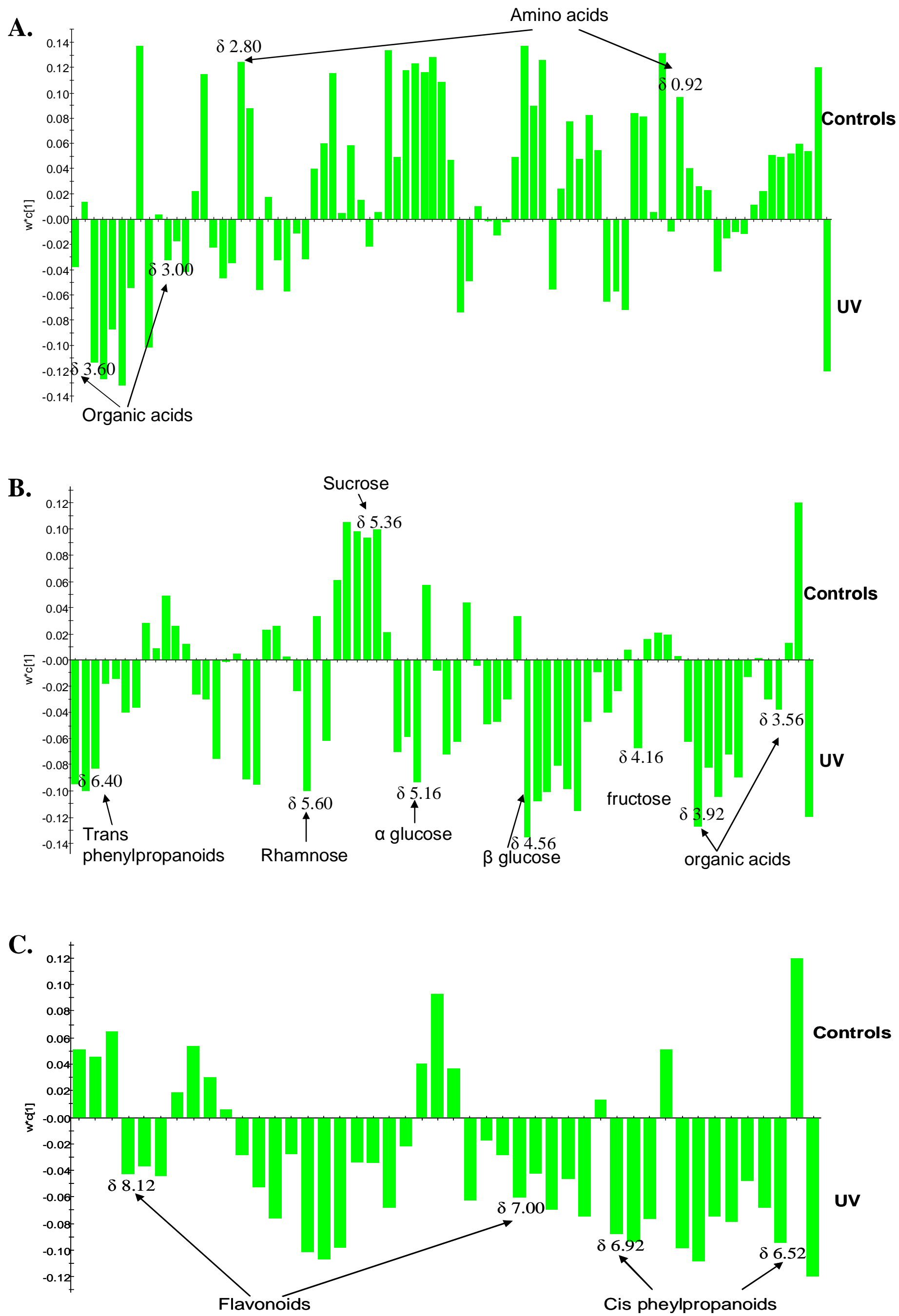

Figure 7.4. PLS-DA loadings column plot of UV-A/blue light treated *Arabidopsis* Col.0 and controls

7.3.2. Metabolic profiling of UV-A/blue light treated CHS transgenic Arabidopsis thaliana Col. 0 by NMR spectroscopy

The ^{1}H-NMR data of UV-A/blue light treated CHS transgenic *Arabidopsis* were analyzed in the same way as the control group but no clear separation was obtained between UV-A/blue light treated and none UV-A/blue light treated plants in the PLS score plot (**Figure 7.5**). So a supervised method PLS-DA was applied to see the effect of UV light on the metabolome of transgenic ACS plants. The PLS-DA score plot (**Figure 7.6**) shows only minor separation between UV-A/blue light treated ACS and control ACS in both PC1 and PC2. In order to identify the corresponding metabolites responsible for the separation we used a PLS-DA loadings Bi plot which resulted in **Figure 7.7**. **Figure 7.7** do show that there are only few metabolites which correspond to separation but those are unknown signals and none of the main identified metabolites contributes to seperation. It means there are no significant metabolome changes in ACS plants after the UV-A/blue light treatments.

In order to see the effect of *CHS* expression on the metabolome of UV-A/blue light treated *Arabidopsis* plants, PCA was applied to analyze only the CHS transgenic Arabidopsis and wild type plants at 24h UV-A/blue light treatment. That time point showed the biggest change of *Arabidopsis* metabolites under UV-A/blue light stress (**Figure 7.2**). The results of PCA analyse are presented in **Figure 7.8** which shows that the UV-A/blue light treated groups are very close to the non-treated UV-A/blue light groups in *CHS* transgenic *Arabidopsis* whereas the UV-A/blue light treated groups is very far from non-treated UV-A/blue light groups in wild type *Arabidopsis.* Also the UV-A/blue light treated groups of wild type *Arabidopsis* are very close to transgenic plants. This result means that UV-A/blue light did not have a clear effect on the CHS transgenic *Arabidopsis* in contrast it has a big effect on wild type *Arabidopsis* in which the metabolites of UV-A/blue light treated wild type plants became similar to CHS transgenic *Arabidopsis.*

Figure 7.5. PLS score scatter plot of UV-A/blue light treated CHS transgenic *Arabidopsis* line ACS 20 (20) and ACS 2 (B) in 6 hr (20U6, BU6), 24 hr (20U24, BU24), 48 hr (20U48, BU48) and controls (20N6, 20N24, 20N48, BN6, BN24, BN48)

Figure 7.6. PLS-DA loading scatter plot of UV-A/blue light treated CHS transgenic *Arabidopsis* line ACS 20 (U20), ACS 2 (UB) and controls (N20, NB)

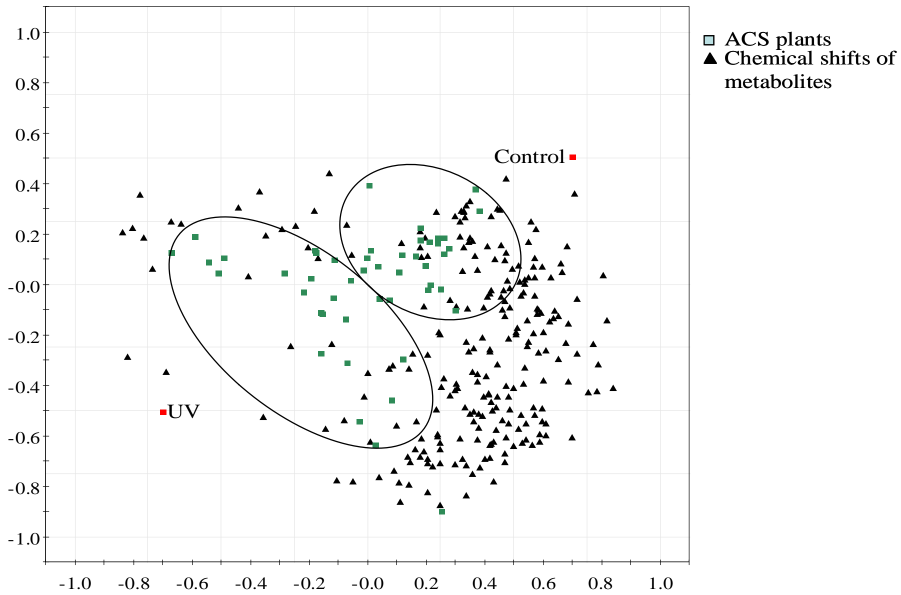

Figure 7.7. The PLS-DA loadings Bi plot of UV-A/blue light treated CHS transgenic *Arabidopsis* (ACS 20 and ACS 2) and controls

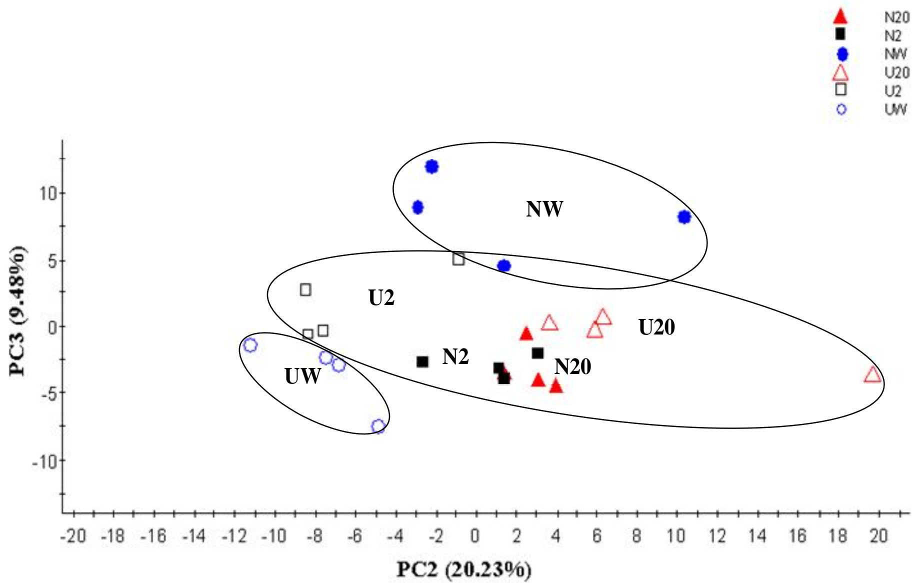

Figure 7.8. The PCA loading scatter plot of UV-A/blue light treated CHS transgenic *Arabidopsis* ACS 20 (U20), ACS 2 (U2), wild type (UW) and controls (N20, N2, NW).

7.4. Discussion

This study shows that UV-A/blue light strongly influences the metabolome of *A. thaliana* Col. 0 wild type while the metabolome of transgenic ACS does not show any specific change under UV-A/blue light treatment. The samples of 6 hr UV-A/blue light treatment showed no metabolic changes. At that time point a response at gene level may have been accomplished which causes the metabolic changes observed at 24 hr after the start of the treatment. That could explain that the biggest separation was obtained between control and UV-A/blue light treated group at 24 hr (**Figure 7.1**). The light treated group shows accumulation of flavonoids, phenylpropanoids, simple sugars such as glucose, fructose and rhamnose and a decrease of sucrose and amino acids. Flavonoids, phenylpropanoids and simple sugars can absorb UV-A/blue light. Several studies have shown that flavonoid production is induced by UV-A/blue light and occurs in the leave epidermal layers [Buchholz *et al.,* 1995, Jenkins *et al.,* 2001]. Those compounds play a role as sun filter for the plants to avoid DNA damage caused by UV-A/blue light. The decrease of sucrose and amino acids in UV-A/blue light treated plants might be due to the major metabolic changes needed from cell homeostasis to cell stress metabolism. The simple sugars are needed for energy and precursors or the biosynthesis of flavonoids and phenylpropanoids. Amino acids are possibly required for the de-novo biosynthesis of enzymes and as precursors.

We did not see any specific effect of UV-A/blue light on the transgenic ACS metabolome. There was no specific change of the metabolome observed for UV-A/blue light treated transgenic plants, if compared with transgenic controls and UV-A/blue light treated wild type (**Figure 7.8**). The ACS transgenic plants have high accumulation of flavonoids and phenylpropanoids itself (**Chapter 6**) so it might be enough to protect the plant from UV light. Very high levels of flavonoids and phenylpropanoids can be toxic for the plant and consequently there will be a limit in the level of metabolites that plants can produce without causing cell death. In the transgenic plants probably the limit of flavonoid and phenylpropanoid production is already achieved, not allowing any further increase of the biosynthesis, whereas in the wild type cells induction is possible. At what level the phenylpropanoid biosynthesis is regulated cannot be concluded from our results, but it might be at the level of CHS. At least our results support that the

phenylpropanoid and flavonoid biosynthetic pathways are the major metabolic pathways involved in the response to UV-A/blue light stress.

Chapter 8

Effect of benzothiadiazole on the metabolome of *Arabidopsis thaliana*

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Abstract

Benzothiadiazole (BTH) is a functional analogue of the plant endogenous hormone-like compound, salicylic acid (SA), which is required for the induction of plant defense genes leading to systemic acquired resistance (SAR). Previous molecular and genetic studies have suggested that BTH itself might potentiate SAR resulting in the induction of several pathogenesis-related (PR) genes. However, the changes in the metabolome, which occur as a result of BTH-treatment, remain unclear. In this study, metabolic alterations in BTH-treated *Arabidopsis thaliana* were investigated using nuclear magnetic resonance (NMR) spectroscopy followed by multivariate data analyses such as principal component analysis (PCA) and partial least square - discriminant analysis (PLS-DA). Both PCA and PLS-DA show that increase of glucose, glutamine, inositol, malic acid, sucrose, and threonine as well as BTH and its degraded metabolites contribute to the clear discrimination of the metabolome of BTH-treated *Arabidopsis* from control plants. However, the levels of phenolic metabolites which have generally been observed to be induced by other signaling molecules were significantly reduced in BTH-treated *Arabidopsis.* In addition to these changes due to BTH-treatment, it was

also found that the ethanol used as a solvent in this treatment may *per se* act as an inducer of the accumulation of a flavonoid.

Keywords: *Arabidopsis thaliana*, Benzothiadiazole, Metabolomics, Nuclear magnetic resonance spectroscopy, Systemic acquired resistance, Principal component analysis, Partial least square - discriminant analysis

8.1. Introduction

Plants interact constantly with the environment, e.g. other organisms, soil, climate, water conditions, or exogenous chemicals. When challenged, plants can switch on their defense mechanism in general, as a response to many stimuli, or specifically, responding to a certain stimulus. Among the plant defense mechanisms, systemic acquired resistance (SAR) is a whole-plant resistance response that follows an earlier local exposure to a pathogen. Fungal, bacterial, or viral pathogenic infections induce SAR, involving different biochemical pathways that produce salicylic acid (SA) among others, transduction signals of pathogenesis-related (PR) proteins, and/or phytoalexins [Bol *et al.,* 1990; Dixon, 1986; Felton *et al.,* 1999; Kombrink *et al.,* 1997; , Métraux *et al.,* 1990]. SAR can also be induced by exposing the plant to virulent, nonpathogenic microbes, or to chemicals such as SA, 2,6-dichloro-isonicotinic acid (INA) or benzo(1,2,3)thiadiazole-7-carbothioic acid-*S*- methyl ester (BTH) [Kuć, 1982; Ryals *et al.,* 1996; Sticher *et al.,* 1997].

The phenomenon of pathogen-induced SAR has been recognized as a plant response to pathogen infection for almost 100 years and has therefore been extensively studied in many plants at a genetic and proteomic level [Ryals *et al.,* 1996]. SAR is associated with the induction of gene expression of defensive factors such as PR proteins, and this activation requires the production of endogenous SA [Métraux *et al.,* 1990]. Several PR proteins including PR-1, PR-2 (β-1,3-glucanases), PR-3 (chitinases), PR-4, and PR-5 (osmotin) were found positively correlated with the onset of SAR although with an expression level of marker genes for SAR that varied between different species [Kessmann *et al.,* 1994;, Vleeshouwers *et al.,* 2000]. In *Arabidopsis thaliana,* the mRNAs for PR-1, PR-2, and PR-5 accumulated in a coordinated manner in tissues that became resistant after pathogen infection [Uknes *et al.,* 1993]. Most PR proteins were found to accumulate in the extracellular space or in the vacuole. The extracellular PR proteins are thought to be directly in contact with the pathogen penetrating the tissue and vacuole PR proteins are probably involved in the following defense reaction after decompartmentalization [Sticher *et al.,* 1997]. Different roles have been attributed to PR proteins, such as antimicrobial or antifungal activities *in vitro* activities [Liu *et al,* 1994; Mauch *et al.,* 1988] or the capacity of releasing elicitors [Kurosaki *et al,* 1986]. However, the exact role of PR proteins in SAR still remains unclear.

Benzo(1,2,3)-thiadiazole-7-carbothioic acid-*S*-methyl ester (BTH, Fig. 1) is a potent SAR activator which provides protection in natural conditions against a broad spectrum of diseases affecting a variety of crops [Friedrich *et al.,* 1996; Görlach *et al.,* 1996; Lawton *et al.,* 1996]. Although BTH is a strong SAR inducer which causes the expression of the same set of SAR genes as those induced by SA, it does not require accumulation of SA but may act downstream of SA [Friedrich *et al.,* 1996; Görlach *et al.,* 1996;, Kuć 1982]. BTH induces SAR in tobacco [Friedrich *et al.,* 1996], wheat [Görlach *et al.,* 1996] and in *Arabidopsis* [Lawton *et al.,* 1996]. In the latter, BTH was found to directly activate PR-1 and to prime the plants for potential phenylalanine ammonia-lyase (PAL) expression in response to the infection by phytopathogenic *Pseudomonas syringae* p.v. tomato (Pst) [Lawton *et al.,* 1996]. It is also an excellent elicitor for the SA-activated defensive pathways in cotton, inducing remarkable levels of activity of PR proteins both locally and systemically [Inbar *et al.,* 2001]. At the metabolome level, BTH proved unable to induce any specific metabolites itself, even though there was a significant induction of PR genes and proteins [Katz *et al.,* 1998]. A remarkable change at this level was detected only after elicitation [Katz *et al.,* 1998], implying that BTH can only potentiate plants following elicitation or infection by induction of PR protein genes. In contrast with these findings, interesting results were recently reported about the metabolic variation of grapevine following BTH-treatment [Iriti *et al.,* 2004]. In this case, total polyphenols such as stilbenoids, flavonoids, anthocyanidins, and proanthocyanidins increased notably in the plants after BTH-treatment. This report awakened our interest in studying possible metabolomic changes in BTH-treated *Arabidopsis* since there is very scarce information on this aspect as compared to the knowledge of transcriptomic and proteomic levels of BTH-treated plants.

Changes at a transcriptomic and proteomic level should necessarily be reflected in the metabolome, since metabolites are the final amplified product of gene and protein expression. In recent years, metabolomics studies have received increasing attention, as a means of acquiring a better insight into the complete biological process, combining this information with that obtained through genomics, transcriptomics and proteomics [Hirai *et al.,* 2004; Kolbe *et al.,* 2006; Oksman and Saito, 2005]. Technological advances in analytical chemistry and instrumentation have accelerated the development

of diverse tools for metabolomics, particularly the information technology and mathematics needed to deal with the handling of large datasets, which have played a major role in developing the full potential of these analytical methods. Among these, it is generally accepted that NMR is the optimal tool for macroscopic metabolomics [Choi *et al.,* 2006; Verpoorte *et al.,* 2007]. This is especially the case when ^{1}H-NMR spectroscopy is applied to metabolomics since a diverse group of metabolites including amino acids, carbohydrates, lipids, phenolics, and terpenoids can be detected simultaneously [Choi *et al.,* 2004; Hendrawati *et al.,* 2006; Liang *et al.,* 2006]. It is also an easier and more robust method for acquiring quantitative raw data when compared to other methods. These positive features of NMR have led many researchers to use NMR as the first choice of plant metabolomics.

In this study NMR spectroscopy and multivariate data analysis including principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied to the analysis of metabolic changes in *Arabidopsis thaliana* treated by the SAR inducing chemical, BTH. Based on the results, the induction or suppression of diverse metabolites following BTH treatment in *Arabidopsis* as compared to control plants was investigated. The information thus obtained is expected not only to provide knowledge on metabolic characteristics but also to advance the understanding of the molecular basis of systemic acquired resistance in plants.

8.2. Methods

8.2.1. Reagents

Analytical grade CH_3OH and $CHCl_3$ were purchased from Merck Biosolve Ltd. (Valkenswaard, The Netherlands). CH_3OH-d_4 and D_2O (99.0%) were obtained from Cambridge Isotope Laboratories Inc (Miami, FL, USA) and NaOD was purchased from Cortec (Paris, France). Potassium dihydrogen phosphate (KH_2PO_4) and trimethylsilane propionic acid sodium salt (TMSP) were bought from Merck (Darmstadt, Germany). Benzo(1,2,3)-thiadiazole-7-carbothioic acid-*S*-methyl ester (BTH) was obtained form Novartis (Basel, Switzerland).

8.2.2. Growing Arabidopsis

Seeds of *Arabidopsis thaliana* ecotype Col-0 were generously donated by Dr. J. Memelink (Institute of Biology, Leiden University, Leiden, The Netherlands). Seeds were sown in soil and kept at 4 °C for 4 days. The *Arabidopsis* plants were grown under identical long-day controlled environment conditions in trays, 25 °C, and 16 h light/8 h dark. Four groups of *Arabidopsis* were prepared including BTH (in 80% EtOH)-treated, 80% EtOH-treated, and two control groups without any treatment which were grown in different locations under the same growing conditions. Four replicates were used for each time point of the groups. BTH or EtOH treatment was performed after 6 weeks growth.

8.2.3. BTH treatment

Treatments were carried out following the method of Lawton et al. with a BTH concentration of 300 µmol in 80% EtOH per plant [Lawton *et al.,* 1996]. Two groups of control *Arabidopsis* plants were prepared: one group was untreated and another was treated with the same amount of 80% EtOH as BTH treated ones. Plants were harvested at 4, 24, 48 and 96 h after treatment and frozen in liquid nitrogen. The whole aerial parts were homogenized and stored at - 80 °C until used.

8.2.4. RNA analysis

Total RNA was isolated using RNeasy plant mini kit (Qiagen, Hilden, Germany). The purified RNA was treated with DNase I using a DNA-free kit (Ambion, Austin, TX, USA) and the purity of RNA integrity was confirmed by running on a 1.5% (w/v) agarose gel. Total RNA (2 µg) was reverse transcribed in a 20 µl reaction using an oligo$(dT)_{18}$ primer and SuperScript™ II reverse transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. The cDNA was diluted 20 times and 1 µl was used as template for a real-time PCR experiment. The primers used to amplify *PR-1* were 5’-GTAGGTGCTCTTGTTCTTCCC-3’ and 5’-CACATAATTCCCACGAGGATC- 3’. The primers used to amplify *actin1* were 5’-ATGAAGCTCAATCCAAACGA-3’ and 5’-CAGAGTCGAGCACAATACCG-3’.
Real-time PCR was performed on ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). 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 3.5 min followed by 40 cycles at 95 °C for 0.5 min and at 60 °C for 1 min. Changes in gene expression as a relative fold difference between BTH treated samples and control ones were calculated using the comparative C_t ($2^{-\Delta\Delta Ct}$) method [Schmittgen *et al.,* 2000; Winer *et al.,* 1999]. *Actin1* was used as a reference gene to normalize for differences of the total RNA amount.

8.2.5. Extraction and fractionation of plant material

For the analysis of polar metabolites, plant material was pulverized in liquid nitrogen using a mortar and pestle and freeze dried. An aliquot of dried material (50 mg) was transferred to a microtube and 1.5 ml of 50% CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% Trimethylsilane propionic acid sodium salt (TMSP, w/v) was added. The mixture was vortexed at room temperature for 1 min, sonicated for 20 min and centrifuged at 13,000 rpm at room temperature for 5 min. A volume of 800 µl of the supernatant was transferred to a 5 mm-NMR tube.

In the case of non-polar metabolite extraction, 20 mg of plant material, were submitted to the same method as that described for polar metabolites extraction and extracted with 4 ml of $CHCl_3$-CH_3OH (1:1, v/v) followed by vortexing for 30 seconds and sonication for 1 min. The sample was then centrifuged at 3,000 rpm for 20 min. The extracts were placed in a 25 ml-round bottom evaporation flask and dried in a rotary vacuum evaporator. The dried fractions were redissolved in 800 µl of CH_3OH-d_4.

8.2.6. NMR spectra measurements

^{1}H-NMR and J-resolved spectra were recorded at 25 °C on a 400 MHz Bruker AV-400 spectrometer. Each ^{1}H-NMR spectrum consisted of 256 scans requiring 17.3 min acquisition time with the following parameters: 0.15 Hz/point, pulse width (PW) = 45° (3.3 µ sec), and relaxation delay (RD) = 2.0 sec and acquisition time (AQ) =2.0 sec. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zero-filled to 32 K points. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, all using XWIN NMR (version 3.5, Bruker). Two dimensional presaturated J-

resolved ^{1}H-NMR spectra were acquired using 16 scans per 64 increments that were collected into 16 K data points, using spectral widths of 5208 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin-spin coupling constant axis). A 1.0 sec relaxation delay and 1.6 sec for acquisition time was employed, giving a total acquisition time of 55.39 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine functions prior to double complex FT. ^{1}H-^{1}H-correlated spectroscopy (COSY) spectra were acquired with 1.0 sec relaxation delay, 4194 Hz spectral width in both dimensions. The heteronuclear multiple quantum coherence (HMQC) spectra were obtained with 1.4 sec relaxation delay, 4401 Hz spectral width in F2 and 20124 Hz in F1. The heteronuclear multiple bond correlation (HMBC) spectra were recorded with 1.4 sec for relaxation delay, 4251 Hz for F2 axis, 20124 for F1 axis. Qsine function was used both for HMQC and HMBC (SSB=2.0)

8.2.7. Multivariate data analysis

The ^{1}H-NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.8, Bruker Biospin). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.30 – δ 8.48. The regions of δ 4.70 - δ 4.90 and δ 3.28 - δ 3.34 in 50% CH_3OH-*d*$_4$ in D_2O (KH_2PO_4 buffer, pH 6.0) and δ 4.90 - δ 5.28 and δ 3.24 - δ 3.34 in CH_3OH-*d*$_4$ were excluded from the analysis because of the residual signal of solvent and water. Principal component analysis (PCA) and partial least square - discriminant analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden). For scaling method Pareto and unit variance method were used for PCA and PLS-DA, respectively.

8.3. Results and Discussion

8.3.1. PR-1 expression in BTH-treated Arabidopsis

Prior to metabolic analysis the expression of the PR-1 gene was confirmed by qPCR since it is a specific marker of SAR in *Arabidopsis* [Ryals *et al.,* 1996]. The accumulation of the PR-1 gene in BTH treated samples was observed 4 h after treatment and increased after 24 h and 48 h, but decreased after 96 h. The ethanol used to dissolve BTH seemed to act as an inducer itself since in EtOH treated samples; the expression of PR-1 was also detected 4 h after treatment. However, after 24 h the level of PR-1

expression was similar again to that of the control plants (non treated plants, data not shown).

8.3.2. Principal component analysis of ^{1}H-NMR spectra of control, ethanol and BTH-treated Arabidopsis

No single extraction method makes it possible to isolate a complete metabolome, i.e. the whole profile of metabolites, owing to its huge diversity in terms of chemical properties. To overcome this problem, two different extraction solvents were used: CH_3OH-H_2O for polar, hydrophilic metabolites and $CHCl_3$-CH_3OH for the less polar ones. This last extract showed no discriminating metabolites between control, EtOH and BTH-treated *Arabidopsis*.

A previous ^{1}H-NMR metabolomic study of *Arabidopsis*, carried out on a CH_3OH-water extract showed a great amount of amino acids, carbohydrates, organic acids, and phenolics that were clearly detected in a single spectrum [Hendrawati *et al,* 2006]. Aside from these constitutive plant metabolites, one more point had to be considered in this study. When a non-volatile chemical is introduced into a plant, residues of the compound itself or eventually its degradation products will also be included in the analysis. The suitability of a selected analytical method for metabolomics is dependent on whether or not those exogenous chemicals will interfere. A typical ^{1}H-NMR spectrum of BTH-treated *Arabidopsis* in a mixture of CH_3OH-d_4 and KH_2PO_4 buffer (pH 6.0) (1:1) is shown in **Figure 8.1**. ^{1}H-NMR will allow the detection of very diverse compounds, without magnifying a certain group of metabolites. Thus, amino acids, carbohydrates, flavonoids, nitrogen-containing metabolites, and phenylpropanoids are observed. A limitation of one dimensional (1D)-NMR spectroscopy is the congestion of signals. It was solved using diverse two dimensional (2D)-NMR techniques. In particular, 2D-J-resolved spectra greatly facilitated the analysis of the phenolic region (**Figure 8.3**). For treated plants, as expected, the signals of residual BTH at δ 8.97 (H-6, d, 9.2 Hz), δ 8.60 (H-4, d, 7.2 Hz), and δ 7.95 (H-5, t, 8.0 Hz) were observed in the spectra (**Figure 8.1A**). Adjacent to these signals, similar types of resonances at δ 8.74, δ 8.28 and δ 7.84 were detected and identified as those of a product of hydrolysis of BTH (**Figure 8.2**). All known *Arabidopsis* metabolites were elucidated based on the chemical shifts and coupling constants observed which were confirmed by diverse 2D-NMR such

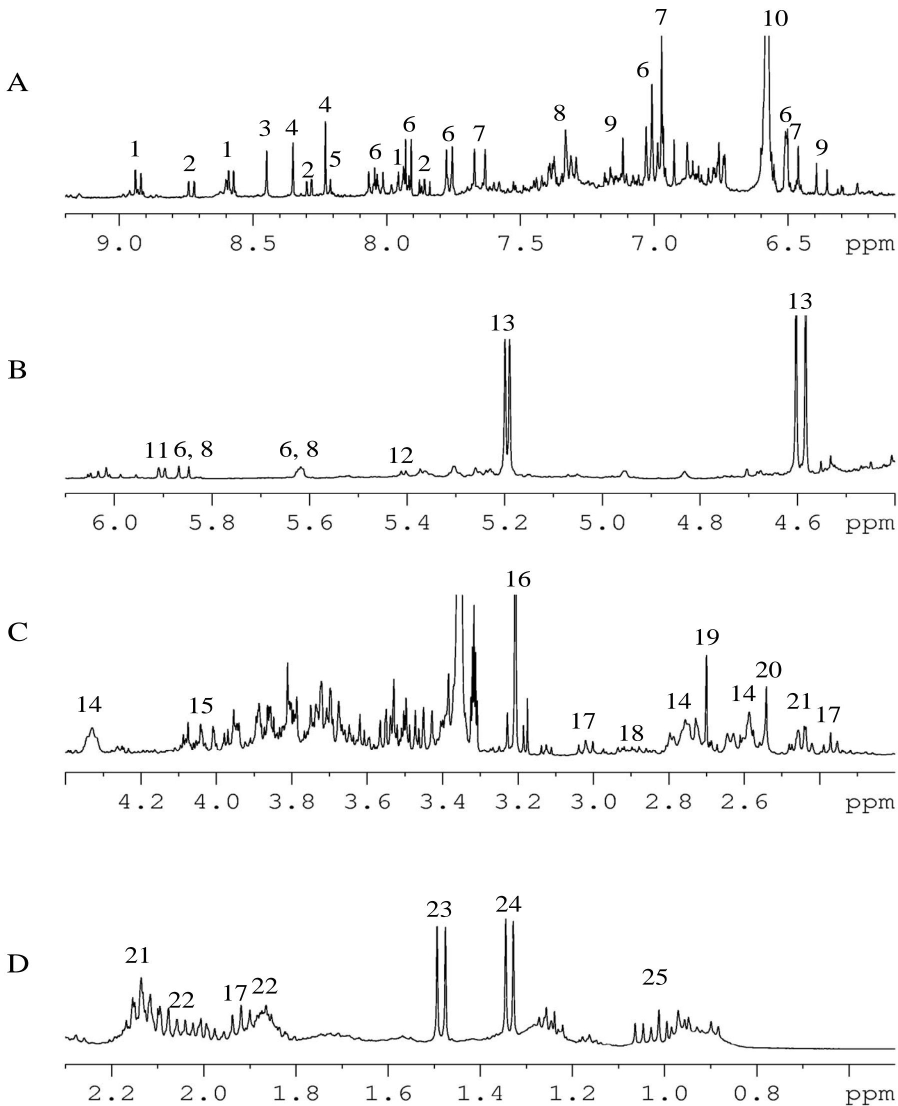


Figure 8.1. ^{1}H-NMR spectrum of *Arabidopsis thaliana* treated with BTH in 50% CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) in the range of δ 6.1 – δ 9.2 (A), δ 4.4 – δ 6.1 (B), δ 2.2 – δ 4.4 (C), δ 0.5 – δ 2.3 (D). 1; BTH, 2; hydrolyzed BTH, 3; formic acid, 4; adenosine, 5; adenine, 6; kaempferol glycosides, 7; sinapoyl malate, 8; quercetin-3-*O*-glycosides, 9; feruloyl malate, 10; fumaric acid, 11; cytosine, 12; sucrose, 13; *α* glucose, 14; malic acid, 15; inositol, 16; choline, 17; GABA, 18; asparagine, 19; diethylamine, 20; succinic acid, 21; glutamine, 22; proline, 23; alanine, 24; threonine, 25; valine

as J-resolved, correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra, and published data in our previous study [Hendrawati *et al.,* 2006].

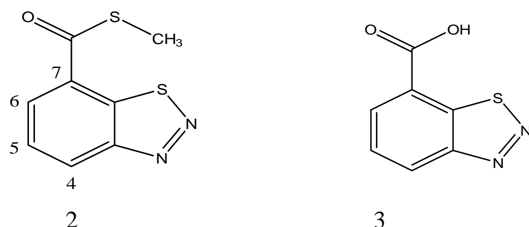


Figure 8.2. Chemical structures of BTH and its hydrolyzed metabolite.

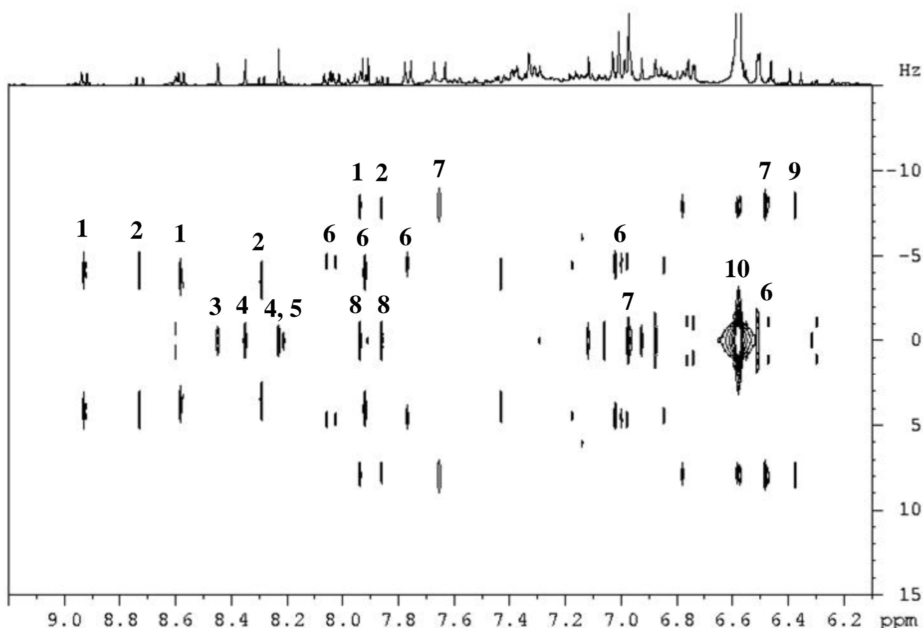


Figure 8.3. Two dimensional J-resolved spectrum of *Arabidopsis thaliana* treated with BTH in CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) in the range of δ 6.1 – δ 9.2). 1; BTH, 2; hydrolyzed BTH, 3; formic acid, 4; adenosine, 5; adenine, 6; kaempferol glycosides, 7; sinapoyl malate, 8; quercetin-3-*O*-glycosides, 9; feruloyl malate, 10; fumaric acid.

For a first overview of the metabolomic changes in BTH treated-*Arabidopsis,* principal component analysis (PCA) was applied to the ^{1}H-NMR data. Of the many multivariate data analysis method used in metabolomics, PCA is the most popular unsupervised grouping method for the reduction of the original data dimensions since the grouping or separation can be achieved simply based on maximum variation of samples without any biased information. In order to evaluate intact metabolic change, all the ^{1}H-NMR signals of BTH and its hydrolyzed metabolite were excluded for PCA.

Figure 8.4 shows the score plot of PCA based on ^{1}H-NMR spectra of the control sample, and 80% EtOH-treated and BTH-treated *Arabidopsis* (4, 24, 48 and 96 h after treatment). Three groups of control *Arabidopsis* plants were compared with BTH-treated ones. BTH treatment was carried out using a solution of BTH in 80% EtOH. It was therefore necessary to submit plants to a treatment with this solvent (as a control) in order to evaluate its activity. For non-treated controls, two batches of *Arabidopsis* were grown in different locations but under the same conditions, in order to assess possible biological variations. Metabolism is quite dynamic, being easily affected by many factors. Even under seemingly controlled conditions, level of metabolites might be affected by unpredictable external factors. However, while no separation was observed in the PCA score plot of the two control groups which had been grown in different locations (**Figure 8.4**), EtOH treated plants were slightly different from the two control groups. In accordance with reported PCR results, there is a possibility that the addition of EtOH may cause a weak induction of PR genes, which can result in a metabolic change. However, the EtOH-treated *Arabidopsis* are also separated from BTH-treated ones. As shown in Fig. 4, the BTH-treated plants are undoubtedly differentiated from others, especially 24 h after treatment. In general, change in metabolomic expression resulting from changes in the level of gene and protein levels can be expected to occur later. In fact most expression of genes related to BTH effect such as PR-1, PR-2, and PR-3 can be shown within 24 h [Wendehenne *et al.,* 1998] and metabolic alteration was clearly first detected after 24 h.

The signals responsible for the difference of BTH-treated *Arabidopsis* could be identified using the loading plot. The cluster of BTH-treated plants 24 h after treatment compared to the untreated controls or EtOH-treated samples, shows lower principal component (PC) 2 in the score plot of PCA. Using the loading plot, alanine, glucose,

glutamine, malic acid, and threonine were found positively related to the lower PC2 whereas aspartic acid, flavonoid glycosides, phenylpropanoids, and succinate were higher in the controls.

8.2.3. Partial least square-discriminant analysis of ^{1}H-NMR spectra of control, ethanol and BTH-treated Arabidopsis

While PCA is an excellent tool for data reduction and grouping multivariate data, it has limitations, which have to be considered. The separation by PCA is obtained only from maximum variations between samples as unsupervised multivariate data analysis is used. However, when information on some classification is available, it may be useful to apply a type of discriminant analysis, i.e. a supervised method in which grouping can be obtained by maximum covariance (e.g. metabolic difference corelated to classification). In this study, partial least square-discriminant analysis (PLS-DA) was employed using two classes; class 1 for control and EtOH-treated and class 2 for BTH-treated *Arabidopsis* plants. One of the advantages derived from the use of PLS-DA is that it concentrates on the effect of the selected factor (e.g. BTH or ETOH treatment) by reducing the influence of uninteresting factors such as the developmental stages of the plant.

The result obtained applying PLS-DA can be observed in **Figure 8.5**. Comparing this to the result of the PCA score plot (**Figure 8.4**), it is clear that a better separation between control (non-treated and EtOH-treated) and BTH-treated *Arabidopsis* plants is achieved. The separation in the score plot of PLS-DA could be obtained from the covariance between metabolites and treatments, disregarding the change due to developmental stages from 4 – 96 h or EtOH effect in which the metabolome were found to be greatly changed. According to PCA, the level of primary metabolites including alanine, glucose, glutamine, inositol, malic acid, sucrose, and threonine in BTH-treated samples were highly increased in a similar way. In previous studies, plants treated with signaling molecules such as analogues of jasmonic or salicylic acid, revealed a great variation in phenolics, which are known to play a role in plant defense [Görlach *et al.,* 1996; Kurosaki *et al.,* 1986; , Liang *et al.,* 2006]. However, BTH-treated plants exhibited a decrease of phenolics which could be explained considering that BTH may cause an accumulation of lignin by enhancing polar auxin transport [Besseau *et al.,* 2007; Katz *et*

al., 1998]. Additionally, numerous previous studies proved that BTH itself does not change metabolism but primes plant defense by activation of some specific genes, e.g. genes encoding peroxidases [Katz *et al.,* 1998] or by the formation of protective layers at the sites of attack [Benhamou and Belanger, 1998]. Resistance in terms of increased biosynthesis levels of defense compounds can be activated only by further elicitation after BTH treatment. For instance, phenylalanine ammonia-lyase (PAL), involved in the biosynthesis of many phenolics, did not increase in BTH-treated cultured parsley (*Petroselinum crispum* L.) but further elicitation greatly increased the mRNA level of PAL activity and coumarin secretion [Katz *et al.,* 1998].

In previous studies, trace amount of EtOH was found to alter the activity of certain enzymes [Li *et al.,* 2004]. For example, the activities of peroxidases and superoxidedismutase in cucumber roots highly increase by the addition of EtOH [Li *et al.,* 2004]. In the case *Ilex paraguariensis cell suspension* culture EtOH is glycosylated. However, the effect of EtOH on plant metabolims is still unclear. In our qPCR experiment, ethanol treated *Arabidopsis* expressed PR-1 at 4 h after treatment. Also, the metabolic change in EtOH-treated *Arabidopsis* was detected in the PCA score plot (**Figure 8.4**). In order to investigate the effect of EtOH on the metabolome of *Arabidopsis*, PLS-DA using two classes such as control and EtOH-treated samples was performed (**Figure 8.6**). When *Arabidopsis* was treated with 80% EtOH, a kaempferol glycoside as well as alanine, GABA, glucose, proline, and threonine, were unexpectedly induced. Two flavonoids, kaempferol- 3-*O*-D-glucopyranoside-7-*O*-L-rhamnopyranoside and kaempferol -3,7-*O*-L-dirhamnopyranoside, are the two major flavonoids reported in *Arabidopsis* [Hendrawati *et al.,* 2006]. Interestingly, only kaempferol 3-*O*-D-glucopyranoside-7-*O*-L-rhamnopyranoside was affected by the EtOH-treatment. However, this flavonoid was not found as a discriminating metabolite in BTH-treated plants although BTH was used in EtOH solution. It might be because BTH attenuate the effects of ethanol on the accumulation of the flavonoid in BTH-treated plants.

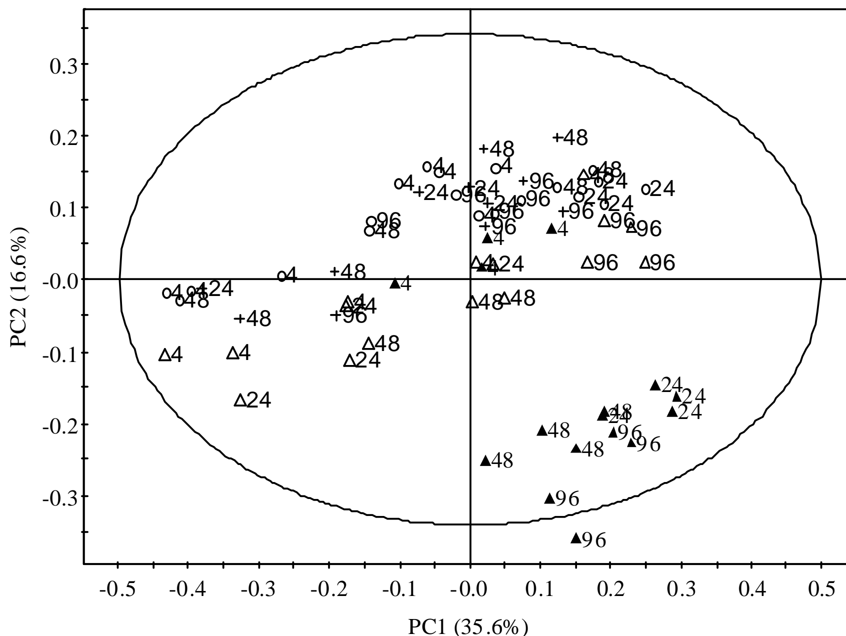
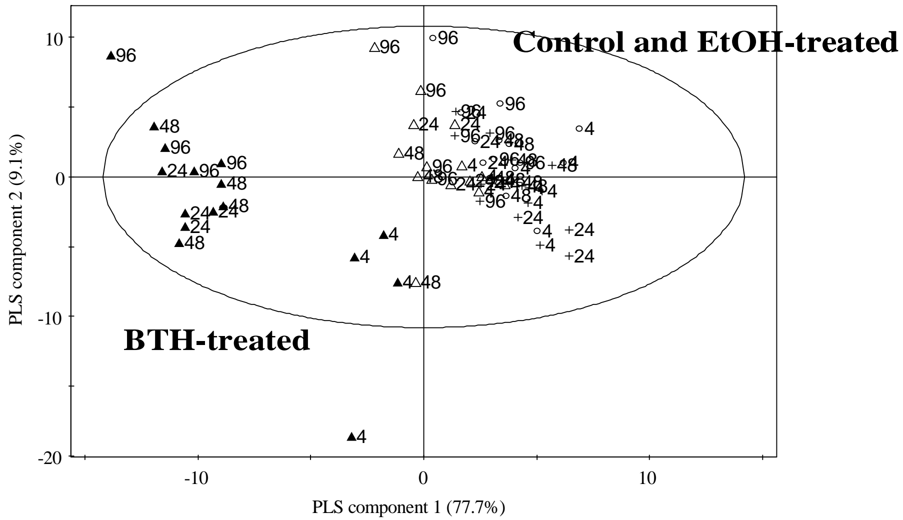
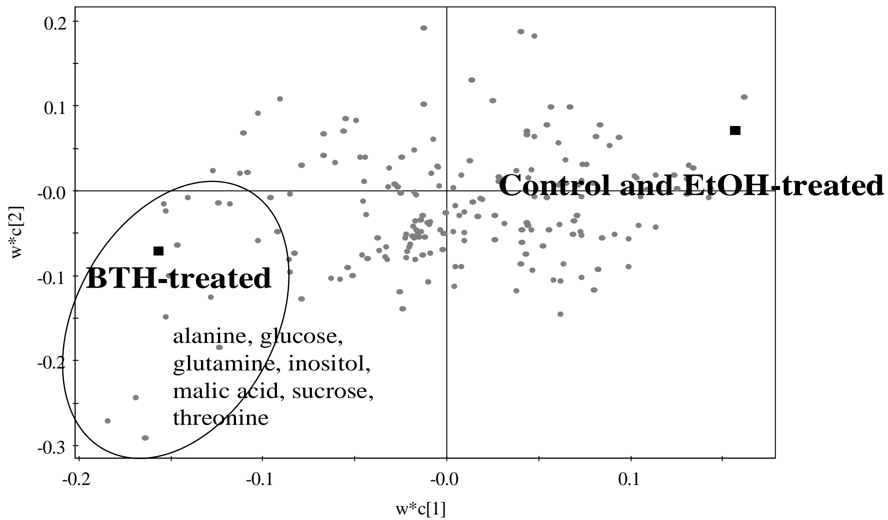


Figure 8.4. Fig. 4. Score plot of principal component analysis (PC1 vs PC2) at each time point after BTH or EtOH treatment based on ^{1}H- NMR spectra of the *Arabidopsis thaliana* (O; control 1, +; control 2, Δ; 80% EtOH-treated, ▲; BTH-treated). The eclipse represents Hotelling's T2 with 95% confidence in score plots. Numberings on the plot are the number of hours after treatment.

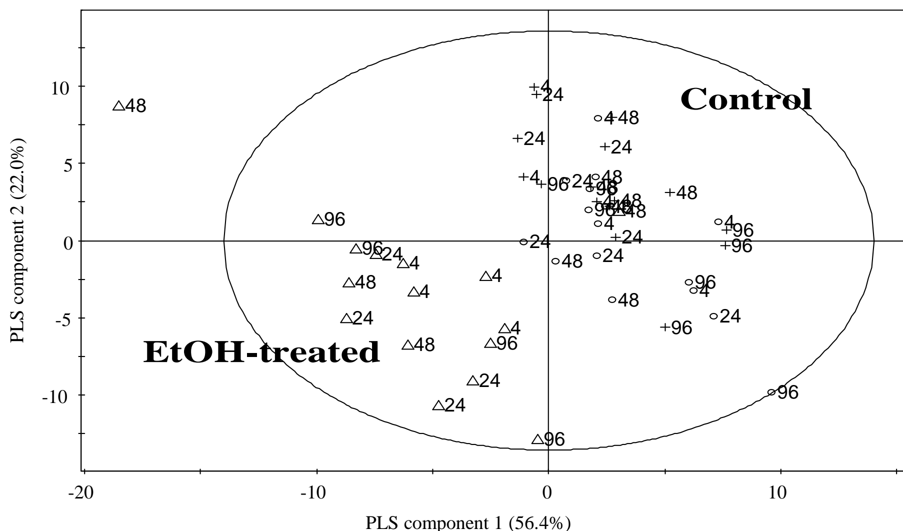


A

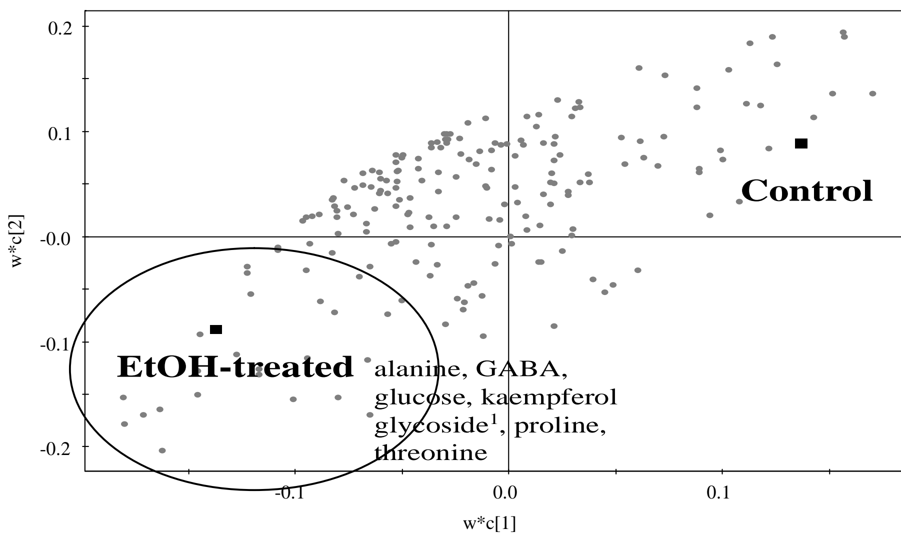


B

Figure 8.5. Score (A) and loading (B) plot of PLS-DA using two classes (class 1; non-treated and EtOH-treated, class 2; BTH-treated *Arabidopsis thaliana*). O; control 1, +; control 2, Δ; 80% EtOH-treated, ▲; BTH-treated. The eclipse represents Hotelling's T2 with 95% confidence in score plots. Numberings on the plot are the number of hours after treatment.



A



B

Figure 8.6. Score (A) and loading (B) plot of PLS-DA using two classes (class 1; non-treated class 2; EtOH-treated *Arabidopsis thaliana*). O; control 1, +; control 2, Δ; 80% EtOH-treated. The eclipse represents Hotelling's T2 with 95% confidence in score plots. Numberings on the plot are the number of hours after treatment.

8.4. Conclusions

The treatment of *Arabidopsis thaliana* with BTH induces PR-1 gene expression followed by an increase in the levels of metabolites such as glucose, glutamine, inositol, malic acid, sucrose, and threonine. This metabolic differentiation was clearly detectable using a combination of NMR spectroscopy and a supervised multivariate data analysis, PLS-DA. However, unlike the changes observed in the levels of primary metabolites, the levels of phenolic metabolites, which are generally induced by other signaling molecules like jasmonic acid or salicylic acid, did not vary. These results are consistent with the previous finding that BTH itself may prime SAR, which is then triggered by subsequent elicitation or infection. Apart from the changes resulting from BTH-treatment, it was found that the EtOH used to dissolve BTH in this study could, *per se,* act as an inducer of some metabolites. In particular, the levels of kaempferol- 3-*O*-D-glucopyranoside-7-*O*-L-rhamnopyranoside were affected by EtOH-treatment.

Chapter 9

Summary and general discussion

Plants metabolites are synthesized from intermediates of primary metabolism via often complex biosynthetic pathways. Flavonoids are a most interesting group of metabolite. They have an important function in the plant defense as well as in the nutritional value of the plant. Also they have a wide variety of pharmacological activities.

The biosynthesis of flavonoids is initiated by an enzymatic step catalysed by chalcone synthase (CHS) resulting in naringenin chalcone, the first intermediate of the flavonoid biosynthesis pathway. The pathway proceeds with several enzymatic steps to produce various classes of flavonoids, such as flavanones, dihydroflavonols, isoflavones and anthocyanins. Those compounds have been shown to function as e.g. flower pigments, UV protectants, phytoalexins, insect and herbivore protectants, allelochemicals, initiators of symbiotic interactions, regulators of auxin transport, and stimulators of pollen germination [Dixon and Paiva, 1995].

Down-regulation or over-expression of structural flavonoid genes in transgenic plants have shown to be useful tools to elucidate the function of flavonoid pathway genes. The aim of this thesis was overexpression of CHS in *Arabidopsis thaliana* and with that model to study the effect of the heterologous CHS in the plant. An overview about CHS, especially in pathogen resistance, is presented in **Chapter 2**. It shows clearly that CHS play a key role in the flavonoid biosynthesis pathway as well as in plant resistance. The cDNA encoding chalcone synthase from *Cannabis sativa* was introduced into *Arabidopsis thaliana* Col. 0 via *Agrobacterium tumefaciens*-mediated transformation and twenty one transgenic *Arabidopsis* line (ACS 1 - 21) were obtained. Six of them were analyzed by RT-PCR and quantitative real-time PCR which indicated that the exogenous gene was successfully integrated into the genome and expressed in *Arabidopsis thaliana* plants. All six transgenic lines contain multi copy numbers of *CHS* gene (**Chapter 3**).

Immunoblot and enzyme activity assay were used (**Chapter 4)** to confirm the expression of CHS in the transgenic *Arabidopsis* plants. Five transformants were checked and it was found that CHS was expressed in all transformants. The activity level of endogenous *Arabidopsis* CHS in 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.

Arabidopsis thaliana Col.0 metabolites were investigated by use of NMR spectroscopy (**Chapter 5**). Methanol-d_4 was found as the best solvent for direct extraction for NMR analysis of phenolic compounds in *Arabidopsis.* Four flavonoids, kaempferol 3-*O*-glucopyranoside-7-*O*-rhamnopyranoside, kaempferol 3-*O*-rhamnosyl (1–2) glucoside-7-*O*-rhamnopyranoside, kaempferol 3,7-*O*-dirhamnopyranoside and quercetine 3-*O*-rhamnopyranoside were isolated and identified using reference compounds. Another twenty metabolites of *Arabidopsis thaliana* Col. 0 including amino acids, organic acids, sugars, phenylpropanoids, and flavonoids were identified as well in this study.

Changes of metabolome in transgenic Arabidopsis are presented in **Chapter 6**. By use of Nuclear Magnetic Resonance (NMR) and Multivariate Data Analysis the changes between the transgenic plants and controls are seen clearly in the PLS-DA plots. This analysis indicated that the level of sugars, flavonoids and phenylpropanoid compounds are higher in the CHS transgenic plants than control plants, which means that chalcone synthase overexpression affects plant secondary metabolism as well as primary metabolism.

Chalcone synthase is strongly stimulated by UV-A/blue light in plants [Chappell and Hahlbrock, 1984; Frohnmeyer *et al.,* 1992]. The metabolome of *Arabidopsis thaliana* Col. 0 and CHS transgenic plants changes upon treatment with UV-A/blue light (**Chapter 7**). The investigation showed a high accumulation of flavonoids, phenylpropanoids, glucose, fructose, rhamnose, and organic acids in *A. thaliana* Col. 0 wild type whereas no significant change was observed in CHS transgenic plants after treatments with UV-A/blue light and the metabolites of UV-A/blue light treated *Arabidopsis thaliana* Col. 0 were similar to CHS transgenic *Arabidopsis*. That means CHS play a major role in UV-A/blue light stress.

Chapter 8 describes the study of the metabolic alterations in BTH-treated *Arabidopsis thaliana* plants (above ground parts). PCA and PLS-DA show that glucose, glutamine,

inositol, malic acid, sucrose, and threonine as well as BTH and its degradation products contribute to the clear discrimination of the metabolome of BTH-treated *Arabidopsis* from control plants. However, there was no significant increase of phenolic metabolites observed, which are generally induced by other signaling molecules. In addition to these changes due to BTH-treatment, it was also found that the EtOH used as a solvent in this treatment may itself act as an inducer of the accumulation of flavonoids and phenylpropanoids.

Over-expression of heterogolous genes involved in flavonoid biosynthesis pathway can be used in metabolic engineering strategies to overcome rate-limiting enzymatic steps in the pathway. In this way, the flux through already existing pathways of the host plant can be increased, which in case of CHS may lead to enhanced levels of specific flavonoids or even new flavonoids. This approach has been used to increase the flavonoid content of tomato fruit, in order to improve the food quality of this important crop [Verhoeyen *et al.,* 2002].

Metabolomics aims at measuring all the metabolites in a cell or biological system and is now one of the core functional genomics tool. It provides a direct link between genome and phenome because metabolites are products of gene expression and components of the phenotype. Metabolomics provides an overview of the metabolic status and global biochemical events associated with a cellular or biological system. Metabolomics not only has direct relevance to fundamental biological studies, but also to areas such as genetic or infectious diseases, cancer biology, nutrition, plant metabolism, crop quality traits, microbial physiology, environmental biology, biotechnology, drug discovery, diagnostics, molecular markers and many more applied areas. Metabolome analysis involves separation, identification and quantitation of as many metabolites as possible from a cell or tissue system.

Conclusions

This study has shown that it is possible to introduce the heterologous *CHS* gene in *Arabidopsis thaliana* and common multicopies of transgenes containing plants were obtained. By analysis of the change in metabolome of CHS transgenic plants, high expression transgenic lines can be identified by markers such as flavonoids and phenylpropanoids (this thesis). From the results it is also clear that UV-A/blue light

stress does not further increase the levels of these marker compounds in CHS transgenic *Arabidopsis* plants, whereas in wild type plants such a treatment results in increased levels of these compounds, in fact similar to that in the transgenic plants. Apparently there are certain physiological limitations in the accumulation of certain products.

After studying the link of genome to metabolome the conclusion is thus that we even have to go one step further to the phenome, in which things like energy, transport and storage, and in fact the logistics of biosynthetic pathways need to be considered.

Samenvatting en algemene discussie

In planten worden metabolieten vaak gemaakt via complexe biosyntheseroutes van stoffen uit het primaire metabolisme. Flavonoïden zijn een zeer interessante groep van metabolieten. Zij hebben een belangrijke functie bij de afweer van planten en zijn voor ons ook belangrijk voor de voedingswaarde van de plant. Daarnaast hebben ze een grote variatie aan farmacologische activiteiten.

De biosynthese van flavonoïden begint altijd met een enzymatische stap gekatalyseerd door het enzym chalconsynthase (CHS) wat resulteert in de chalcon naringenine, de eerste intermediair van de flavonoïd biosyntheseroute. De biosyntheseroute gaat vervolgens verder via meerdere enzymatische stappen waarbij de verschillende klassen van flavonoïden, zoals dihydro flavonolen, isoflavonen en anthocyanen worden gevormd. Deze stoffen hebben in de plant verschillende functies zoals bloemkleurpigment, UV-bescherming, fytoalexine, bescherming tegen insecten en herbivoren, allelochemicaliën, initiatoren van symbiotische interacties, regulatoren van het auxine transport en stimulatoren van de kieming van pollen [Dixon en Paiva, 1995].

Het remmen of het stimuleren van de genexpressie van structurele genen welke betrokken zijn bij de flavonoïd biosynthese in transgene planten iseen geschikte methode gebleken voor het onderzoek naar de functie van genen uit de flavonoïd biosyntheseroute. Het doel van dit promotieonderzoek was de overexpressie van CHS in *Arabidopsis thaliana* en om met dit model het effect van deze heterologe CHS in de plant te bestuderen. Een overzicht over CHS, met name in resistentie tegen pathogenen wordt gepresenteerd in **hoofdstuk 2**. Hieruit blijkt duidelijk dat CHS een sleutelrol speelt in de flavonoïd biosyntheseroute en in de resistentie van planten.

Het cDNA dat het chalconsynthase van *Cannabis sativa* codeert werd met behulp van *Agrobacterium tumefaciens* in *A. thaliana Col. 0* geïntroduceerd. Dit resulteerde in eenentwintig transgene *Arabidopsis* lijnen (ACS 1-21). Zes van deze lijnen zijn met behulp van RT-PCR en kwantitatieve real time PCR geanalyseerd en hieruit kon worden geconcludeerd dat het exogene gen succesvol in het genoom was geïntegreerd en in *A. thaliana* planten tot expressie werd gebracht. Alle zes de transgene lijnen

bevatten meerdere copiën van het *CHS* gen (**hoofdstuk 3**). Immunoblotten en enzym assays (**hoofdstuk 4**) zijn gebruikt om de expressie van CHS in de transgene *Arabidopsis* planten te bevestigen en om de enzymactiviteit te bepalen. Er zijn vijf transformanten per lijn onderzocht en dit toonde aan dat CHS in alle transgene planten tot expressie kwam. De enzymactiviteit van het endogene CHS van *Arabidopsis* in wildtype planten was lager dan dat van de transgene lijn ACS 20, terwijl de CHS activiteit van lijn ACS 2 vergelijkbaar was aan die van het wildtype.

De metabolieten van *Arabidopsis thaliana* Col. 0 werden onderzocht met behulp van NMR spectroscopie (**hoofdstuk 5**). Voor de NMR analyse van fenolische verbindingen was MeOD het meest geschikte oplosmiddel voor de directe extractie uit *Arabidopsis*. Vier flavonoïden, kaempferol 3-*O*-glucopyranoside-7-*O*-rhamnopyranoside, kaempferol 3-*O*-rhamnosyl (1,2) glucoside-7-*O*-rhamnopyranoside, kaempferol 3,7-*O*-dirhamnopyranoside en quercetine 3-*O*-rhamnopyranoside werden geïsoleerd en geïdentificeerd met behulp van referentie stoffen. Daarnaast zijn er bij dit onderzoek ongeveer twintig metabolieten uit *A. thaliana* geïdentificeerd, waaronder aminozuren, organische zuren, suikers, fenylpropanoïden en flavonoïden.

De veranderingen in het metaboloom van de transgene *Arabidopsis* planten zijn beschreven in **hoofdstuk 6**. Na het gebruik van Nucleaire Magnetische Resonantie (NMR) in combinatie met Multivariate Data Analyse werden de verschillen tussen de transgene planten en de controle planten in de PLS-DA grafieken duidelijk zichtbaar. Deze analyse toonde aan dat de gehaltes van suikers, flavonoïden en fenylpropanoïden in de CHS transgene planten ten opzichte van de controle planten hoger zijn. Dit betekent dat de overexpressie van chalconsynthase zowel het primaire als het secundaire metabolisme beïnvloed.

Chalconsynthase wordt in planten sterk gestimuleerd door UV-A/blauw licht [Chappell en Hahlbrock, 1984; Frohnmeyer *et al.*, 1992]. Het metaboloom van *A. thaliana* Col. 0 en van CHS transgene planten verandert door behandeling van de planten met UV-A/blauw licht (**hoofdstuk 7**). Het onderzoek toonde na behandeling met UV-A/blauw licht in de *A. thaliana* Col. 0 een hoge accumulatie aan van flavonoïden, fenylpropanoïden, glucose, fructose, rhamnose, en organische zuren terwijl er geen significante verschillen werden waargenomen in de CHS transgene planten na behandeling met UV-A/blauw licht. De metabolieten van UV-A/blauw licht behandelde

A. thaliana Col. 0 waren vergelijkbaar aan de CHS transgene *Arabidopsis*. Dit geeft aan dat CHS een belangrijke rol speelt in UV-A/blauw licht stress.

In **hoofdstuk 8** wordt het onderzoek beschreven naar de metabole veranderingen in BTH-behandelde *A. thaliana* planten (bovengrondse delen). De Principale Componenten Analyse en PLS-DA toonde aan dat glucose, glutamine, inositol, malonzuur, sucrose en threonine maar daarnaast ook BTH en zijn afbraakproducten, bijdragen aan het duidelijke verschil tussen het metaboloom van BTH behandelde *Arabidopsis* planten en controle *Arabidopsis* planten. Desondanks werd er geen significante toename aan fenolische metabolieten waargenomen welke doorgaans wel worden geïnduceerd door andere signaalstoffen. Naast de veranderingen veroorzaakt door de BTH-behandeling, werd duidelijk dat EtOH wat gebruikt werd als een oplosmiddel bij deze behandeling zelf ook de accumulatie van flavonoïden en fenylpropanoïden kan stimuleren.

Overexpressie van heterologe genen welke betrokken zijn bij de flavonoïdbiosyntheseroute kunnen worden gebruikt bij "**metabolic engineering strategies"** om snelheidsbeperkende enzymstappen te verhelpen. Op deze manier kan de flux door reeds bestaande biosyntheseroutes van de plant worden vergroot, wat in het geval van CHS kan resulteren in hogere gehaltes aan specifieke flavonoïden of zelfs nieuwe flavonoïden. Deze manier van aanpak is gebruikt bij het verhogen van het flavonoïd gehalte van tomaten om op deze manier de voedselkwaliteit van dit belangrijke gewas te verhogen [Verhoeyen *et al.,* 2002].

Metabolomics heeft als doel om alle metabolieten in een cel of biologisch systeem te meten en is nu een van de belangrijkste "functional genomics" instrumenten. Het verschaft een directe link tussen genoom en fenoom omdat metabolieten de producten zijn van genexpressie en componenten van het fenotype. Metabolomics verschaft een overzicht van de metabole status en de globale biochemische gebeurtenissen welke worden geassocieerd met een cellulair of biologisch systeem. Metabolomics is niet alleen direct relevant voor fundamenteel biologische studies, maar ook voor gebieden als genetische of infectie ziektes, biologie van kanker, voeding, het metabolisme van planten, behandelingen voor de kwaliteit van een gewas, microbiële fysiologie, milieu biologie, biotechnologie, ontdekking van geneesmiddelen, diagnostiek, moleculaire markers en op nog veel meer toegepaste gebieden. Metaboloomanalyse omvat de

scheiding, identificatie en kwantificatie van zoveel mogelijk metabolieten als mogelijk van een cel of een weefsel.

Conclusies

Dit onderzoek heeft aangetoond dat het mogelijk is om een heteroloog *CHS* gen in *Arabidopsis thaliana* te introduceren en dat er daarbij normale planten werden verkregen waarbij er meerdere copiën van het transgen aanwezig waren. Bij het analyseren van de metabole veranderingen in de CHS transgene planten kunnen transgene lijnen met een hoog expressie niveau geïdentificeerd worden met behulp van markers zoals flavonoïden en fenylpropanoïden (dit proefschrift). Van de verkregen resultaten is duidelijk dat UV-A/blauw licht stress de gehaltes aan deze marker in CHS transgene *Arabidopsis* planten niet verder verhoogt, terwijl in wildtype planten eenzelfde behandeling wel resulteert in hogere gehaltes aan deze stoffen, vergelijkbaar aan het gehalte van de CHS transgene planten. Waarschijnlijk zijn er bepaalde fysiologische limitaties in de accumulatie van bepaalde producten.

Na het bestuderen van de stap van genoom naar metaboloom moeten we concluderen dat we zelfs nog een stap verder moeten gaan, namelijk naar het fenoom, waar ook zaken als energie, transport en opslag, en in feite de logistiek van de biosyntheseroutes moeten worden betrokken.

References

Abdel-Farid I.B., Kim H.K., Choi Y.H., Verpoorte R (2007). Metabolic characterization of *Brassica rapa* leaves by NMR spectroscopy. J. Agric. Food Chem. 55, 7936-7943.

Ahmad M., Jarillo J.A., Smirnova O., Cashmore A.R. (1998) Cryptochrome blue-light photoreceptors of *Arabidopsis* implicated in phototropism. Nature 392, 720–723.

Amedeo P., Habu Y., Afsar K., Scheid O. M., Paszkowski J. (2000) Disruption of the plant gene *MOM* releases transcriptional silencing of methylated genes. Nature 405, 203–206.

An C., Ichinose Y., Yamada T., Tanaka Y., Shiraishi T., Oku H. (1993) Structure and organization of the genes encoding chalcone synthase in *Pisum sativum.* Plant Mol. Biol. 21, 789–803.

Arakawa O. (1988) Photoregulation of anthocyanin synthesis in apple fruit under UV-B and red light. Plant Cell Physiol. 29, 1385–1390.

Arias J.A., Dixon R.A., Lamb C.J. (1993) Dissection of the Functional Architecture of a Plant Defense Gene Promoter Using a Homologous in Vitro Transcription Initiation System. Plant Cell 5, 485–496.

Askerlund P., Sommarin M. (1996) Calcium efflux tranporters in higher plants. *In* M Smallwood, JP Knox, DJ Bowles, eds, Membranes: Specialized Functions in Plants. BIOS Scientific Publishers Ltd., Oxford, UK, 281–299.

Baker S.M., White, E.E. (1996) A chalcone synthase/stilbene synthase DNA probe for conifers. Theor. Appl. Genet. 92, 827–831.

Balandrin M.J. and Klocke J.A. (1988) Medicinal, aromatic and industrial materials from plants. *In* Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plant, vol. 4. Springer-Verlag, Berlin, Heidelberg, pp. 1-36.

Baum G., Long J.C., Jenkins G.I., Trewavas A.J. (1999) Stimulation of the blue light phototropic receptor NPH1 causes a transient increase in cytosolic Ca^{2+}. Proc. Natl. Acad. Sci. USA 96, 13554-13559.

Beggs C.J., Jehle A.S., Wellmann E. (1985) Isoflavonoid formation as an indicator of UV-stress in bean (*Phaseolus vulgaris* L.) leaves: the significance of photorepair in assessing potential damage by increased solar UV-B radiation. Plant Physiol. 79, 630-634.

Bell A.A. (1981) Biochemical mechanisms of disease resistance. Annu. Rev. Plant Physiol. 32, 21-81.

Bell J.N., Dixon R.A., Bailey J.A., Rowell P.M., Lamb C.J. (1984) Differential induction of chalcone synthase mRNA activity at the onset of phytoalexin accumulation in compatible and incompatible plant: pathogen interactions. Proc. Natl. Acad. Sci. USA 81, 3384–3388.

Bell J.N., Ryder T.B., Wingate V.P.M., Bailey J.A., Lamb C.J. (1986) Differential accumulation of plant defense gene transcripts in a compatible and an incompatible plant: pathogen interaction. Mol. Cell Biol. 6, 1615–1623.

Benhamou N., Belanger R. R., (1998) Benzothiadiazole-mediated induced resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in Tomato. Plant Physiol. 118, 1203-1212.

Besseau S., Hoffmann L., Geoffroy P., Lapierre C., Pollet B., Legrand M. (2007) Flavonoid Accumulation in *Arabidopsis* Repressed in Lignin Synthesis Affects Auxin Transport and Plant Growth, Plant Cell 19, 148-162.

Block A., Dangl J.L., Hahlbrock K., Schulze L.P. (1990) Functional borders, genetic fine structure, and distance requirements of cis elements mediating light responsiveness of the parsley chalcone synthase promoter. Proc. Natl. Acad. Sci. USA 87(14), 5387–5391.

Bloor S.J., Abrahams S. (2002) The structure of the major anthocyanin in *Arabidopsis thaliana*. Phytochemistry 59, 343–346.

Bohm B.A. (1998) Introduction to flavonoids, Vol. 2. Amsterdam, Netherlands: Hardwood Academic Publishers

Bol J. F., Linthorst H. J. M., Cornelissen B. J. C. (1990) Plant pathogenesis-related proteins induced by virus infection, Annu. Rev. Phytopathol. 28, 113-138.

Bomati E.K., Austin M.B., Bowmann M.E., Dixon R.A., Noel J.P. (2005) Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis. J. Biol. Chem. 280, 30496–30503.

Bongue B.M.; Phillips D.A. (1995) Nitrogen stress regulates gene expression of enzymes on the flavonoid biosynthetic pathway of tomato *Plant Physiol. Biochem.* 33, 539– 546.

Bosco D., Meda P., Iynedjian P.B. (2000) Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. Biochem. J. 348 , 215-222.

Bowles D. (1990) Defense-related proteins in higher plants. Annu. Rev. Biochem. 59, 873–907.

Brignolas F., Lacroix B., Lieutier F., Sauvard D., Drouet A., Claudot A.C., Yart A., Berryman A.A., Christiansen E. (1995) Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark beetle-associated fungus. Plant Physiol. 109, 821– 827.

Brosché M., Strid A. (2003) Molecular events following perception of ultraviolet-B radiation by plants. Physiol. Planta. 117, 1-10.

Brown D.E., Rashotte A.M., Murphy A.S., Tague B.W., Peer W.A., Taiz L., Muday G.K. (2001). Flavonoids act as negative regulators of auxin transport *in vivo* in *Arabidopsis thaliana*. Plant Physiol. 126, 524–535.

Bruns B., Hahlbrock K., Schafer E. (1986). Fluence dependente of the ultraviolet-light-induced accumulation of chalcone synthase mRNA and effects of blue and far-red light in cultured parsley cells. Planta 169, 393-398.

Buck N., Callaghan T.V. (1999) The direct and indirect effects of enhanced UV-B on the moth caterpillar *Epirrita autumnata*. Ecol. Bull. 47, 68-76.

Buer C.S., Muday G.K. (2004) The *transparent testa4* mutation prevents flavonoid synthesis and alters auxin transport and the response of *Arabidopsis* roots to gravity and light. Plant Cell 16, 1191-1205.

Buchholz G., Ehmann B., Wellmann E. (1995) Ultraviolet light inhibition of phytochromeinduced flavonoid biosynthesis and DNA photolyase formation in mustard cotyledons (*Sinapsis alba* L.). Plant Physiol. 108, 227-234.

Burbulis I.E., Winkel S.B. (1999) Interactions among enzymes of the *Arabidopsis* flavonoids biosynthesis pathway. Proc. Natl. Acad. Sci. USA 96, 12929-12934.

Burbulis I.E., Iacobucci M., Shirley B.W. (1996) A null mutation in the first enzyme of flavonoid biosynthesis does not affect male fertility in *Arabidopsis*. Plant Cell 8, 1013-1025.

Bush D.S. (1995) Calcium regulation in plant cells and its role in signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95-122

Cameron D.C., Altaras N.E., Hoffman M.L., Shaw, A.J. (1998) Metabolic engineering of propanediol pathways. Biotechnol. Prog. 14**,** 116-125.

Campos A.D., Ferreira A.G., Hampe M.M.V., Antunes I.F., Brancao N., Silvera E.P., Silva J.B., Osorio V.A. (2003) Induction of chalcone synthase and phenylalanine ammonia-lyase by salicylic acid and *Colletotrichum lindemuthianum* in common bean. Braz. J. Plant Physiol., 15(3), 129-134.

Carrari F., Nunes N.A., Gibon Y., Lytovchenko A., Ehlers L. M., Fernie A.R. (2003) Reduced expression of aconitase results in an enhanced rate of photosynthesis and marked shifts in carbon partitioning in illuminated leaves of *Lycopersicon pennellii*. Plant Physiol. 133, 1322-1335

Chappell J., Hahlbrock K. (1984) Transcription of plant defense genes in response to UV light or funga1 elicitor. Nature 311, 76-78.

Chet I. (1993) Biotechnology in plant disease control. Wiley-Liss, New York.

Cheynier V. (2005) Polyphenols in foods are more complex than often thought. Am. J. Clin. Nutr. 81, 223S-229S.

Choi Y.H., Kim H.K., Verpoorte R. (2006) Metabolomics, in : Kayser O., Quax W. (Eds), Medicinal Plant Biotechnology From Basic Research to Industrial Applications, Wiley-VCH, Weinheim, pp. 9-28.

Choi Y.H., Tapias E.C., Kim H.K., Lefeber A.W.M., Erkelens C., Verhoeven J.T.J. Verhoeven, Brzin J., Zel J., Verpoorte R. (2004) Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using ^{1}H-NMR spectroscopy and multivariate data analysis. Plant Physiol. 135, 2398–2410.

Choudary A.D., Lamb C.J., Dixon, R.A. (1990) Stress responses in alfalfa (*Medicago sativa* L.). VI. Differential responsiveness of chalcone synthase induction to

fungal elicitor or glutathionein electroporated protoplasts. Plant Physiol. 94, 1802–1807.

Christensen A.B., Gregersen P.L., Olsen C.E., Collinge D.B. (1998a) A flavonoid 7-O-methyltransferase is expressed in barley leaves in response to pathogen attack. Plant Mol. Biol. 36, 219-227.

Christensen A.B., Gregersen P.L., Schroder J., Collinge D.B. (1998b) A chalcone synthase with an unusual substrate preference is expressed in barley leaves in response to UV light and pathogen attack. Plant Mol. Biol. 37: 849–857.

Christie J.M., Jenkins G.I. (1996) Distinct UVB and UV-A/Blue light signal transduction pathways induce chalcone synthase gene expression in Arabidopsis cells. Plant Cell 8, 1555–1567.

Clark T.A., Zeyen R.J., Smith A.G., Carver T.L.W., Vance C.P. (1994) Phenylalanine ammonialyase mRNA accumulation, enzyme activity, and cytoplasmic responses in barley isolines, differing in *Ml-a* and *ml-o* loci attacked by *Erysiphe graminis* f. sp. *hordei.* Physiol. Mol. Plant Path. 44, 171-185.

Clough S.J., Bent A.F., (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J.16 (6), 735-743.

Creelman R.A., Mullet J.E. (1997) Biosynthesis and action of jasmonate in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 355–381.

Creelman R.A., Tierney M.L., Mullet J.E. (1992) Jasmonic acid/methyl jasmonate accumulated in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl Acad. Sci. USA 89, 4938–4941.

Cuadra P., Harborne J.B. (1996) Change in epicuticular flavonoids and photosynthetic pigment as a plant respone to UV-radiation, Naturforsch. 51c, 671-680.

Dalkin K., Edwards R., Edington B., Dixon R.A. (1990) Stress responses in alfalfa (*Medicago sativa* L.). I. Induction of phenylpropanoid biosynthesis and hydrolytic enzymes in elicitor-treated cell suspension culture. Plant Physiol. 92, 440-446.

Davies C.R., Wareing P.F. (1965) Auxin-directed transport of radiophosphorus in stems. Planta 65, 139–156.

De Bruyne T., Pieters L., Deelstra H., Vlietinck A. (1999) Condensed vegetable tannins: biodiversity in structure and biological activities. Biochem. Syst. Ecol. 27, 445-459.

De Buck S., Jacobs A., Van Montagu M., Depicker A. (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. Plant J. 20, 295–304.

De Neve M., De Buck S., Jacobs A., Van Montagu M., Depicker A. (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from ligation of separate T-DNAs. Plant J. 11, 15–29.

Debeaujon I., Peeters A.J.M., Léon-Kloosterziel K.M., Koornneef M. (2001) The *TRANSPARENT TESTA12* gene of Arabidopsis encodes a multidrug secondary transporter–like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell 13, 853-871.

Dhawale S., Souciet G., Kuhn, D.N. (1989) Increase of chalcone synthase mRNA in pathogen-induced soybeans with race-specific resistance is different in leaves and roots. Plant Physiol. 91, 911–916.

Dixon R.A., Strack D. (2003) Phytochemistry meets genome analysis, and beyond. Phytochemistry 62, 815–816.

Dixon R.A. (2001) Natural products and plant disease resistance. Nature 411, 843–847

Dixon R.A., Paiva N. (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097.

Dixon R.A., Harrison M.J., Lamb C.J. (1994) Early events in the activation of plant defense responses. Annu. Rev. Phytopath. 32, 479-501.

Dixon R.A., (1986) The phytoalexin response: elicitation, signaling, and control of host gene expression. Biol. Rev. Camb. Philos. Soc. 61, 239-192.

Dooner H.K. (1983) Co-ordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Genetics 91, 309-315.

Dröge L.W., Kaiser A., Lindsay W.P., Halkier B.A., Loake G.J., Doerner P., Dixon R.A., Lamb C. (1997) Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses. EMBO J. 16, 726–738.

Dron M., Clouse S.D., Dixon R.A., Lawton M.A., Lamb C.J. (1988) Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts. Proc. Natl. Acad. Sci. USA 85(18), 6738–6742.

Durbin M.L., McCaig B., Clegg M.T. (2000) Molecular evolution of the chalcone synthase multigene family in the morning glory genome. Plant Mol. Biol. 42, 79-92.

Edwards M.L., Stemerick D.M., Sunkara P.S. (1990) Chalcones: a new class of antimitotic agents. J. Med. Chem. 33, 1948 – 1954.

Ehmann B., Ocker B., Schafer E. (1991) Development and light dependent regulation of the expression of two different chalcone synthase transcripts in mustard cotyledons. Planta 183, 416-422.

Elomaa P., Helariutta Y., Kotilainen M., Teeri T.H. (1996) Transformation of antisense constructs of the chalcone synthase gene superfamily into *Gerbera hybrida*: differential effect on the expression of family members. Mol. Breed. 2, 41–50.

Estabrook E.M., Sengupta G.C. (1991) Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development. Plant Cell 3, 299–308.

Faktor O., Kooter J.M., Dixon R.A., Lamb C.J. (1996) Functional dissection of a bean chalcone synthase gene promoter in transgenic tobacco plants reveals sequence motifs essential for floral expression. Plant Mol. Biol. 32, 849-859.

Faktor O., Kooter J.M., Loake G.J., Dixon R.A., Lamb C.J. (1997b) Differential utilization of regulatory cis-elements for stress-induced and tissue-specific activity of a French bean chalcone synthase promoter. Plant Sci. 124, 175- 182.

Faktor O., Loake G., Dixon R.A., Lamb C.J. (1997a) The G-box and H-box in a 39 bp region of a French bean chalcone synthase promoter constitute a tissue-specific regulatory element. Plant J. 11, 1105–1113.

Fan T.W.M. (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. Progress in Nuclear Magnetic Resonance Spectroscopy 28, 161–219. Fernie A.R., Trethewey R.N., Krotzky A.J., Willmitzer L. (2004) Metabolite profiling: from diagnostics to systems biology. Nat. Rev. Mol. Cell Biol. 5, 763–769.

Farmer E.E., Johnson R.R., Ryan C.A. (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol. 98, 995–1002.

Faulkner I.J., Rubery P.H. (1992). Flavonoids and flavonoid sulphates as probes of auxin-transport regulation in *Cucurbita pepo* hypocotyl segments and vesicles. Planta 186, 618–625.

Feinbaum R.L., Storz G., Ausubel F.M. (1991) High intensity and blue light regulated expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants. Mol Gen Genet 226, 449-456.

Feinbaum R.L., Ausubel F.M. (1988). Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol. Cell. Biol. 8, 1985–1992.

Feldbrügge M., Sprenger M., Hahlbrock K., Weisshaar B. (1997) PcMYB1, a novel plant protein containing a DNA-binding domain with one MYB repeat, interacts in vivo with a light-regulatory promoter unit. Plant J. 11, 1079–1093.

Felton G.W., Korth K.L., Bi J.L., Wesley S.V., Huhman D.V., Mathews M.C., Murphy J.B., Lamb C., Dixon R.A. (1999) Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory, Curr. Biol. 9, 317-320.

Ferrer J.L., Jez J.M., Bowman M.E., Dixon R.A., Noel, J.P. (1999) Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat. Struct. Biol. 6, 775–784.

Feucht W., Treutter D., Polster J. (2004) Flavanol binding of nuclei from tree species. Plant Cell Rep. 22, 430-436.

Fiehn O., Kopka J., Trethewey R.N., Willmitzer L. (2000). Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. Anal. Chem. 72, 3573–3580.

Flavell R.B. (1994). Inactivation of gene expression in plants as a consequence of novel sequence duplications. Proceedings of the National Academy of Sciences of the United States of America 91, 3490-3496.

Fliegmann J., Schröder G., Schanz S., Britsch L., Schröder 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.

Friedrich L., Lawton K.A., Ruess W., Masner P., Specker N., Rella M.G., Meier B., Dincher S., Staub T., Uknes S., Metraux J.P., Kessmann H., Ryals J. (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco, Plant J. 10, 61-70.

Fritze K., Staiger D., Czaja, I., Walden R., Schell J., Wing D. (1991). Developmental and UV light regulation of the snapdragon chalcone synthase promoter. Plant Cell 3**,** 893-905.

Frohnmeyer H., Bowler C., Schaefer E. (1997) Evidence for some signal transduction elements involved in UV-light dependent responses in parsley protoplasts. J. Exp. Bot. 48, 739–750.

Frohnmeyer H., Ehmann B., Kretsch T., Rocholl M., Harter K., Nagatani A., Furuya M., Batschauer A., Hahlbrock K., Schaefer E. (1992) Differential usage of photoreceptors for chalcone synthase gene expression during plant development. Plant J. 2, 899–906.

Frohnmeyer H., Staiger D. (2003) Ultraviolet-B radiation-mediated responses in plants. Balancing damage and protection. Plant Physiol. 133, 1420–1428.

Fuglevand G., Jackson J.A., Jenkins G.I. (1996) UV-B, UV-A, and blue light signal transduction pathways interact synergistically to regulate chalcone synthase gene expression in Arabidopsis. Plant Cell 8, 2347–2357.

Furner I.J., Sheikh M.A., Collett C.E. (1998) Gene silencing and homology-dependent gene silencing in *Arabidopsis*: genetic modifiers and DNA methylation. Genetics 149, 651–662.

Garcia I., Rodgers M., Pepin R., Hsieh T.F., Matringe M. (1999) Characterization and Subcellular Compartmentation of Recombinant 4-Hydroxyphenylpyruvate Dioxygenase from *Arabidopsis* in Transgenic Tobacco. Plant Physiol. 119, 1507-1516.

Gehm B.D., McAndrews J.M., Chien P.Y., Jameson J.L. (1997) Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc. Natl. Acad. Sci. USA 94, 14138–14143.

Gläßgen W.E., Rose A., Madlung J., Koch W., Gleitz J., Seitz H.U. (1998) Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. Planta 204, 490–498.

Görlach J., Volrath S., Knauf-Beiter G., Hengy G., Beckhove U., Kogel K.H., Staub M.T., Ward E., Kessmann H., Ryals J. (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates expression and disease resistance in wheat, Plant Cell 8, 629–643.

Graham T. (1998) Flavonoid and flavonol glycoside metabolism in *Arabidopsis*. Plant Physiol. Biochem. 36, 165-144.

Grandmaison J., Ibrahim, R.K. (1996) Evidence for nuclear protein binding of flavonol sulfate esters in *Flaveria chloraefolia*. J. Plant Physiol. 147, 653-660.

Gregersen P.L. Christensen A.B. Sommer-Knudsen J. Collinge D.B. (1994) A putative O-methyltransferase from barley is induced by fungal pathogens and UV light. Plant Mol. Biol. 26, 1797-1806.

Grotewold E., Chamberlin M., Snook M., Siame B., Butler L., Swenson J., Maddock S., St. Clair G., Bowen B. (1998) Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. Plant Cell 10, 721-740.

Guetsky R., Kobiler I., Wang X., Perlman N., Gollop N., Avila-Quezada G., Hadar I., Prusky D. (2005) Metabolism of the flavonoid epicatechin by laccase of *Colletotrichum gloeosporioides* and its effect on pathogenicity on avocado fruits. Phytopathology 95, 1341–1348.

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

Hahlbrock K. (1981) Flavonoids. In The Biochemistry of Plants, Vol. 7, P.K. Stumpf and E.E. Conn, eds (New York: Academic Press), 425–456.

Hahlbrock K., Grisebach H . (1979) Enzymatic controls in the biosynthesis of lignin and flavonoids . Annu. Rev. Plant Physiol. 30,105-130

Hahlbrock K., Zilg H., Grisebach H. (1970) Stereochemistry of the enzymatic cyclisation of 4,2',4'-trihydroxychalcone to 7,4'-dihydroxyflavanone by isomerases from Mung Bean seedlings. Eur. J. Biochem. 15, 13-18.

Hammond S.M., Caudy A.A., Hannon G.J. (2001) Post-transcriptional gene silencing by double-stranded RNA. Nat. Rev. Genet. 2, 110–119.

Hannon G. J. (2002) RNA interference. Nature 418, 244–251.

Harborne J.B., Grayer R.J. (1994) Flavonoids and insects. In: The Flavonoids, Advances in Research since 1986, Chapman & Hall, London, 589–618.

Harborne J.B., Williams C.A. (2000) Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.

Harker C.L., Ellis T.H., Coen, E.S. (1990). Identification and genetic regulation of the chalcone synthase multigene family in pea. Plant Cell 2, 185–194.

Harker C.L., Ellis T.H.N., Coen E.S. (1990) ldentification and genetic regulation of the chalcone synthase multigene family in pea. Plant Cell 2, 185-194.

Harrison M.J, Dixon. R.A (1993) Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula*. Mol. Plant Microb. Interact. 5, 643–654.

Harrison M.J., Lawton M.A., Lamb C.J., Dixon R.A. (1991) Characterization of a nuclear protein that binds to three elements within the silencer region of a bean chalcone synthase gene promoter. Proc. Natl. Acad. Sci. USA 88**,** 2515–2519.

Hartmann U., Valentine W.J., Christie J.M., Hays J., Jenkins G.I., Weisshaar B. (1998) Identification of UV/blue light-response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. Plant Mol. Biol. 36, 741–754.

Haussuehl K.K., Rohde W., Weissenboeck G. (1996) Expression of chalcone synthase genes in coleoptiles and primary leaves of *Secale cereale* L. after induction by UV radiation: evidence for a UV protective role of the coleoptiles. Bot. Acta. 109, 229–238.

Helariutta Y., Kotilainen M., Elomaa P., Kalkkinen N., Bremer K., Teeri T.H., Albert V.A. (1996) Duplication and functional divergence in the chalcone synthase gene family of Asteraceae: evolution with substrate change and catalytic simplification. Proc. Natl. Acad. Sci. USA 93, 9033–9038.

Hendrawati O., Yao Q., Kim H.K., Linthorst H.J.M., Erkelens C., Lefeber A.W.M., Choi Y.H., Verpoorte R. (2006) Metabolic differentiation of *Arabidopsis*

treated with methyl jasmonate using nuclear magnetic resonance spectroscopy, Plant Sci.170, 1118-1124.

Hinderer W., Seitz H.U. (1985) Chalcone synthase from cell suspension cultures of Daucus carota L. Arch. Biochem. Biophys. 240, 265-272.

Hipskind J, Hanau R, Leite B, Nicholson RL (1990) Phytoalexin synthesis in sorghum: identification of an apigeninidin acyl ester. Physiol. Mol. Plant. Pathol. 36, 381-396.

Hirai M.Y. Hirai, Yano M. Yano, D.B. Goodenowe, S. Kanaya, T. Kimura, M. Awazuhara, Arita M. Arita, Fujiwara T. Fujiwara, Saito K. Saito(2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. USA 101, 10205-10210.

Holton T.A., Cornish E.C. (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7, 1071–1083.

Hrazdina G., Jensen R. A. (1992) Spatial Organization of Enzymes in Plant Metabolic Pathways. Ann. Rev. Plant Physiol. Plant Mol. Biol. 43, 241-267.

Hrazdina G., Kreuzaler F., Hahlbrock K, Grisebach H. (1976) Substrate specificity of flavanone synthase from cell suspension cultures of parsley and structure of release products *in vitro.* Arch. Biochem. Biophys. 175, 392-399.

Hrazdina G., Marx F.A., Hoch H.C. (1982) Distribution of secondary plant metabolites and their biosynthetic enzymes in pea (*Pisum sativum* L.) leaves. Plant Physiol. 70, 745-748.

Hrazdina G., Wagner G. J. (1985) Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. Annu. Proc. Phytochem. Soc. Europe 25, 120-133.

Hutzler P., Rischbach R., Heller W., Jungblut T. P., Reuber S., Schmitz R., Veit M., Weissenbck G., Schmitzler J.-P. (1998) Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. J. Exp. Bot. 49, 953-965.

Inbar, M., Doostdar H., Gerling D., Mayer R. T. (2001) Induction of systemic acquired resistance in cotton by BTH has a negligible effect on phytophagous insects, Entomol. Exp. Appl. 99, 65-70.

Ingelbrecht I., Van Houdt H., Van Montagu M., Depicker A. (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. USA 91, 10502–10506.

Ingham D.J., Beer S., Money S., Hansen G. (2001). Quantitative real-time PCR assay for determining transgene copy number in transformed plants. Biotechniques 31, 132–140.

Ingham J.L. (1976) Induced and constitutive isoflavonoids from stems of chickpeas (*Cicer arietinum* L.) inoculated with spores of *Helminthosporium carbonum* Ullstrup. J. Phytopathol. 87, 353 – 367.

Iriti M., Rossoni M., Borgo M., Faoro F. (2004) Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis cinerea*, J. Agric. Food Chem. 52, 4406- 4413.

Ito M., Ichinose Y., Kato H., Shiraishi T., Yamada T. (1997) Molecular evolution and functional relevance of the chalcone synthase genes of pea. Mol. Gen. Genet. 255: 28–37.

Iyer L.M., Kumpatla S.P., Chandrasekharan M.B., Hall T.C. (2000) Transgene silencing in monocots. Plant Mol. Biol. 43, 323–346.

Jacobs M., Rubery P.H. (1988) Naturally occurring auxin transport regulators. Science 241, 346-349.

Jahangir M., Kim H.K., Choi Y.H., Verpoorte R. (2008) Metabolomic response of *Brassica rapa* submitted to pre-harvest bacterial contamination. Food Chemistry 107, 362-368.

Jang M., Cai L., Udeani G.O., Slowing K.V., Thomas C.F., Beecher C.W., Fong H.H.S., Farnsworth N.R., Kinghorn A.D., Mehta R.G., Moon R.C., Pezzuto J.M. (1997) Cancer chemo preventive activity of resveratrol, a natural product derived from Grape, Science 275, 218-220.

Jang M.S., Cai E.N., Udeani G.O., Slowing K.V., Thomas C.F., Beecher C.W.W., Fong H.H.S., Fransworth N.R., Kinghorn A.D., Mehta R.G., Moon R.C., Pezzuto J.M. (1997) Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes. Science 275, 218-220.

Jenkins G.I. (1997) UV and blue light signal transduction in Arabidopsis. Plant Cell Environ 20, 773-778.

Jenkins G.I., Long J.C., Wade H.K., Shenton M.R., Bibikova T.N. (2001) UV and blue light signalling: pathways regulating chalcone synthase gene expression in *Arabidopsis.* New Phytol. 151 (1), 121–131.

Jez J.M., Austin M.B., Ferrer J.L., Bowman M.E., Schröder J., Noel J.P. (2000) Structural control of polyketide formation in plant-specific polyketide synthases. Chem. Biol. 40, 1–12.

Jez J.M., Bowman M.E., Noel J.P. (2001a) Structure-guided programming of polyketide chain-length determination in chalcone synthase. Biochemistry 40, 14829–14838.

Jez J.M., Ferrer J.L., Bowman M.E., Austin M.B., Schröder J., Dixon R.A., Noel J.P. (2001b) Structure and mechanism of chalcone synthase-like polyketide synthases. J. Ind. Microbiol. Biotechnol. 27, 393–398.

John D. Hipskind J.D., Robert Hanau R., Breno Leite B., Ralph L. Nicholson R.L. (1990) Phytoalexin accumulation in sorghum: identification of an apigeninidin acyl ester. Physiol. Mol. Plant Path. 36, 381–396.

Johnson G., Maag D.D., Johnson D.K., Thomas R.D. (1976) The possible role of phytoalexins in the resistance of sugarbeet (Beta vulgaris) to Cercospora beticola [Fungal pathogens]. Physiol. Plant Path. 8, 225-230

Jorgensen R.A. (1995) Cosuppression, flower color patterns, and metastable gene expression states. Science 268, 686-691.

Junghans H., Dalkin K., Dixon R.A. (1993) Stress responses in alfalfa (*Medicago sativa* L.). 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol. Biol. 22, 239–253.

Kaiser T., Batschauer A. (1995) *Cis*-acting elements of the *CHS1* gene from white mustard controlling promoter activity and spatial patterns of expression. Plant Mol. Biol. 28, 231-243.

Kaiser T., Emmler K., Kretsch T., Weisshaar B., Schafer E., Batschauer A. (1995). Promoter elements of the mustard CHS7 gene are sufficient for light-regulation in transgenic plants. Plant Mol. Biol. 28, 219-229

Kataoka K., Uemachi A., Yazawa S. (2003) Fruit growth and pseudoembryo development affected by uniconazole, an inhibitor of gibberellin biosynthesis, in *pat-2* and auxin-induced parthenocarpic tomato fruits Sci. Hort. 98, 9-16.

Katz V.A., Thulke O.U., Conrath U. (1998) A benzothiadiazole primes parsley cells for augmented elicitation of defense responses, Plant Physiol. 8, 1333–1339.

Kaulen H., Schell J., Kreuzaler F. (1986) Light-induced expression of the chimeric chalcone synthase-NPTII gene in tobacco cells. EMBO J. 5, 1–8.

Keasling J.D., Vandien S.J., Pramanik J. (1998) Engineering polyphosphate metabolism in *Escherichia coli* - implications for bioremediation of inorganic contaminants. Biotech. Bioeng. 58, 231-239.

Kerhoas L., Aouak D., Cingoz A., Routaboul J.M., Lepiniec L., Einhorn J., Birlirakis N. (2006) Structural characterization of the major flavonoid glycosides from *Arabidopsis thaliana* seeds. J. Agric. Food Chem. 54, 6603–6612.

Kessmann H., Staub T., Hofmann C., Maetzke T., Herzog J., Ward E., Ukness S., Ryals J. (1994) Induction of systemic acquired resistance in plants by chemicals. Annu. Rev. Phytopathol. 32, 439-459.

Knogge W., Schmelzer E., Weissenböck G. (1986) The role of chalcone synthase in the regulation of flavonoid biosynthesis in developing oat primary leaves. Arch. Biochem. Biophys. 250, 364–372.

Kodama O., Miyakawa J., Akatsuka T., Kiyosawa S. (1992) Sakuranetin, a flavanone phytoalexin from ultraviolet-irradiated rice leaves. Phytochemistry 31, 3807–3809.

Koes R.E., Spelt C.E., Mol J.N.M. (1989) The chalcone synthase multigene family of *Petunia hybrida* (V30): differential, light-regulated expression during flower development and UV light induction. Plant Mol. Biol. 12, 213–225.

Koes R.E., Spelt C.E., Mol J.N.M., Gerats A.G.M. (1987) The chalcone synthase multigene family of *Petunia hybrida*: sequence homology, chromosomal localization and evolutionary aspects. Plant Mol. Biol. 10, 159–169.

Koes R.E., Spelt C.E., Reif H.J., van den Elzen P.J.M., Veltkamp E., Mol J.N.M. (1986) Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family. Nucleic Acids Res. 11 (14), 5229 - 5239.

Kohli A., Leech M., Vain P., Laurie D.A., Christou P. (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc. Natl. Acad. Sci. USA 95, 7203–7208.

Kolbe A., Oliver S.N., Fernie A.R., Stitt M., van Dongen J.T., Geigenberger P. (2006) Combined Transcript and Metabolite Profiling of Arabidopsis Leaves Reveals Fundamental Effects of the Thiol-Disulfide Status on Plant Metabolism, Plant Physiol. 141, 412-422.

Kombrink E., Somissich I. E. (1997) Pathogenesis-related proteins and plant defense in: Carrol G. C., Tudzynski P. (Eds), The Mycota V part A, plant Relationships, Springer-Verlag, Berlin, 107-128.

Kong J.M., Chia L.S., Goh N.K., Chia T.F., Brouillard R. (2003) Analysis and biological activities of anthocyanins. Phytochemistry 64, 923–933.

Kraemer K.H., Schenkel E.P., Verpoorte R. (2002) *Ilex paraguariensis* cell suspension culture characterization and response against ethanol, Plant Cell Tissue Organ Cult. 68, 257-263.

Kreuzaler F., Hahlbrock K. (1975) Enzymatic synthesis of an aromatic ring from acetate units. Partia1 purification and some properties of flavanone synthase from cell-suspension cultures of Petroselinum hortense. Eur. J. Biochem. 56, 205-213.

Kreuzaler F., Ragg H., Fautz E., Kuhn D.N., Hahlbrock K. (1983) UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. Proc. Natl. Acad. Sci. USA 80, 2591–2593.

Krishnan P., Kruger N.J., Ratcliffe R.G. (2005) Metabolite fingerprinting and profiling in plants using NMR. J. Exp. Bot. 56, 255-265.

Kubasek W.L., Shirley B.W., Mc Killop A., Goodman H.M., Briggs W., Ausubel F.M. (1992) Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. Plant Cell 4, 1229-1236.

Kuć J. (1982) Induced immunity to plant disease, Bioscience 32, 854-856.

Kuhn D.N. (1988) Plant stress responses: Discussion of models for race-specific resistance. Recent. Adv. Phytochem. 22, 127-161.

Kuhn D.N., Chappell J., Boudet A., Hahlbrock K. (1984) Induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in cultured plant cells by UV light or fungal elicitor. Proc. Natl. Acad. Sci. USA 81, 1102-1106.

Kuras M., Stefanowska-Wronka M., Lynch J.M., Zobel A.M. (1999) Cytochemical localization of phenolic compounds in columella cells of the root cap in seeds of *Brassica napus*—changes in the localization of phenolic compounds during germination. Ann. Bot. 84, 135-143.

Kurosaki F., Amin M., Nishi A. (1986) Induction of phytoalexin production and accumulation of phenolic compounds in cultured carrot cells. Pysiol. Mol. Plant Pathol. 28, 359-370.

Langcake P., Pryce R.J. (1977*a*) A new class of phytoalexins from grapevines. Experientia 33, 151–152.

Langcake P., Pryce R.J. (1977*b*) The production of resveratrol and the viniferins by grapevines in response to ultraviolet irradiation. Phytochemistry 16, 1193–1196.

Lanz T., Tropf S., Marner F.J., Schröder J., Schröder G. (1991) The role of cysteines in polyketide synthases: site-directed mutagenesis of resveratrol and chalcone synthases, two key enzymes in different plant-specific pathways. J. Biol. Chem. 266, 9971–9976

Lawson C.G.R., Djordjevic M.A., Weinman J.J., Rolfe B.G. (1994) *Rhizobium* inoculation and physical wounding result in the rapid induction of the same chalcone synthase copy in *Trifolium subterraneum.* Mol. Plant Microb. Interact. 7, 498 507.

Lawton K.A., Friedrich L., Hunt M., Weymann K., Delaney T., Kessmann H., Staub T., Ryals J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway, Plant J. 10, 71-82.

Lawton M.A., Clouse S.D., Lamb C.J. (1990). Glutathione-elicited changes in chromatin structure within the promoter of the defense gene chalcone synthase. Plant Cell Rep. 8, 561−564.

Lawton M.A., Lamb C.J. (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol. Cell. Biol. 7, 335 341.

Le Gall G., Metzdorff S.B., Pedersen J., Bennett R.N., Colquhoun I.J. (2005) Metabolite profiling of *Arabidopsis thaliana* (L.) plants transformed with an antisense chalcone synthase gene. Metabolomics 1, 181-198.

Lessard R., Rochette P., Gregorich E.G., Pattey E., Desjardins R.L., (1996)_ Nitrous oxide fluxes from manure-amended soil under maize. J. Environ. Qual. 25, 1371–1377.

Leyva A., Jarillo T.A., Salinas J., Martinez-Zapater J.M. (1995) Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis thaliana* in a light-dependent manner. Plant Physiol. 108, 39–46.

Li J., Lee T.M.O., Raba R.., Amundson R.G., Last R.L. (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell 5, 171-179.

Li R.S., Kenyon G.L., Cohen F.E., Chen X.W., Gong B.Q., Dominquez J.N., Davidson E., Kurzban G., Miller R.E., Nuzum E.O., Rosenthal P.J., McKerrow J.H. (1995) In Vitro Antimalarial Activity of Chalcones and Their Derivatives J. Med. Chem. 38, 5031 - 5037.

Li X.Q., Li S.H., Chen D.F., Meng F.R. (2004) Induced Activity of Superoxide Dismutase and Peroxidase of *in vitro* Plants by Low Concentrations of Ethanol, Plant Cell Tissue Organ Cult. 79, 83-86.

Li Z., Hansen J.L., Liu Y., Zemetra R.S., Berger P.H. (2004) Using real-time PCR to determine transgene copy number in wheat. Plant Mol. Bio. Rep. 22, 179–188.

Liang Y.S., Kim H. K., Lefeber A. W. M., Erkelens C., Choi Y. H., Verpoorte R. (2006) Identification of phenylpropanoids in *Brassica rapa* leaves treated with methyl jasmonate using two-dimensional nuclear magnetic resonance spectroscopy. J. Chromatogr. A 1112, 148-155.

Liang Y.S., Kim H.K., Linthorst H.J.M., Choi Y.H., Verpoorte R., Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by 2-dimensional NMR spectroscopy, Phytochemistry 67 (2006) 2503-2511.

Lin C. (2000) Plant blue-light receptors. Trends Plant Sci. 5, 337-342.

Lin Y., Irani N.G., and Grotewold E. (2003) Sub-cellular trafficking of phytochemicals explored using auto-fluorescent compounds in maize cells. BMC Plant Biol. 3, 1011-1012.

Lipphardt S., Brettschneider R., Kreuzaler F., Schell J., Dangl J.L. (1988) UV-inducible transient expression in parsley protoplasts identifies regulatory *cis*-elements of

a chimeric *Antirrhinum majus* chalcone synthase gene. EMBO J. 7, 4027–4033.

Liu D., Raghothama K. G., Hasegawa P. M., Bressan R. A., Osmotin overexpression in potato delays development of disease symptoms, Proc. Natl. Acad. Sci. USA 91 (1994) 1888-1892.

Livak K.J., Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.

Lo S.C., Hipskind J.D., Nicholson R.L. (1998) cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defense-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. Mol. Plant Microb. Interact. 12, 479-489.

Loake G.J., Faktor O., Lamb C.J., Dixon R.A. (1992) Combination of H box (CCTACCN7CT) and G box (CACGTG) *cis* elements is necessary for feed-forward stimulation of a chalcone synthase promoter by the phenylpropanoid pathway intermediate p-coumaric acid. Proc. Natl. Acad. Sci. USA 89, 9230–2934.

Logemann E., Tavernaro A., Schulz W., Somssich I. E., Hahlbrock K. (2000) UV light selectively coinduces supply pathways from primary metabolism and flavonoid secondary product formation in parsley. Proc. Natl. Acad. Sci. USA 97, 1903-1907.

Lois R. (1994) Accumulation of UV-absorbing flavonoids induced by UV-B radiation in *Arabidopsis thaliana* L. I. Mechanisms of UV resistance in Arabidopsis. Planta 194, 498-503.

Lois R., Dietrich A., Hahlbrock K., Schulz W. (1989) A phenylalanine ammonia-lyase from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. EMBO J. 8, 1641–1648.

Long J.C., Jenkins G.I. (1998) Involvement of Plasma Membrane Redox Activity and Calcium Homeostasis in the UV-B and UV-A /Blue Light Induction of Gene Expression in Arabidopsis. Plant Cell 10, 2077–2086.

Loyall L., Uchida K., Braun S., Furuya M., Frohnmeyer H. (2000) Glutathione and a UV light-induced glutathione *S*-transferase are involved in signaling to

chalcone synthase in cell cultures. The first indication that tau GSTs are involved in plant cell stress signaling. Plant Cell. 12, 1939–1950.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., Wessler, S.R. (1989) *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcription activators and contains the *myc*-homology region. Proc. Natl. Acad. Sci. USA 86, 7092-7096.

Lue W.L., Kuhn D., Nicholson R.L. (1989) Chalcone synthase activity in sorghum mesocotyls inoculated with *Colletotrichum graminicola*. Physiol. Mol. Plant Pathol. 35, 413-422.

Mackerness S., John C.F., Jordan B.R., Thomas B. (2001) Early signaling componets in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. FEBS Lett. 489, 237–242.

Manach C., Scalbert A., Morand C., Rémésy C., Jimenez L. (2004) Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79, 727-747.

Markham, K.R., Gould, K.S., Ryan K.G. (2001) Cytoplasmic accumulation of flavonoids in flower petals and its relevance to yellow flower colouration. Phytochemistry 58, 403-413.

Marles M.A., Ray H., Gruber M.Y. (2003) New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry 64, 367–383.

Martin C.R. (1993) Structure, function and regulation of the chalcone synthase. Int. Rev. Cytol 147, 233–284.

Mathews H., Clendennen S.K., Caldwell C.G., Liu XL., Connors, K., Matheis N., Schuster D.K., Menasco D.J., Wagoner W., Lightner J., Wagner D.R. (2003) Plant Cell 15, 1689-1703.

Matthews D.E., Matthews P.S. (1989) Phytoalexin detoxification: Importance for pathogen city and practical implications. Annu. Rev. Phytopath. 27, 143–164.

Mauch F., Mauch-Mani B., Boller T. (1988) Antifungal hydrolases in pea tissue. 2. Induction of chitinase and b-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol. 76, 936-942.

Meier B.M., Shaw N., Slusarenko A.J. (1993) Spatial and temporal accumulation of defense gene transcripts in bean (*Phaseolus vulgaris*) leaves in relation to

bacteria-induced hypersensitive cell death. Mol. Plant Microb. Interact. 6, 453-466.

Mert T.F., Bennet M.H., Mansfield J.W., Holub E.B. (2003) Camalexin accumulation in *Arabidopsis thaliana* following abiotic elicitation or inoculation with virulent or avirulent *Hyaloperonospora parasitica.* Physiol. Mol. Plant Pathol. 62, 137–145.

Métraux J.P., Signer H., Ryals J., Ward E., Wyss-Benz M., Gaudin J. Raschdorf K., Schmid E., Blum W., Inveradi B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber, Science 250, 1004-1006.

Mitrecic D., Huzak M., Curlin M., Gajovic S. (2005) An improved method for determination of gene copy numbers in transgenic mice by serial dilution curves obtained by real-time quantitative PCR assay. J. Biochem. Biophys. Methods 64(2), 83-98.

Mol J. N. M., Robbins M. P., Dixon R. A., Veltkamp E.. (1985). Spontaneous and enzymic rearrangement of naringenin chalcone to flavanone. Phytochemistry 24, 2267-2269.

Mol J.N.M., Stuitje A., Gerats A., Koes R. (1988) Cloned genes of plant phenylpropanoid metabolism. Plant Mol. Biol. Rep. 6, 274–279.

Mol N.J.J., Schram A.W., de Vlaming P., Gerats A.G.M., Kreuzaler F., Hahlbrock K., Reif H.J., Veltkamp E. (1983) Regulation of flavonoid gene expression in *Petunia hybrida*: description and partial characterization of a conditional mutant in chalcone synthase gene expression. Mol. Gen. Genet. 192, 424–429.

Monache G.D., Botta B., Vinciguerra V., de Mello J.F., Andrade Chiapetta de A. (1996) Antimicrobial isoflavanones from *Desmodium canum.* Phytochemistry 41, 537-544.

Moriguchi T., Kita M., Tomono Y., Endo-Inagaki T., Omura M. (1999) One type of chalcone synthase gene expressed during embryogenesis regulates the flavonoid accumulation in citrus cell cultures. Plant Cell Physiol. 40, 651–655.

Morita H., Takahashi Y., Noguchi H., Abe I. (2000) Enzymatic formation of unnnatural aromatic polyketides by chalcone synthase. Biochem. Biophys. Res. Commun. 279, 190–195.

Morkunas I., Marczak L., Stachowiak J., Stobiecki M. (2005) Sucrose-induced lupine defense against *Fusarium oxysporum*: Sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum.* Plant Physiol. Biochem. 43, 363-373.

Nagy N.E., Fossdal C.G., Krokene P., Krekling T., Lonneborg A., Solheim H. (2004) Induced responses to pathogen infection in Norway spruce phloem: changes in polyphenolic parenchyma cells, chalcone synthase transcript levels and peroxidase activity. Tree Physiol. 24, 505-515.

Naidu R.A., Collins G.B., Ghabrial S.A. (1991) Nucleotide sequence analysis of a cDNA clone encoding the coat protein gene of peanut stunt virus. Plant Mol. Biol. 16, 175–185.

Nakamura N., Nakamae H., Maekawa L. (1980) Effects of light and kinetin on anthocyanin accumulation in the petals of *Rosa* hy*brida,* Hort cv. Ehigasa. Z. Pflanzenphysiol. 98, 263-270.

Napoli C., Lemieux C., Jorgensen R.A. (1990) Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in trans.Plant Cell 2, 279–289.

Nicholson R.L., Kollipara S.S. Vincent J.R., Lyons P.C., Cadena-Gomez G. (1987) Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. Proc. Natl. Acad. Sci. USA 84**,** 5520-5524.

Nijveldt R.J., van Nood E., van Hoorn D.E.C., Boelens P.G., van Norren K., van Leeuwen P.A.M. (2001) Flavonoids : A review of probable mechanisms of action and potential applications. Am. J. Clin. Nutr. 74, 418–425.

Novák P., Krofta K., Matoušek J. (2006) Chalcone synthase homologues from *Humulus lupulus*: some enzymatic properties and expression. Biol. Plant. 50, 48–54.

Ohl S., Hahlbrock K., Schafer, E. (1989) A stable blue-lightderived signal modulates ultraviolet-light-induced activation of the chalcone-synthase gene in cultured parsley cells. Planta 1**77,** 228-236.

Ohto M.A., Onai K., Furukawa Y., Aoki E., Araki T., Nakamura K. (2001) Effects of sugar on vegetative development and floral transition in Arabidopsis. Plant Physiol. 127, 252–261.

Oksman-Caldentey K.M., Saito K. (2005) Integrating genomics and metabolomics for engineering plant metabolic pathways, Curr. Opin. Biotech. 16, 174-179.

Oku H., Ouchi S., Shiraishi T., Komoto Y., Oki K. (1975) Phytoalexin activity in barley powdery mildew. Ann. Phyto. Path. Soc. Japan 41, 185–191

Panke S., Sanchezromero J. M. (1998) Engineering of quasi-natural *Pseudomonas putida* strains for toluene metabolism through an ortho-cleavage degradation pathway. Appl. Environ. Microbiol. 64, 748-751.

Pare P.W., Mischke C.F., Edwards R., Dixon R.A., Norman H.A., Mabry T.J. (1992) Induction of phenylpropanoid pathway enzymes in elicitor-treated cultures of *Cephalocereus senilis,* Phytochemistry 31, 149-153.

Pare, P.W., : Mischek, C.F., : Edwards, R., : Dixon, R.A., : Norman, H.A., : Mabry, T.J. (1992) Induction of phenylpropanoid pathway enzymes in elicitro-treated cultures of Cephalocereus senilis. Phytochemistry 31, 149-153.

Paz-Ares J., Wlenand U., Peterson P.A., Saedler H. (1986). Molecular cloning of the c locus of *Zea* mays: A locus regulating the anthocyanin pathway. EMBO J. 5, 829-833.

Peer W.A., Brown D.E., Tague B.W., Muday G.K., Taiz L., Murphy A.S. (2001) Flavonoid accumulation patterns of transparent testa mutants of *Arabidopsis*. Plant Physiol. 126, 536–548.

Perrot G.H., Cone K.C. (1989) Nucleotide sequence of the maize R-S gene. Nucl. Acids Res. 17**,** 8003.

Peters A., Schneider-Poetsch H.J., Schwarz H., Weissenböck G. (1988) Biochemical and immunological characterization of chalcone synthase from rye leaves. J. Plant Physiol. 133, 178-182.

Peterson G.L. (1977) A simplification of the protein assay method of Lowry et al. which is generally more applicable. Anal. Biochem. 83, 346-356.

Qian W., Tan G., Liu H., He S., Gao Y., An C. (2007) Identification of a bHLH-type G-box binding factor and its regulation activity with G-box and Box I elements of the *PsCHS1* promoter. Plant Cell Rep. 26, 85–93.

Raharjo T. J., (2004) Studies of cannabinoid biosynthesis in *Cannabis sativa* L.: The polyketide synthase, PhD thesis, Leiden University, Leiden, The Netherlands.

Rasmussen S., Dixon R.A. (1999) Transgene-mediated and elicitor-induced perturbation of metabolic channeling at the entry point into the phenylpropanoid pathway. Plant Cell 11, 1537-1551.

Reuber S., Bornman J.F., Weissenoeck G. (1996) A flavonoid mutant of barley (*Hordeum vulgare* L.) exhibits increased sensitivity to UV-B radiation in the primary leaf. Plant Cell Envir. 19, 593–601

Reymond P., Short T., Briggs W.R., Poff K.L. (1992). Light induced phosphorylation of a membrane protein plays an early role in signal transduction for phototropism in *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA 89, 4718-4721.

Richard S., Lapointe G., Rutledge R. G., Seguin A. (2000) Induction of chalcone synthase expression in white spruce by wounding and jasmonate. Plant Cell Physiol. 41, 982–987.

Rocholl M., Talke-Messerer C., Kaiser T., Batschauer A. (1994) Unit I of the mustard chalcone synthase promoter is sufficient to mediate light responses from different photoreceptors. Plant Sci. 97, 189–198.

Roessner U., Luedemann A., Brust D., Fiehn O., Linke T., Willmitzer L., Fernie A.R. (2001) Metabolic profiling and phenotyping of genetically and environmentally modified plant systems. Plant Cell 13, 11-29.

Roessner U., Wagner C., Kopka J., Trethewey R.N., Willmitzer L. (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J. 23, 131-142.

Rohde A., Morreel K., Ralph J. (2004) Molecular phenotyping of the pal1 and pal2 mutants of Arabidopsis thaliana reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell 16, 2749–2771.

Rolland F., Moore B., Sheen J. (2002) Sugar sensing and signalling in plants. The Plant Cell 14, S185–S205.[

Ryals J.A., Neuenschwander U.H., Willits M.G., Molina A., Steiner H.Y., Hunt M.D. (1996) Systemic acquired resistance, Plant Cell 8, 1809-1819.

Ryan C. (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. 28, 425–449.

Ryder T.B., Hedrick S.A., Bell J.N., Liang X., Clouse S. D., Lamb C.J. (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.

Ryder T.B., Cramer C.L., Bell J.N., Robbins M.P., Dixon R.A., Lamb C.J. (1984) Elicitor rapidly induces chalcone synthase mRNA in *Phaseolus vulgaris* cells at the onset of the phytoalexin defense response. Proc. Natl. Acad. Sci. USA 81, 5724–5728.

Sablowski R., Moyano E., Culianez-Macia F., Schuch W., Martin C., Bevan M. (1994) A flower-specific Myb protein activates transcription of phenylpropanoid synthetic genes. EMBO J. 13**,** 128–137.

Sakuta M. (2000) Transcriptional control of chalcone synthase by environmental stimuli. J. Plant Res. 113, 327–333.

Sambrook J., Fritsch E.F., Maniatis T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanchez I.J.F. (2008) Polyketide synthase in *Cannabis sativa* L., PhD thesis, Leiden University, Leiden, The Netherlands.

Sankawa U., Hakamatsuka T. (1997) Biosynthesis of isoflavone and related compounds in tissue cultures of *Pueraria lobata*, p . 25-48 . *In* K . Ogura and U . Sankawa (ed.), Dynamic aspects of natural products chemistry . Kodansha, Tokyo, Japan.

Saslowsky D., Winkel S.B. (2001) Localization of flavonoid enzymes in Arabidopsis roots. Plant J. 27, 37-48

Schäfer E., Kunkel T., Frohnmeyer H. (1997) Signal transduction in the photocontrol of chalcone synthase gene expression. Plant Cell Environ. 20, 722–727.

Schenk P.M., Kazan K., Wilson I., Anderson J.P., Richmond T., Somerville S.C., Manners J.M. (2000) Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proc. Natl. Acad. Sci. USA 97**,** 11655–11660.

Schmelzer E., Jahnen W., Hahlbrock K. (1988) *In situ* localization of light-induced chalcone synthase mRNA, chalcone synthase, and flavonoid end products in epidermal cells of parsley leaves. Proc. Natl. Acad. Sci. USA 85, 2989-2993.

Schmittgen T.D., Zakrajsek B.A., Mills A.G., Gorn V., Singer M.J., Reed M.W. (2000) Quantitative reverse transcription polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods. Anal. Biochem. 285, 194-204.

Schnitzler J.P., Jungblut T.P., Heller W., Hutzler P., Heinzmann U., Schmelzer E., Ernst D., Langebartels C., Sandermann H. (1996) Tissue localisation of UV-B screening pigments and chalcone synthase mRNA in Scots pine (*Pinus sylvestris* L.) needles. New Phythol. 132, 247–258.

Schröder J. (2008) http://www.biologie.unifreiburg.de/data/bio2/schroeder/CHS_Reaction_Mechanism.html

Schröder J. (1997) A family of plant - specific polyketide synthases: facts and predictions. Trends Plant Sci. 2, 373– 378.

Schröder J., Schäafer E. (1980) Radioiodinated antibodies, a tool in studies on the presence and role of inactive enzyme forms: regulation of chalcone synthase in parsley cell suspension cultures. Arch. Biochem. Biophys. 203, 800-808.

Schulze L.P., Becker A.M., Schulr W., Hahlbrock K., Dangl J.L. (1989) Functional architecture of the light-responsive chalcone synthase promoter from parsley. Plant Cell 1**,** 707-714.

Schuez R., Heller W., Hahlbrock K. (1983) Substrate specificity of chalcone synthase from *Petroselinum hortense*. J. Biol. Chem. 258, 6730–6734.

Seikel M.K., Bushnell A.J., Birzgalis R. (1962) The flavonoid constituents of Barley (Hordeum vulgare). III. Lutonarin and its 3'-methyl ether. Arch. Biochem. Biophys. 99, 451–457.

Seki H., Ichinose Y., Ito M., Shiraishi T., Yamada T. (1997) Combined effects of multiple cis-acting elements in elicitor-mediated activation of *PSCHSl* gene. Plant Cell Physiol. 38, 96-100.

Seki H., Nagasugi Y., Shiraishi T., Yamada T. (1999) Changes in vivo DNA-protein interactions in pea phenylalanine ammonia-lyase and chalcone synthase gene promoter induced by fungal signal molecules. Plant Cell Physiol. 40, 88-95.

Shimada H, Kondo K, Fraser P D, Miura Y, Saito T, Misawa N (1998) Increased carotenoid production by the food yeast *Candida utilis* through metabolic

engineering of the isoprenoid pathway. Appl. Environ. Microbiol. 64, 2676, 2676–2680.

Shimizu T., Akada S., Senda M., Ishikawa R., Harada T., Niizeki M., Dube S.K. (1999) Enhanced expression and differential inducibility of soybean chalcone synthase genes by supplemental UV-B in dark-grown seedlings. Plant Mol. Biol. 39, 785–795.

Shvarts M., Borochov A., Weiss D. (1997) Low temperature enhances petunia flower pigmentation and induces chalcone synthase gene expression. Physiol. Plant. 99, 67 - 72.

Smirnoff N. (1998). Plant resistance to environmental stress. Curr. Opinion Biotechnol. 9, 214-219.

Snyder B.A., Nicholson R.L. (1990) Synthesis of phytoalexins in sorghum as a site specific response to fungal ingress. Science 248, 1637-1639.

Soylu S. (2006) Accumulation of cell-wall bound phenolic compounds and phytoalexin in *Arabidopsis thaliana* leaves following inoculation with pathovars of *Pseudomonas syringae*. Plant. Sci. 170, 942-952.

Srivastava V., Anderson O.D., Ow D.W. (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc. Natl. Acad. Sci. USA 96, 11117–11121.

Stafford H.A. (1974) Possible multi-enzyme complexes regulating the formation of C_6-C_3 phenolic compounds and lignins in higher plants. Rec. Adv. Phytochem. 8, 53-79.

Stafford H.A. (1991) Flavonoid evolution: an enzymic approach. Plant Physiol. 96, 680-685.

Staiger D., Kaulen H., Schell J. (1989) A CACGTG motif of the *Antirrhinum majus* chalcone synthase promoter is recognised by an evolutionary conserved nuclear protein. Proc. Natl. Acad. Sci. USA 86, 6930–6934.

Stassi D.L., Kakavas S.J., Reynolds K.A., Gunawardana G., Swanson S., Zeidner D., Jackson M., Liu H., Buko A., Katz L. (1998). Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. Proc. Natl. Acad. Sci. USA. 95, 7305-7309.

Stephanopoulos G. (1999) Metabolic fluxes and metabolic engineering. Metab. Eng. 1, 1-11.

Sticher L., Mauch-Mani B., Métraux J.P. (1997) Systemic acquired resistance, Annu. Rev. Phytopathol. 35, 235-270.

Stobiecki M., Kachlicki P. (2005). Metabolomics and metabolite profiling – can we achieve the goal? Acta Physiol. Plant. 27, 109–116.

Strid A., Porra R.J. (1992) Alterations in pigment content in leaves of *Pisuvn sativum* after exposure to supplementary UV-B. Plant Cell Physiol. 33, 1015-1023.

Sullivan M.L. (2009) A Novel red clover hydroxycinnamoyl transferase has enzymatic activities consistent with a role in phaselic acid (2-*O*-[caffeoyl]-L-malate) biosynthesis. Plant Physiol._150, 1866–1879.

Sumner L.W., Mendes P., Dixon R.A. (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 62, 817–836.

Taylor L.P., Briggs W.R. (1990) Genetic regulation and photocontrol of anthocyanin accumulation in maize seedlings. Plant Cell 2, 115-127.

Taylor R.M., Tobin A.K., Bray C.M. (1997) DNA damage and repair in plants. *In* PJ Lumsden, ed, Plants and UV-B. Cambridge University Press, Cambridge, UK, 53-75.

Terzaghi W.B., Cashmore A.R. (1995). Light-regulated transcription. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46**,** 445–474.

Tevini M., Braun J., Fieser G. (1991) The protective function of the epidermal layer of rye seedlings against ultraviolet-B radiation. Photochem. Photobiol. 53, 329-333.

Thain S.C., Murtas G., Lynn J.R., McGrath R.B., Millar A.J. (2002) The circadian clock that controls gene expression in *Arabidopsis* is tissue specific. Plant Physiol. 130, 102–110.

Tian L., Wan S.B., Pan Q.H., Zheng Y.J., Huang W.D. (2008) A novel plastid localization of chalcone synthase in developing grape berry. Plant Sci. doi: 10.1016/j.plantsci.2008.03.012.

Tropf S., Kärcher B., Schröder G., Schröder J. (1995) Reaction mechanisms of homodimeric plant polyketide synthase (stilbenes and chalcone synthase). A single active site for the condensing reaction is sufficient for synthesis of

stilbenes, chalcones, and 6'-deoxychalcones. J. Biol. Chem. 270(14), 7922–7928.

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

Tsukaya H., Ohshima T., Naito S., Chino M., Komeda Y. (1991). Sugar-dependent expression of the *CHS-A* gene for chalcone synthase from petunia in transgenic *Arabidopsis*. Plant Physiol. 97, 1414-1421.

Tuteja J.H., Clough S.J., Chan W.C., Vodkin L.O. (2004) Tissue-specific gene silencing mediated by a naturally occurring chalcone synthase gene cluster in *Glycine max*. Plant Cell 16, 819–835.

Tzfira T., Citovsky V. (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr. Opin. Biotechnol. 17, 147–154.

Uknes S., Winter A.M., Delaney T., Vernooij B., Morse A., Friedrich L., Nye Potter G., Ward E., Ryals J. (1993) Biological induction of systemic acquired resistance in *Arabidopsis*, Mol. Plant-Microbe Interact. 6, 692-698.

Van der Krol A.R., Mur L.A., Beld M., MoI J.N.M., Stuitje A.R. (1990a). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2, 291-299.

Van der Krol A.R., Mur L.A., de Lange P., Gerats A.G.M., MOI J.N.M., Stuitje A.R. (1990b). Antisense chalcone synthase genes in petunia: Visualization of variable of transgene expression. MoI. Gen. Genet. 220, 204-212.

Van der Krol AR., Lenting P.E., Veenstra J., van der Meer I.M., Koes R.E., Gerats A.G.M., MOI, J.N.M., and Stuitje, A.R. (1988). An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature 333, 866-869.

Van der Meer I.M., Stuitje A.R., Mol J.N.M. (1993) Regulation of general phenylpropanoid and flavonoid gene expression. *In* Control of Plant Gene Expression. Edited by Verma, D.P.S. pp. 125–155. CRC Press, Boca Raton.

Van der Meer I.M., Spelt C.E., Mol J.N.M., Stuitje A.R (1990) Promoter analysis of the chalcone synthase (chsA) gene of Petunia hybrida: A 67 bp promoter region directs flower-specific expression. Plant Mol. Biol. 15, 95–109.

Van Etten H.D., Pueppke S.G. (1976) Isoflavonoid phytoalexins, pp. 239–289, *in* J. Friend and D.R. Threlfall (eds.). Biochemical Aspects of Plant-Parasite Relationships. Academic Press, New York.

Van Tunen A.J., Koes R.E., Spelt C.E., van der Krol A.R., Stuitje A.R., Mol J.N.M. (1988). Cloning of the two chalcone flavanone isomerase genes from Petunia *hybfida:* Coordinate, light-regulated, and differential expression of flavonoid genes. EMBO J. 7, 1257-1263.

Vaucheret H., Béclin C., Elmayan T., Feuerbach F., Godon C., Morel J.B., Mourrain P., Palauqui J.C., Vernhettes S. (1998) Transgene-induced gene silencing in plants. Plant J. 16, 651-659.

Veit M., Pauli G.F. (1999) Major flavonoids from *Arabidopsis thaliana* leaves. J. Nat. Prod. 62, 1301–1303.

Verhoeyen M.E., Bovy A., Collins G., Muir S., Robinson S., de Vos C.H.R., Colliver S. (2002) Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. J. Exp. Bot. 53, 2099–2106.

Verpoorte R., Choi Y.H., Kim H.K. (2007) NMR-based metabolomics at work in phytochemistry, Phytochem. Rev. 6, 3-14.

Verpoorte R., Van der Heijden R., Memelink J. (2000) Engineering the plant cell factory for secondary metabolite production. Transgenic Res. 9, 323-343.

Vleeshouwers V.G.A.A., van Dooijeweert W., Govers F., Kamoun S., Colon L.T. (2000) Does basal *PR* gene expression in *Solanum* species contribute to non-specific resistance to *Phytophthora infestans*?, Physiol. Mol. Plant Pathol. 57, 35-42.

Von Roepenack L.E., Degenkolb T., Zerjeski M., Franz M., Roth U., Wessjohann L., Schmidt J., Scheel D., Clemens S. (2004) Profiling of Arabidopsis secondary metabolites by capillary liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry. Plant Physiol 134, 548–559.

Wade H.K., Bibikova T.N., Valentine W.J., Jenkins G.I. (2001) Interactions within a network of phytochrome, cryptochrome and UV-B phototransduction pathways regulate chalcone synthase gene expression in Arabidopsis leaf tissue. Plant J. 25, 675–685.

Wagner C., Sefkow M., Kopka J. (2003) Construction and application of a mass spectral and retention time index database generated from plant GC/EI–TOF–MS metabolite profiles. Phytochemistry 62, 887–900.

Wang C.K., Chen P.Y., Wang H.M., To K.Y. (2006) Cosuppression of tobacco chalcone synthase using *Petunia* chalcone synthase construct results in white flowers. Botanical Studies 47, 71-82.

Wasternack C., Parthier B. (1997) Jasmonate-signalled plant gene expression. Trends Plant Sci. 2, 302–307.

Wegulo S. N., Yang X. B., Martinson C. A., Murphy P. A. (2005) Effects of wounding and inoculation with *Sclerotinia sclerotiorum* on isoflavones concentrations in soybean. Canadian J. plant sci. 85, 749-760.

Weiergang I., Hipskind J.D., Nicholson R.L. (1996) Synthesis of 3-deoxyanthocyanidin phytoalexins in sorghum occurs independent of light. Physiol. Mol. Plant Pathol. 49, 377-388.

Weisshaar B., G . Jenkins I . (1998) . Phenylpropanoid biosynthesis and its regulation. Curr . Opin . Plant Biol . 1, 251-257.

Weisshaar B., Armstrong G.A., Block A., da Costa e Silva O., Hahlbrock K. (1991) Light inducible and constitutively expressed DNAbinding proteins recognising a plant promoter element with functional relevance in light responsiveness. EMBO J. 10, 1777-1786.

Wendehenne D., Durner J., Chen Z., Klessig D. F. (1998) Benzothiadiazole, an inducer of plant defenses, inhibits catalase and ascorbate peroxidase. Phytochemistry 47, 651-657.

Whitehead J.M., Dixon R.A. (1983) Chalcone synthase from cell suspension cultures of *Phaseolus vulgaris*. Biochem. Biophys. Acta. 747, 298-303.

Williams M.E., Foster R., Chua N.H. (1992) Sequences flanking the hexameric G-box core CACGTG affects the specificity of protein binding. Plant Cell 4, 485-496.

Winer J., Jung C.K.S., Shackel I., Williams P.M. (1999) Development and validation of real-time quantitative reverse transcriptase–polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro, Anal. Biochem. 270, 41-49.

Winer J., Jung C.K.S., Shackel I., Williams P.M. (1999) Development and Validation of Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction for Monitoring Gene Expression in Cardiac Myocytes in Vitro. Anal. Biochem. 270, 41-49.

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

Winkel S.B. (2004) Metabolic channeling in plants. Annu. Rev. Plant Biol. 55, 85 –107.

Winkel S.B. (2002) Biosynthesis of flavonoids and effects of stress. Curr. Opinion in Plant Biol. 5 (3), 218-223.

Winkel S.B. (2001) Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology and bio technology. Plant Physiol. 126, 485–493.

Winkel S.B. (1999) Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. Physiol. Plant 107, 142–149.

Wollenweber E., Dietz V.H. (1981) Occurrence and distribution of free flavonoid aglycones in plants. Phytochemistry 20, 869-932.

Wrolstad R.E. (2000) Anthocyanins. In: F.J. Francis and G.J. Lauro, Editors, Natural Food Colorants, Marcel Dekker, New York pp. 237–252.

Xu B.W., Wild J.R., Kenerley C.M. (1996) Enhanced expression of a bacterial gene for pesticide degradation in a common soil fungus. J. Ferment. Bioeng. 81, 473-481.

Xu Y., Chang P.F.L., Liu D., Narasimhan M.L., Raghothama K.G., Hasegawa P.M., Bressan R.A. (1994) Plant defence genes are synergistically induced by ethylene and methyl jasmonate. Plant cell 6, 1077-1085.

Yamaguchi T., Kurosaki F .F., Suh D . D. Y ., Sankawa U ., Nishioka M ., Akiyama T ., Shibuya, M ., Ebizuka Y . (1999) Cross-reaction of chalcone synthase and stilbene synthase overexpressed in *E . coli* . FEBS Lett . 460, 457-461.

Yang W.C., Canter C.H.C. J., Hogendijk P., Katinakis P., Wijffelman C.A., Franssen H., van Kammen A., Bisseling T. (1992) *In-situ* localization of chalcone synthase mRNA in pea root nodule development. Plant J. 2, 143-152.

Yilmaz Y., R. T. Toledo (2004) Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. J. Agric. Food Chem. 52, 255–260.

Yoshida K., Wakamatsu S., Sakuta M. (2008) Characterization of SBZ1, a soybean bZIP protein that binds to the chalcone synthase gene promoter. *Plant* Biotechnol. 25, 131–140.

Yu L.M., Lamb C.J., Dixon R.A. (1993) Purification and biochemical characterization of proteins which bind to the H-box *cis*-element implicated in transcriptional activation of plant defense genes. Plant J. 3, 805–816.

Yuan J.S., Burris J., Stewart N.R. (2007) Mentewab A., Stewart C.N. Jr., Statistical tools for transgene copy number estimation based on real-time PCR. BMC Bioinformatics, 8(7), S6.

Zabala G., Zou J.J., Tuteja J., Gonzalez D.O., Clough S.J., Vodkin L.O. (2006) Transcriptome changes in the phenylpropanoid pathway of Glycine max in response to Pseudomonas syringae infection. BMC Plant Biol. 6: 26.

Zeier J., Delledonne M., Mishima T., Severi E., Sonoda M., Lamb C. (2004) Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions. Plant Physiol. 136, 2875–2886.

Zhou B., Li Y., Xu Z., Yan H., Homma S. and Kawabata S. (2007) Ultraviolet A-specific induction of anthocyanin biosynthesis in the swollen hypocotyls of turnip (*Brassica rapa*). J. Exp. Bot. doi:10.1093/jxb/erm036

Zhu Q., Dröge L.W., Dixon R.A., Lamb C. (1996) Transcriptional activation of plant defense genes. Curr. Opin. Genet. Dev. 6, 624–630.

Zufall R., Rausher M.D. (2001) Diffuse coevolution and anthocyanin production. Botany 2001: "Plants and People", Albuquerque, New Mexico.

Zuurbier K.W.M., Lesser J., Berger T., Hofte A.J.P., Schröder G., Verpoorte R., Schröder J. (1998) 4-hydroxy-2- pyrone formation by chalcone synthase and stylbene synthase with nonphysiological substrates. Phytochemistry 49,1945-1951.

Zuurbier K.W.M., Fung S.Y., Scheffer J.J.C., Verpoorte R. (1995) Formation of aromatic intermediates in the biosynthesis of bitter acids in *Humulus lupulus*. Phytochemistry 38, 77-82.

Zuurbier K.W.M., Fung S.Y., Scheffer J.J.C., Verpoorte R. (1993) Assay of chalcone synthase activity by high-performance liquid chromatography. Phytochemistry 34, 1225-1229.

Zwaagstra M.E., Timmerman H., Tamura M., Tohma T., Wada Y., Onogi K., Zhang M.Q. (1997) Synthesis and Structure-Activity Relationships of Carboxylated Chalcones: A Novel Series of $CysLT_1$ (LTD_4) Receptor Antagonists. J. Med. Chem. 40, 1075-1089.

Curriculum vitae

The author of this thesis, Thi Thanh Hien Dao (30/01/1976), was born and grew up in Hanoi, Vietnam - the country of rice fields. In 1992, she completed her higher education from the Hanoi-Amsterdam high school, Hanoi, Vietnam and continued her studies at the Hanoi University of Pharmacy. She obtained her pharmacist diploma in 1997 and master degree in 2000 at the same university. From 2000 to 2004 she worked as lecturer in the Traditional Pharmacy Department at Hanoi University of Pharmacy. In 2004 she started her PhD program at the Division of Pharmacognosy, Section Metabolomics, Institute of Biology Leiden with a fellowship of the Vietnamese Oversea Scholarship Program. In 2009 she completed her PhD project under supervision of Prof. Dr. Rob Verpoorte.

List of publications

1. Thi Thanh Hien Dao, Roberto Chacon Puig, Hye Kyong Kim, Cornelis Erkelens, Alfons W.M. Lefeber, Huub J.M. Linthorst, Young Hae Choi, Robert Verpoorte**,** **Effect of benzothiadiazole on the metabolome of *Arabidopsis thaliana*.** Plant Physiology and Biochemistry, Volume 47, Issue 2, February 2009, Pages 146-152.
2. Thi Thanh Hien Dao, Huub J.M. Linthorst, Robert Verpoorte**, Chalcone synthase and its functions in plant resistance: A review.** Phytochemistry reviews (submitted)
3. Thi Thanh Hien Dao, Huub J.M. Linthorst, Hye Kyong Kim,Young Hae Choi, Robert Verpoorte**, The metabolic changes in *Arabidopsis thaliana* under UV/blue light** (in preperation)

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