Metabolic changes in Arabidopsis thaliana plants overexperssing chalcone synthase

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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.

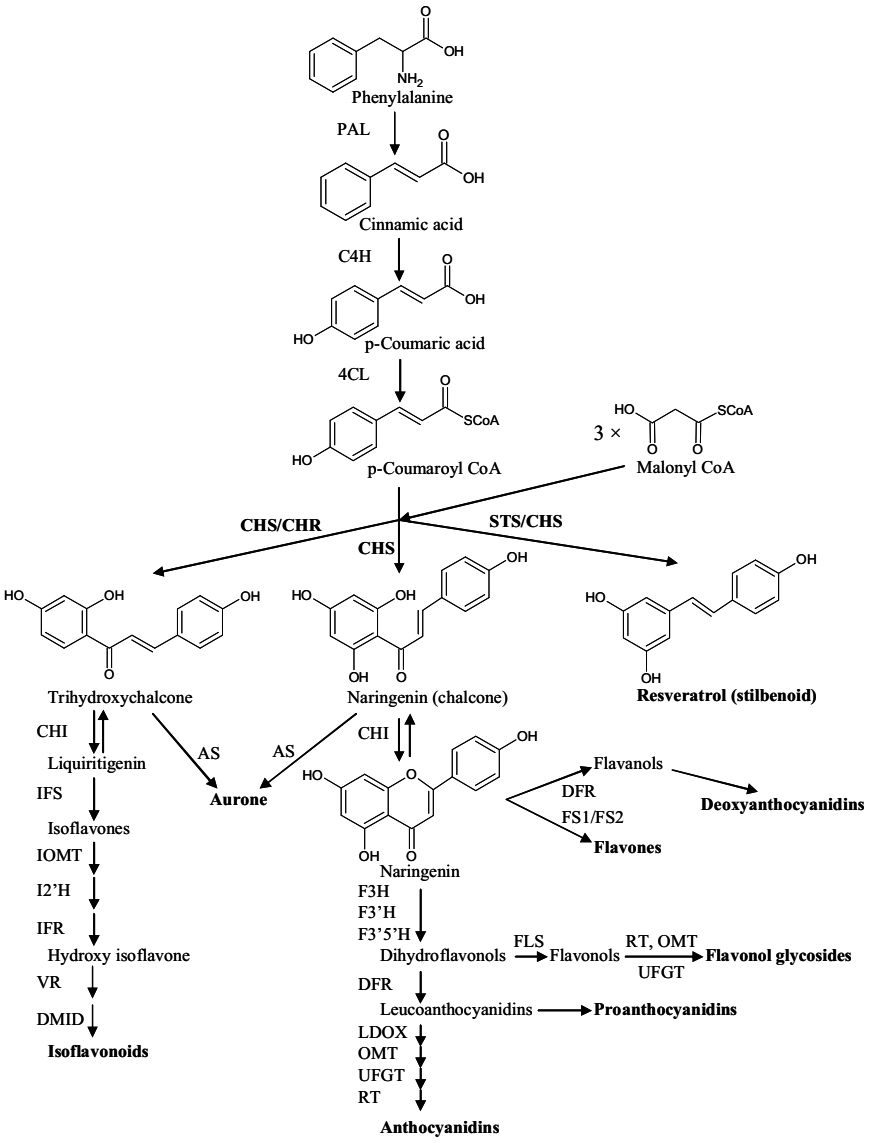

Figure 2.1. Flavonoid biosynthetic pathway

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 (*2S*)-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.**

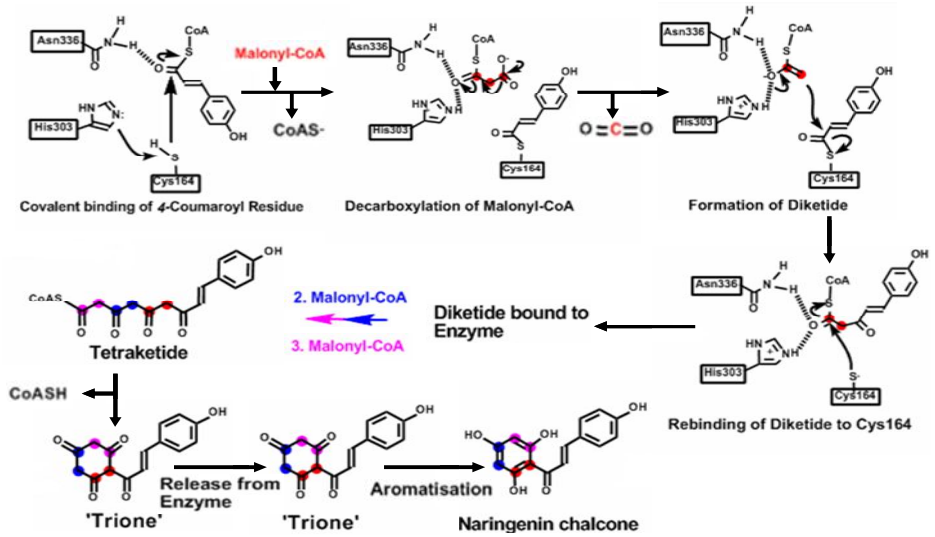

Figure 2.2. Reaction Mechanism of Chalcone Synthase (CHS)
In CHS, three amino acids play key roles in the catalytic functions of type III PKS: Cys164: active site, covalent binding site of starter residues and intermediates, His303 and Asn336: stabilization/activation of both starter (e.g. 4-coumarate) 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

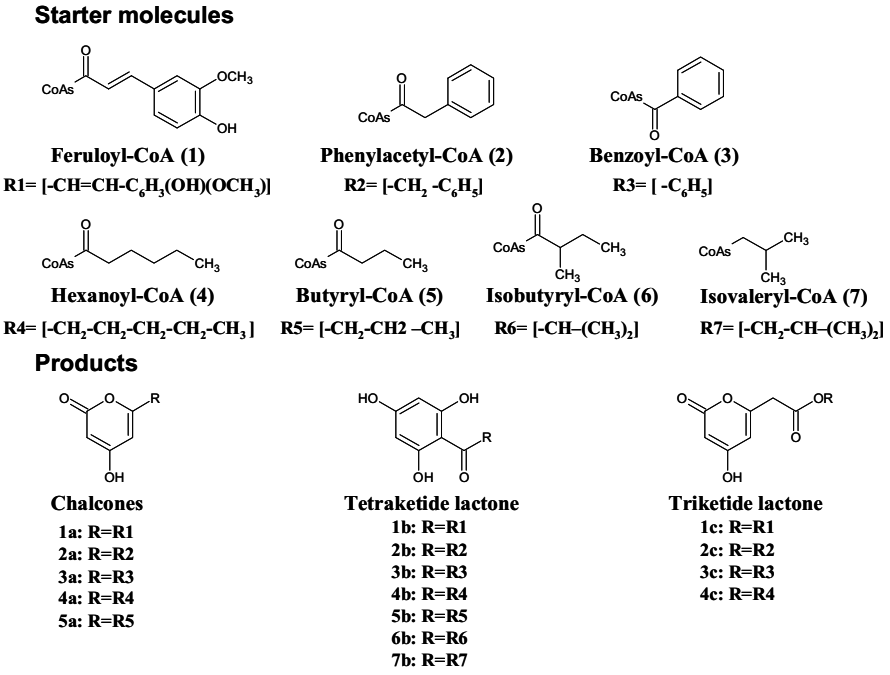

Figure 2.3. Alternate starter molecules and their predicted reaction products catalyzed by CHS

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].

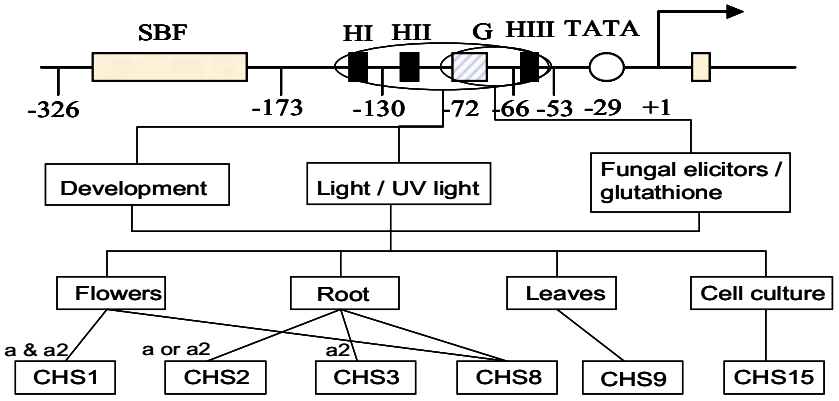

Figure 2.4. Bean *CHS15* promoter and regulators

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* $CuCl_2$ 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.