

**DEVELOPMENT OF ANTIBODIES FOR CHARACTERIZING THE
ARABIDOPSIS FLAVONOID BIOSYNTHETIC PATHWAY**

by

Cody Christopher Cain

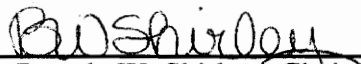
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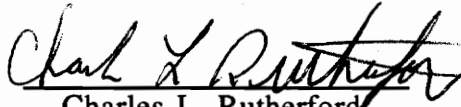
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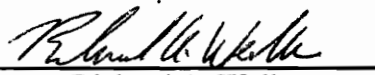
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(ABSTRACT)

Polyclonal antibodies against the first two enzymes of the *Arabidopsis thaliana* flavonoid biosynthetic pathway were developed using conventional and phage antibody technology. cDNAs from *Arabidopsis* coding regions of chalcone synthase (CHS) and chalcone isomerase (CHI) were sub-cloned in-frame into a bacterial expression vector as fusions with glutathione S-transferase (GST) using standard directional cloning techniques. Analysis of crude extracts of *Escherichia coli* containing GST-CHS or GST-CHI fusion protein indicated that the cells expressed equivalent amounts per volume of culture. CHS and CHI were purified to near homogeneity, yielding approximately 100 micrograms of GST-CHS and 1 milligram of GST-CHI per liter of culture. The purified fusion proteins were injected into chickens and polyclonal IgY's were purified from egg yolk. Accumulation of CHS and CHI, as well as products of the pathway, were compared during the first eight days of *Arabidopsis* development. CHS and CHI are sequentially induced and reach maximal accumulation levels by day 5. Anthocyanidin levels are offset by one day, reaching maximal levels at day 6. The fusion proteins were also used to screen a phage-display library for Fab' fragments that recognize CHS and CHI epitopes. Preliminary data indicated that enrichment of phage displaying

antibodies against CHS and CHI was successful. Phage-derived antibodies against CHS and CHI provide valuable tools for future experiments addressing Western blot analysis, immunolocalization experiments, and disruption of the flavonoid biosynthetic pathway by introduction of the corresponding genes into transgenic *Arabidopsis* plants.

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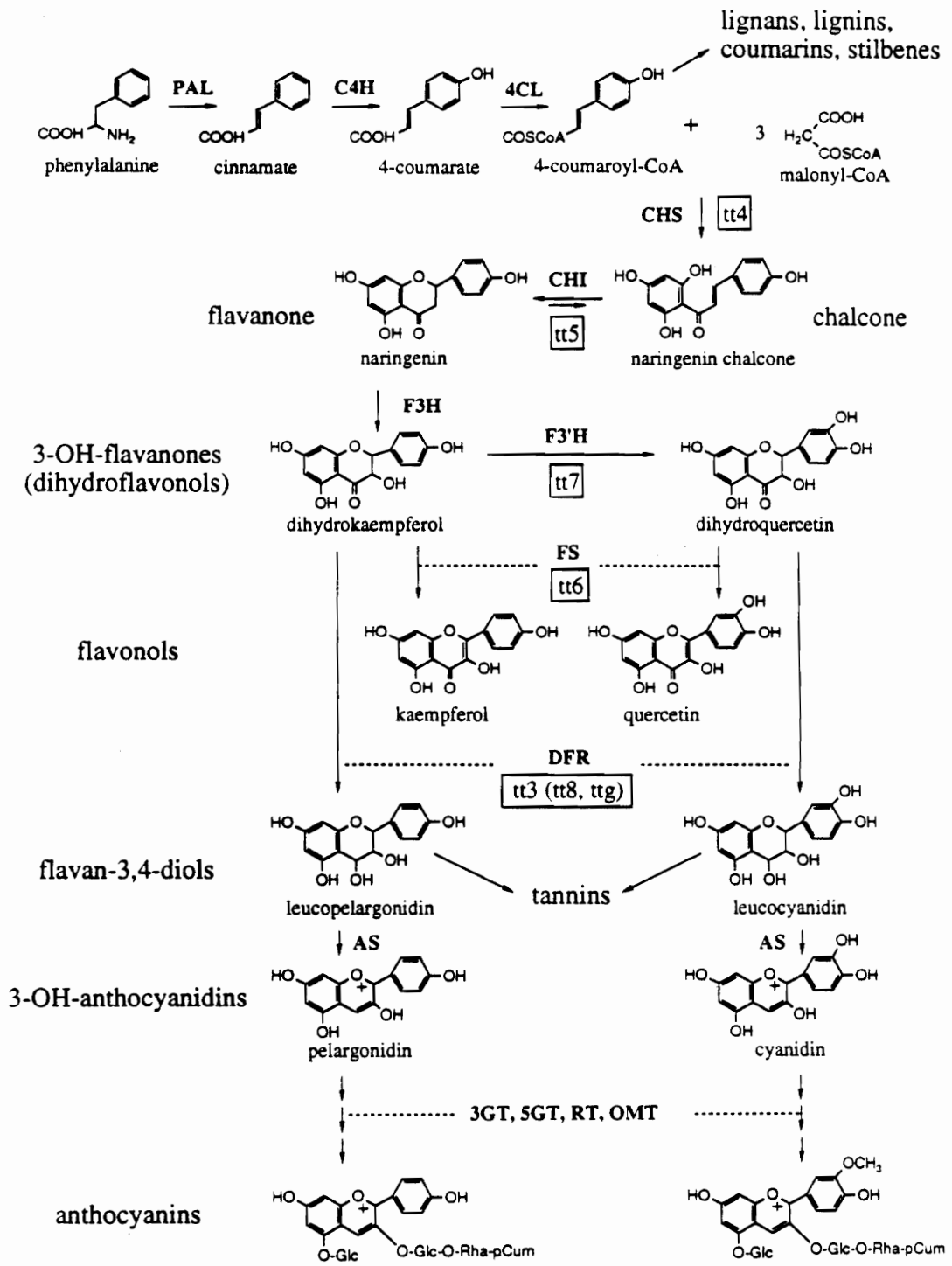
Chapter 1

Literature Review

The long-term goal of our research is to characterize the role of protein-protein interactions in regulating and facilitating cellular metabolism in multienzyme complexes. To this end we have chosen the flavonoid biosynthetic pathway (Figure 1) of *Arabidopsis thaliana* to serve as our model system. A wealth of genetic, biochemical, physiological, and molecular knowledge of flavonoid biosynthesis provides the fundamental foundation for our investigations of metabolism. Flavonoids are an important group of plant secondary metabolites that carry out many diverse functions in the plant kingdom (Stafford, 1990). Flavonoid-deficient strains of *Arabidopsis* have been isolated on the basis of altered seed coat color (Koornneef, 1990), and provide invaluable tools for probing the relationships between enzymes of this pathway. My focus on this project has dealt with the study of accumulation patterns of end products of flavonoid metabolism, more specifically anthocyanins, and of the first two committed enzymes for flavonoid biosynthesis in *Arabidopsis*, chalcone synthase (CHS) and chalcone isomerase (CHI).

Flavonoids carry out a diverse spectrum of functions in higher plants including pigmentation of leaves, seeds, fruits, and flowers, signaling in plant-microbe interactions, protection from ultraviolet radiation protection, and fertility (reviewed in Shirley, *et al.*, 1995). Pigmentation is important to the plant in two respects: 1) it

Figure 1 Schematic of the flavonoid biosynthetic pathway. Enzymatic steps affected in specific *transparent testa* mutants are indicated, with putative regulatory loci given in parenthesis. Adapted from Shirley, *et al.*, 1995.



attracts pollinators such as birds or insects, and 2) attracts seed dispersers; animals that consume the plant's fruits will deposit the seeds elsewhere. Most flower pigments are red, blue, and purple anthocyanins, or yellow aurones and chalcones. In addition, many plants accumulate colorless flavonones and flavonols that can alter petal pigmentation by interaction with metal ions or anthocyanins through a process known as co-pigmentation (reviewed in Koes, *et al.*, 1994).

While pigmentation is a visual signal to pollinators, flavonoids have been implicated as chemical signals for mediating plant-microbe interactions. Symbiotic and pathogenic plant-microbe interactions involve differential expression of flavonoid compounds. Isoflavonoids, flavans, flavanones, 3-hydroxyflavonones, and flavonols all have anti-fungal phytoalexin properties (Stafford, 1990), while the flavanols, kaempferol and quercetin, and the *Rhizobium nod* genes actually induce *Agrobacterium vir* genes, providing host-microbe specificity (Zerback et al., 1989).

Flavonoids provide plants protection against ultraviolet radiation. Plants require sunlight to carry out photosynthetic reactions. Elaborate mechanisms for trapping light have evolved that capture specific wavelengths of light to power reactions leading up to the Calvin cycle, and ultimately carbohydrate production from carbon dioxide and water. However, all wavelengths of light are not beneficial to plants. Ultraviolet radiation is known to cause chromosomal alterations such as inversions and deletions,

which if not repaired can cause severe, if not lethal, mutagenesis (Shirley et al., 1992). Protection against ultraviolet radiation is essential for plant systems that require prolonged exposure to direct sunlight. Two basic strategies are available to minimize DNA damage: DNA repair and shielding to minimize DNA damage. Flavonoids may play a critical role in shielding plants from ultraviolet radiation. Anthocyanins and flavonols, in particular, have partially overlapping absorption spectra across a broad range of the ultraviolet spectrum (Stapleton and Walbot, 1994). While expression of flavonoid genes in response to ultraviolet radiation has been shown in a variety of monocots and dicots alike, (Kreuzaler et al., 1983; Schmelzer et al., 1988; Koes et al., 1989; Feinbaum et al., 1991; Kubasek et al., 1992) the role of flavonoid protection against ultraviolet light has only been demonstrated in *Arabidopsis* (Li et al., 1993) and maize (Stapleton and Walbot, 1994). Recently, it has been demonstrated that flavonoids are necessary for male fertility in maize and petunia (Mo et al., 1992; Taylor and Jorgensen, 1992) Chalcone synthase mutants of maize and petunia have dysfunctional pollen (Mo et al., 1992). Subsequent complementation studies with these mutants showed that pollen function can be restored after crosses with wild-type plants. The enormous evolutionary distance that separates maize (monocot) from petunia (dicot) might suggest that this is a universal requirement for male fertility in higher plants. However, molecular genetic evidence from *Arabidopsis* (Burbulis, Iacobucci, and Shirley, unpublished data)

suggests that *Arabidopsis* exhibits flavonoid-independent male fertility.

The fertility of flavonoid-deficient lines of *Arabidopsis* make it ideal for molecular and genetic studies of flavonoid biosynthesis. Many plant model systems, monocots and dicots alike, have been employed to study flavonoid biosynthesis. Most if not all of the initial research was carried out in *Petroselinum hortense* (parsley), *Zea mays* (maize), *Petunia hybrida* (petunia), and *Antirrhinum majus* (snapdragon). Other systems that have been utilized include *Avena sativa* (oat), *Callistephus chinensis* (florists' aster), *Cicer arietinum* (garbanzo bean), *Cosmos sulphureus*, *Daucus carota* (carrot), *Dianthus carophyllus* (carnation), *Fagopyrum esculentum* (buckwheat), *Glycine max* (soybean), *Helichrysum bracteatum* (everlastings), *Hordeum vulgare* (barley), *Ipomea platensis* (morning glory), *Matthiola incana* (common stock), *Medicago sativa* (alfalfa), *Phaseolus vulgaris* (bean), *Picea excelsa* (a gymnosperm), *Pisum sativum* (pea), *Secale cereale* (rye), *Sinapis alba* (mustard), *Spinacia oleraceae* (spinach), *Tulipa* cv., *Verbena hybrida*, and *Arabidopsis thaliana*.

Arabidopsis thaliana has many useful characteristics that make it ideal for the study of flavonoid biosynthesis. *Arabidopsis* is a relatively small higher plant with a genome size of 1×10^8 base pairs, only about five times that of yeast (*Sacchromyces cerevisiae*). *Arabidopsis* grows well under artificial conditions, and goes from seed to seed in about 8 weeks. A single plant will produce up to ten

thousand seeds. Seedlings can be germinated and grown on agar plates for sterile tissue culture. *Agrobacterium*-mediated transformation has proven successful for *Arabidopsis*, and a new *in planta* method that avoids tissue-culture has recently been developed (Bechtold et al., 1993). *Arabidopsis* is easily crossed and mutations in flavonoid genes do not seem to affect fertility, as has been demonstrated in petunia and maize (Taylor and Jorgensen, 1992). Mutations in the flavonoid biosynthetic genes in *Arabidopsis* have been extensively characterized at the DNA, RNA, and end-product levels (reviewed in Shirley, et al., , 1995) and a collection of flavonoid-deficient mutants of *Arabidopsis* are available to further characterize the pathway.

Flavonoid-deficient mutants of *Arabidopsis* were first identified based on altered seed color by Bürger in 1971. Since then, eleven loci have been reported that are responsible for flavonoid biosynthesis in *Arabidopsis* (Koornneef, 1990). They are collectively named transparent testa (*tt*) because plants carrying mutations at these loci lack pigments in the brown testa, or seed coat, which allow visualization of the underlying yellow cotyledons (Koornneef, 1981). The identities of the loci for 5 structural and 2 regulatory genes have been determined. *tt4* and *tt5* correspond to the loci for CHS and CHI, respectively (Shirley et al., 1995). Mutants at *tt4* (W85 and 2YY6) and *tt5* (W86), have been well characterized at the DNA and RNA levels (Feinbaum et al., 1991; Shirley et al., 1992; Burbulis et al., In preparation).

A *tt4* CHS mutant, W85, was first characterized at the DNA level by Feinbaum and Ausubel in 1988 (Figure 2). This mutant was generated by soaking Landsberg ecotype seeds in ethylene methyl sulfonate (EMS), a potent mutagen (Koornneef, 1990). At amino acid 323 in CHS, a glycine to serine transition occurs due to a single G to A transition at the DNA level (Feinbaum and Ausubel, 1988). This single point mutation is enough to stop production of anthocyanins in seeds, seedlings, and leaves (Shirley et al., 1995). However, wild-type levels of CHS protein have been detected in Western blots with polyclonal antibody against *Arabidopsis* CHS (Cain and Shirley, unpublished data). Whether this mutation affects the active site, substrate binding domains, or disrupts protein-protein interaction with other flavonoid enzymes is unknown.

Another allele of *tt4* generated by EMS mutation, 2YY6, has also been characterized at the protein level (Cain and Shirley, unpublished data). The 2YY6 allele contains a single G to A transition at the 3' splice site of the single intron in chalcone synthase (Shirley et al., 1995) (Figure 2). This mutation is the result of a shifting of the normal splice site by one base pair, and translation of a premature stop codon terminating translation of CHS message. It has been shown that there is no correctly-spliced CHS mRNA in 2YY6 (Burbulis et al., In preparation). The product of this aberrant splicing would be approximately 9 kDa if translated, as compared to 43 kDa for wild-type CHS deduced from sequence. To date, no 9 kDa or wild-type sizes of CHS protein have been detected

Figure 2 Sequence of CHS genes in wild-type plants and two *tt4* alleles.

The CHS nucleotide sequence in ecotype Landsberg *erecta* (Feinbaum and Ausubel, 1988) is given , with the two nucleotide differences in ecotype Columbia (open box) and the A to G transitions in each of the two *tt4* alleles (black boxes) shown above. The arrow indicates the transcription start site for this gene (Feinbaum and Ausubel, 1988) and the shaded circle identifies the active-site cysteine (Lanz *et al.*, 1991). From Shirley, *et al.*, 1995.

GTTTGTATATAAGCTCTCACTCTCCGGTATATTTCCAAATACACCTAACTTGTTTAGTA
 CACAACAGCAACATCAAACCTCTAATA^AACCCAAGTTGGTGATACTATA ATGGTGATGGC
 M V M A
 TGGTGCTTCTCTTTGGATGAGATCAGACAGGCTCAGAGAGCTGATGGACCTGCAGGCAT
 G A S S L D E I R Q A Q R A D G P A G I
 CTTGGCTATTGGCACTGCTAACCCCTGAGAACCATGTGCTTCAGGCGGAGTATCCTGACTA
 L A I G T A N P E N H V L Q A E Y P D Y
 CTACTCCGCATACCAACAGTGAACACATGACCCACCTCAAGGAGAAGTTCAGCGCAT
 Y F R I T N S E H M T D L K E K P K R M
 GT GTACGTCTTATTAACCTTCTACTTTCATTTCCTTTGGCATATATCTTCATTCACATAGT
 C
 TTAGCTAACAAAGTATTTACTATTTCAG^AGGACAAAGTGGACAATTCGGAAACGTCACATG
 D K S T I R K R H M
 CATCTGACGGAGGAATTCTCTCAAGGAAAACCCACACATGTGTGCTTACATGGCTCCTTCT
 H L T E E F L K E N P H M C A Y M A P S
 CTGGACACCAGACAGGACATCGTGGTGGTGGAAAGTCCCTAAGCTAGGCAAAGAAGCGGCA
 L D T R Q D I V V V E V P K L G K E A A
 GTGAAGGCCATCAAGGAGTGGGGCCAGCCCAAGTCAAAGATCACTCATGTCTCTTCTGCG
 V K A I K E W G Q P K S K I T H V V P C
 ACTACCTCCGGCGTCGACATGGCTGGTGGCTGACTACCAGCTCACCAAGCTTCTTGGTCTC
 T T S G V D M P G A D Y Q L T K L L G L
 CGTCTTCGGTCAAGCGTCTCATGATGTACCAGCAAGGTTGCTTCGCCGGCGGTACTGTG
 R P S V K R L M M Y Q Q G P A G G T V
 CTCGGTATCGCTAAGGATCTCGCCGAGAACA^ACGTGGAGCACGTCTCTCGTTGTCTGCG
 L R I A K D L A E N N R G A R V L V V C
 TCTGAGATCACAGCCGTTACCTTCCGTTGGTCCCTCTGACACCCACCTTGACTCCCTCGTC
 S E I T A V T F R G P S D T H L D S L V
 GGTACGGCTCTTTTCAAGTATGGCGCCCGCCACTCATTGTGGGGTGGGACCCCTGACACA
 G Q A L F S D G A A A L I V G S D P D T
 TCTGTCCGGAGAGAAACCCATCTTTGAGATGGTGTCTGCCGCTCAGACCATCCTTCCAGAC
 S V G E K P I F E M V S A A Q T I L P D
 TCTGATGGTGCCATAGACGGACATTTGAGGGAAGT^AGGTCTCACCTTCCATCTCCTCAAG
 S D G A I D G H L R E V G L T P H L L K
 GATGTTCCCGGCCATCTCCAAGAACATTTGTGAAGAGTCTAGACGAAGCGTTTAAACCT
 D V P G L I S K M I V K S L D E A F K P
 TTGGGGATAAGTGAAGTCCCTCTTCTGGATAGCCCAACCCCTGGAGGTCCAGCGCATC
 L G I S D W N S L F W I A H P G G P A I
 CTAGACCAGGTGGAGATAAAGCTAGGACTAAAGGAAGAGAAGATGAGGGCGACACGTGAC
 L D Q V E I K L G L K E E K M R A T R H
 GTGTTGAGCGAGTATGGAACATGTGAGCCCGTGGTCTCTCTCATACTAGACGAGATG
 V L S E Y G N M S S A C V L F I L D E M
 AGGAGGAAGTCAAGTAAAGGATGGTCTGGCCACGACAGGAGAAGGGTGGAGTGGGGTGTG
 R R K S A K D G V A T T G E G L E W G V
 TTGTTTGGTTTCCGACAGGTTCTCACTGTTGAGACAGTGGTCTTGCACAGCGTTCCTCTC
 L F G F G P G L T V E T V V L H S V P L
 TAA ACAGAAGCGTTCCCTTCTATCTCCCTACCTACCTAGCCAAAACCTTAACTCTGTCTTATG

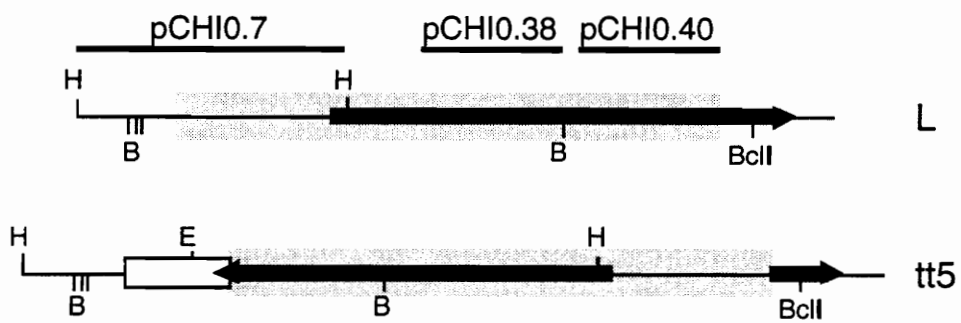
in 2YY6 with polyclonal CHS antiserum (Cain and Shirley, unpublished data) *tt4* (2YY6) contains no detectable levels of anthocyanins in seeds, seedlings, or leaves (Shirley et al., 1995).

The only identified *tt5* mutant to date, W86, was first characterized at the DNA level by Shirley et al., in 1992. W86 was isolated from a Landsberg population mutagenized using fast neutron radiation (Koorneef, 1990). This mutation is a result an inversion of the gene. The inversion removes 40 c-terminal amino acid residues and part of the promoter (Figure 3) (Shirley et al., 1992). W86 has reduced levels of CHI mRNA as compared to wild-type (Shirley et al., 1992), and has no detectable wild-type size CHI protein (Cain and Shirley, unpublished data). *tt5* (W86), like *tt4* (W85 and 2YY6), contains no detectable levels of anthocyanins in seeds, seedlings, or leaves (Shirley et al., 1995).

Studies involving levels and localizations of proteins necessarily require antibodies directed against them. Antibodies against CHS have been generated for buckwheat (Hrazdina et al., 1986), maize (Pollak et al., 1993), parsley (Kreuzaler et al., 1983), and rye (Peters et al., 1988), while CHI antisera have only been generated against petunia (van Tunen et al., 1988) and bean proteins (Robbins and Dixon, 1984). All of these antibodies are polyclonal, with the exception of rye, for which monoclonal antibodies have also been generated. CHS seems to be highly conserved, both structurally and functionally, in many plant species (Stafford, 1990). Cross-reactivity of anti-CHS antibodies to CHS from multiple species has

Figure 3 Structure of the *tt5* locus.

Maps of the CHI gene in Landsberg (wild-type) and *tt5* plants derived from restriction mapping and sequencing. Open boxes correspond to the four CHI exons (I to IV). Horizontal arrows indicate the locations of the CHI transcripts. Abbreviations: H - HindIII, B - BamHI, E - EcoRI. From Shirley, *et al.*, 1992.



been observed. The anti-CHS from maize will cross-react with CHS from petunia (Pollak et al., 1993), and rye CHS monoclonal recognizes rye, pea, maize, spinach, parsley, and oat CHS enzymes (Peters et al., 1988). The first anti-CHS antibody, generated in parsley, cross reacts with *Matthiola incana* (Rall and Hemleben, 1984), petunia (Koes et al., 1986), and pea (Harker et al., 1990). The anti-CHI antibody against petunia protein does not cross react with bean (Blyden et al., 1991). No anti-CHS or -CHI antibodies have been generated using *Arabidopsis* proteins, and none of the above mentioned antibodies has ever been published as having cross-reactivity with *Arabidopsis* CHS or CHI.

In order to generate antibodies, sufficient quantities of the antigen (protein) must be purified in order to elicit an immune response. Endogenous enzyme purifications are costly and labor intensive, but many enzymes, prokaryotic and eukaryotic, can be cheaply and easily generated using prokaryotic expression systems (Smith and Johnson, 1988).

There are many prokaryotic vectors for controlled expression of gene products, and most, if not all, rely on fusion proteins as a method for purification. In the majority of cases, fusion proteins are soluble in aqueous solutions and can be purified from crude bacterial lysates under non-denaturing conditions (Smith and Johnson, 1988). The expressed protein's fusion partner either has high affinity for particular metals, engineered peptide sites recognized by monoclonal antibodies, or affinity for other non-

metallic compounds that are immobilized in chromatography columns or on beads. Glutathione S-transferase (GST) is routinely used in these expression systems as a fusion partner to assist in the purification of the desired gene product. GSTs are enzymes that detoxify xenobiotics by covalently linking the substrates to glutathione (Marrs et al., 1995). The affinity of GST for glutathione provides the basis of purification of GST-fusion proteins. The GST gene in the vector, pGEX-KT, encodes a 26-kDa protein and is derived from *Schistosoma japonicum*, a parasitic helminth (Smith and Johnson, 1988). However, GST and GST-like proteins have been identified in a number of systems (Simons and Jagt, 1977), including *Arabidopsis*. The pGEX-KG vector allows for single step purification and cleavage of fusion protein. This vector contains a glycine linker region between GST and the multiple cloning site, which enhances the ability of thrombin to cleave the fusion partner away from GST, which remains attached to the glutathione-beaded agarose (Guan and Dixon, 1991).

Convincing evidence is accumulating in the literature for the existence of consecutively-assembled, membrane-associated enzyme complexes (Hrazdina and Wagner, 1985). A multienzyme complex is defined as an aggregate of different, functionally related enzymes bound together by non-covalent forces into a highly organized structure (Ginsburg and Stadtman, 1970). Definitive evidence for specific protein-protein interactions in many systems such as those mediating DNA, purine, pyrimidine, and protein

biosynthesis, glycolysis, the tri-carboxylic acid cycle, and the urea cycle has been published (Srere, 1987). Unlike components of protein and nucleic acid biosynthetic machinery that have strong interactions, less processive pathways such as glycolysis are characterized by "looser" complexes that are more difficult to detect and isolate (Mathews, 1993). However, substantial evidence for the existence of multienzyme complexes suggests that their interactions have a fundamental role in overall reaction processes (Srivastava and Bernhard, 1986).

Traditional enzymological studies that focus primarily on the catalytic activity of individual enzymes are almost always performed under conditions that are far from physiological (Sumegi, 1991). Intracellular protein concentrations are far higher than the concentrations at which enzymes are usually studied. Weak protein associations that might maintain organized intracellular structure *in vivo* could well disappear at the far lower protein concentrations *in vitro* (Mathews, 1993). Effects of enzyme-enzyme interactions are not evident at nanomolar concentrations. At the high concentrations of proteins *in vivo*, enzyme-enzyme interactions are far more likely (Srivastava and Bernhard, 1986).

The cytoplasm is not simply a highly concentrated protein solution bathing a set of organelles, but rather a diverse, organized network of protein channels, substrates, and support media (Mathews, 1993). In a number of cases it has been demonstrated that pools of exogenously-supplied precursors do not mix with pools

of endogenous precursors. Such compartmentalization of intermediates in metabolic pathways can best be described by channeling (Hrazdina and Jensen, 1992).

Channeling involves the preferential transfer of an intermediate substrate from one enzyme to a physically adjacent enzyme, with restricted diffusion into the surrounding environment (Mathews, 1993). Two types of experiments have been used to establish evidence for channeling: (i) the inability of metabolic intermediates to compete with precursors in the formation of final product, and (ii) the inability of a specific enzyme to use metabolic precursors synthesized by one of a diverse pair of enzyme pathways (Srivastava and Bernhard, 1986).

There are several explanations that favor the channeling hypothesis over random interactions. Channeling could serve to protect unstable or scarce metabolites by maintaining them in a protein-bound state (Mathews, 1993). Second, channeling could provide a metabolic advantage by maintaining concentration gradients. Moreover, if sequential enzymes are involved in channeling, then effective control of the pathway can be exerted by regulation of one or two key enzymes, and these need not be the first enzymes in a sequence.

Immunocytochemical and enzymological evidence indicates that flavonoid synthesis is catalyzed by a multienzyme complex loosely associated with the endoplasmic reticulum (Hrazdina et al., 1987; Schmelzer et al., 1988). Flavonoid pathways have shown signs

of channeling (Hrazdina, 1988), the best documented example is the formation of *p*-coumaric acid on microsomal membranes from potato discs (Czichi and H., 1975). Synthesis of flavonoids appears to take place on a complex consisting of membrane-associated, consecutively-assembled enzymes. The easy solubilization of CHS and CHI, and the difficulty encountered in demonstrating channeling beyond cinnamate 4-hydroxylase (Figure 1) suggests that the enzyme complex is on the cytoplasmic face of the endoplasmic reticulum membrane, and is held there, and held together, by weak forces (Hrazdina, 1988). Recent evidence for sequestering of flavonoid products in vacuoles has been demonstrated in maize. The *Bronze-2* gene of maize, which catalyzes the last genetically defined step in anthocyanin biosynthesis, encodes a GST-like enzyme that is responsible for anthocyanin import into vacuoles (Marrs et al., 1995).

This thesis describes a series of experiments initiated for the purposes of generating antibodies towards CHS and CHI, using conventional and phage-display technologies. With these antibodies, we can begin to look at sequential induction patterns at the protein level, and effects of mutations on other enzymes in the pathway. These antibodies provide the necessary tools that can finally take full advantage of the *tt* mutants to examine effects on subcellular localization of flavonoid biosynthetic enzymes in *Arabidopsis*.

References

- Bechtold, N., Ellis, J., and Pelletier, G.** (1993) *In Planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris, **316**, 1194-1199.
- Blyden, E.R., Doerner, P.W., Lamb, C.J., and Dixon, R.A.** (1991) Sequence analysis of a chalcone isomerase cDNA of *Phaseolus vulgaris* L. Plant Mol. Biol., **16**, 167-169.
- Burbulis, I.E., Iacobucci, M., and Shirley, B.W.** (In preparation) Molecular analysis of the *Arabidopsis tt4 (2YY6)* allele: evidence for flavonoid-independent male fertility. Plant Cell .
- Czichi, A.J., and H., K.** (1975) Formation of *p*-coumaric acid and *o*-coumaric acid from L-phenylalanine by microsomal membrane fractions from potato: evidence of membrane bound enzyme complexes. Planta, **125**, 115-125.
- Feinbaum, R.L., and Ausubel, F.M.** (1988) Transcriptional Regulation of The *Arabidopsis thaliana* Chalcone Synthase Gene. Mol. Cell. Biol., **8**, 1985-1992.
- Feinbaum, R.L., Storz, G., and 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.
- Ginsburg, A., and Stadtman, E.R.** (1970) Multienzyme systems. Ann. Rev. Biochem., **39**, 429-472.
- Guan, K., and Dixon, J.E.** (1991) Eukaryotic Proteins Expressed in *Escherichia coli*: An Improved Thrombin Cleavage and Purification

Procedure of Fusion Proteins with Glutathione S-Transferase. *Anal. Biochem.*, **192**, 262-267.

Harker, C.L., Ellis, T.H.N., and Coen, E.S. (1990) Identification and Genetic Regulation of the Chalcone Synthase Multigene Family in Pea. *Plant Cell*, **2**, 185-194.

Hrazdina, G. (1988) Compartmentation in Aromatic Metabolism. In *Phenolic Metabolism in Plants*, 26, (H.A. Stafford and R.K. Ibrahim, eds.). New York: Plenum Press, pp. 1-23.

Hrazdina, G., and Jensen, R.A. (1992) Spatial organization of enzymes in plant metabolic pathways. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **43**, 241-267.

Hrazdina, G., Lifson, E., and Weeden, N.F. (1986) Isolation and Characterization of Buckwheat (*Fagopyrum esculentum* M.) Chalcone Synthase and Its Polyclonal Antibodies. *Arch. Biochem. Biophys.*, **247**, 414-419.

Hrazdina, G., and Wagner, G.J. (1985) Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. *Arch. Biochem. Biophys.*, **237**, 88-100.

Hrazdina, G., Zobel, A.M., and Hoch, H.C. (1987) Biochemical, immunological and immunocytochemical evidence for the association of chalcone synthase with endoplasmic reticulum membranes. *Proc. Natl. Acad. Sci. USA*, **84**, 8966-70.

Koes, R.E., Quattrocchio, F., and Mol, J.N.M. (1994) The Flavonoid Biosynthetic Pathway in Plants: Function and Evolution. *Bioessays*, **16**, 123-132.

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

Koornneef, M. (1981) The Complex Syndrome of TTG Mutants. *Arabidopsis Info. Serv.*, **18**, 45-51.

Koornneef, M. (1990) Mutations affecting the testa color in *Arabidopsis*. *Arabidopsis Inf. Serv.*, **28**, 1-4.

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

Kubasek, W.L., Shirley, B.W., Mckillop, A., Goodman, H.M., Briggs, W., and Ausubel, F.M. (1992) Regulation of Flavonoid Biosynthetic Genes in Germinating *Arabidopsis* Seedlings. *Plant Cell*, **4**, 1229-1236.

Li, J., Ou-Lee, T.-M., Raba, R., Amundson, R.G., and Last, R.L. (1993) *Arabidopsis* Flavonoid Mutants Are Hypersensitive to UV-B Irradiation. *Plant Cell*, **5**, 171-179.

Marrs, K.A., Alfenito, M.R., Lloyd, A.M., and Walbot, V. (1995) A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. *Nature*, **375**, 397-400.

Mathews, C.K. (1993) The Cell---Bag of Enzymes or Network of Channels? *Bacteriology*, **175**, 6377-6381.

Mo, Y., Nagel, C., and Taylor, L.P. (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc. Natl. Acad. Sci. USA*, **89**, 7213-7217.

Peters, A., Schneider-Poetsch, H.A.W., Schwarz, H., and Weissenböck, G. (1988) Biochemical and Immunological Characterization of Chalcone Synthase from Rye Leaves. *J. Plant Phys.*, **133**, 178-182.

Pollak, P.E., Vogt, T., Mo, Y., and Taylor, L.P. (1993) Chalcone Synthase and Flavonol Accumulation in Stigmas and Anthers of *Petunia hybrida*. *Plant Phys.*, **102**, 925-032.

Rall, S., and Hemleben, V. (1984) Characterization and expression of chalcone synthase in different genotypes of *Matthiola incana* R. Br. during flower development. *Plant Mol. Biol.*, **3**, 137-145.

Robbins, M.P., and Dixon, R.A. (1984) Induction of chalcone isomerase in elicitor-treated bean cells. Comparison of rates of synthesis and appearance of immunodetectable enzyme. *Eur. J. Biochem.*, **145**, 195-202.

Schmelzer, E., Jahnen, W., and 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.

Shirley, B.W. (In preparation) Flavonoid biosynthesis: "new" functions for an "old" pathway. *Trends Biochem. Sci.*

- Shirley, B.W., Hanley, S., and Goodman, H.M.** (1992) Effects of ionizing radiation on a plant genome: analysis of two *Arabidopsis transparent testa* mutations. *Plant Cell*, **4**, 333-347.
- Shirley, B.W., Kubasek, W.L., Storz, G., Bruggemann, E., Koorneef, M., Ausubel, F.M., and Goodman, H.M.** (1995) Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J.*, **8**, 659-671.
- Simons, P.C., and Jagt, D.L.V.** (1977) Purification of Glutathione S-Transferases from Human Liver by Glutathione Affinity Chromatography. *Anal. Biochem.*, **82**, 334-341.
- Smith, D.B., and Johnson, K.S.** (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*, **67**, 31-40.
- Srere, P.A.** (1987) Complexes of sequential metabolic enzymes. *Ann. Rev. Biochem.*, **56**, 89-124.
- Srivastava, D.K., and Bernhard, S.A.** (1986) Metabolite Transfer via Enzyme-Enzyme Complexes. *Science*, **234**, 1081-1086.
- Stafford, H.A.** (1990) *Flavonoid Metabolism*, Boca Raton: CRC Press.
- Stapleton, A.E., and Walbot, V.** (1994) Flavonoids Can Protect Maize DNA from the Induction of Ultraviolet Radiation Damage. *Plant Phys.*, **105**, 881-889.
- Sumegi, B., Porpaczy Z., and I. Alkonyi** (1991) Kinetic advantage of the interaction between the fatty acid B-oxidation enzymes and the complexes of the respiratory chain. *Biochem. Biophys. Acta*, **1081**, 121-128.

Taylor, L.P., and Jorgensen, R. (1992) Conditional Male Fertility in Chalcone Synthase-Deficient Petunia. J. Heredity, 83, 11-17.

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

Zerback, R., Dreesler, K., and Hess, D. (1989) Flavonoids induction of *Agrobacterium Vir* genes. Plant Science, 62, 83-91.

Chapter 2

Developmental Expression of Chalcone Synthase and Chalcone Isomerase Proteins in *Arabidopsis thaliana*

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Summary

Arabidopsis thaliana cDNA for chalcone synthase (CHS) and chalcone isomerase (CHI) were independently sub-cloned into pGEX-KG, a GST-fusion prokaryotic expression vector. Recombinant protein expression in *Escherichia coli* DH5-alpha reached maximal levels at four hours after induction with IPTG. Polyclonal chicken IgY was generated against purified GST-CHS and GST-CHI. Antibodies raised against the recombinant proteins were used to determine the developmental expression and accumulation patterns of endogenous CHS and CHI in *Arabidopsis* seedlings. Proteins of predicted molecular weights for CHS (43 kDa from sequence) and CHI (26 kDa from sequence) were detected in 3-day-old seedlings of *Arabidopsis* using anti-CHS and anti-CHI IgY, respectively. mRNA levels are known to peak at day 3, however, both proteins reached maximal

levels around day 5. Anthocyanidin extractions from samples of tissue correlated to previously identified levels in *Arabidopsis*, which reach maximal levels a day after protein levels peak. These polyclonal antibodies are the first generated against *Arabidopsis* CHS and CHI, and provide powerful tools for investigations of the flavonoid biosynthetic pathway.

Key Words: *Arabidopsis* - Flavonoids - Chalcone synthase - Chalcone isomerase - Polyclonal antibodies

Introduction

Flavonoids are an important group of plant secondary metabolites that carry out a diverse spectrum of functions in higher plants. Flavonoid genes in a variety of higher plants have been shown to respond to environmental stimuli such as temperature, wounding, pathogens, and ultraviolet light (reviewed in Hahlbrock, *et al.*, 1989). All flavonoids are 15-carbon compounds with a cyclic ring structure that is derived from phenylalanine and acetyl CoA. Of the 12 known classes of flavonoids, the anthocyanins, flavones, and flavonols are the most abundant and widespread (Stafford, 1990). Anthocyanins are responsible for the red, purple and blue pigments found in leaves, seeds, and fruits that provide visual cues to potential pollinators and seed dispersers (reviewed in Koes, *et al.*, 1994). Ultraviolet light protection and male fertility in maize and petunia is attributable to flavonols (Mo *et al.*, 1992).

Chalcone synthase (CHS) and chalcone isomerase (CHI) are the first two enzymes in the flavonoid biosynthetic pathway, a branch of the general phenylpropanoid pathway in higher plants. CHS catalyzes the condensation of 4-coumaroyl-CoA and malonyl-CoA to produce naringenin chalcone which is, in turn, reversibly modified into naringenin by CHI (Hahlbrock, 1981).

Chalcone synthase is encoded by single copy gene in *Arabidopsis*, as well as in snapdragon, bean, and parsley. In contrast, maize, petunia, oat, and rye all have multiple copies of the

CHS gene (reviewed in Shirley, *et al.*, 1995a). Genes encoding CHI have been identified in *Zea mays*, *Phaseolus vulgaris*, *Pisum sativum*, *Petunia hybrida*, *Rutaceae*, *Glycine max*, and *Arabidopsis thaliana*. Only *Phaseolus* and *Arabidopsis* have demonstrated a single copy gene for CHI (Mehdy and Lamb, 1987; Shirley *et al.*, 1992). The single copy genes for CHS and CHI make *Arabidopsis* ideal for genetic studies of the flavonoid pathway.

Arabidopsis offers other advantages as a model system for this pathway; it has provided the first direct evidence for the role of flavonoids in ultraviolet light protection (Kubasek *et al.*, 1992), as well as evidence that flavonols are not essential for pollen germination in all species. Mutations in *Arabidopsis* CHS and CHI genes at corresponding *tt4* and *tt5* loci have been identified and characterized at the DNA, RNA, and end-product levels (Feinbaum and Ausubel, 1988; Kubasek *et al.*, 1992; Shirley *et al.*, 1992; Shirley *et al.*, 1995). Mutations at these loci result in easily scorable seed or flower phenotypes, and are non-lethal which can provide powerful tools for understanding the roles and functions of flavonoids in higher plants. CHS is differentially regulated by levels of high intensity, ultraviolet, and blue light (Feinbaum *et al.*, 1991; Kubasek *et al.*, 1992). CHI is also induced by high intensity light (Shirley *et al.*, 1992). In developing *Arabidopsis* seedlings, mRNA levels peak at day 3 for both CHS and CHI, and anthocyanidins reach maximal levels at day 5 (Kubasek *et al.*, 1992).

Despite what is known at the DNA, RNA, and end-product levels, little is known about expression levels of the pathway at the protein level. CHS and CHI proteins have been characterized in other plant species. Antibodies against CHS have been generated for buckwheat (Hrazdina et al., 1986), maize (Pollak et al., 1993), parsley (Kreuzaler et al., 1983), and rye (Peters et al., 1988), while CHI antibody has only been generated against petunia CHI (van Tunen et al., 1988) and bean (Robbins and Dixon, 1984). All of these antibodies are polyclonal, with exception of rye, for which monoclonal antibodies have also been generated. CHS seems to be highly conserved, both structurally and functionally, based on cross-reactivity of the polyclonal antibodies with CHS from many plant species (Stafford, 1990). Antisera against maize CHS will cross-react with CHS from petunia (Pollak et al., 1993). The first anti-CHS antibody, generated in parsley, cross-reacts with *Matthiola incana* (Rall and Hemleben, 1984), *Petunia hybrida* (Koes 1986), and *Pisum sativum* (Harker et al., 1990). The monoclonal antibody generated against rye CHS also recognizes pea, maize, spinach, parsley, and oat CHS enzymes (Peters et al., 1988).

CHI may not share the conservation that CHS does with other plant species. Antisera against *Petunia hybrida* CHI protein does not cross-react with *Phaseolus vulgaris* (Blyden et al., 1991), or *Arabidopsis* (Cain and Shirley, unpublished data). Until now, no antisera against CHS or CHI proteins from *Arabidopsis* have been

generated, and none of the above mentioned antibodies has ever been published as having cross-reactivity with *Arabidopsis* CHS or CHI.

The over-expression and purification of eukaryotic proteins can be greatly facilitated using prokaryotic expression systems. Eukaryotic proteins expressed as fusions with glutathione S-transferase (GST) have routinely been used for this purpose (Smith 1988). Many active and non-active plant enzymes have been cloned and purified in *Escherichia coli*, including those of *Arabidopsis* (Singh et al., 1991; Bernasconi et al., 1994; Dörmann et al., 1995). We have over-expressed and purified CHS-GST and CHI-GST fusion proteins in *E. coli* DH5-alpha and generated polyclonal antibodies against the fusion proteins using chickens as the host animal. These antibodies cross-react with endogenous CHS and CHI from *Arabidopsis*, and have provided insight into the expression and accumulation of CHS and CHI proteins during development and in flavonoid-deficient lines.

Materials and methods

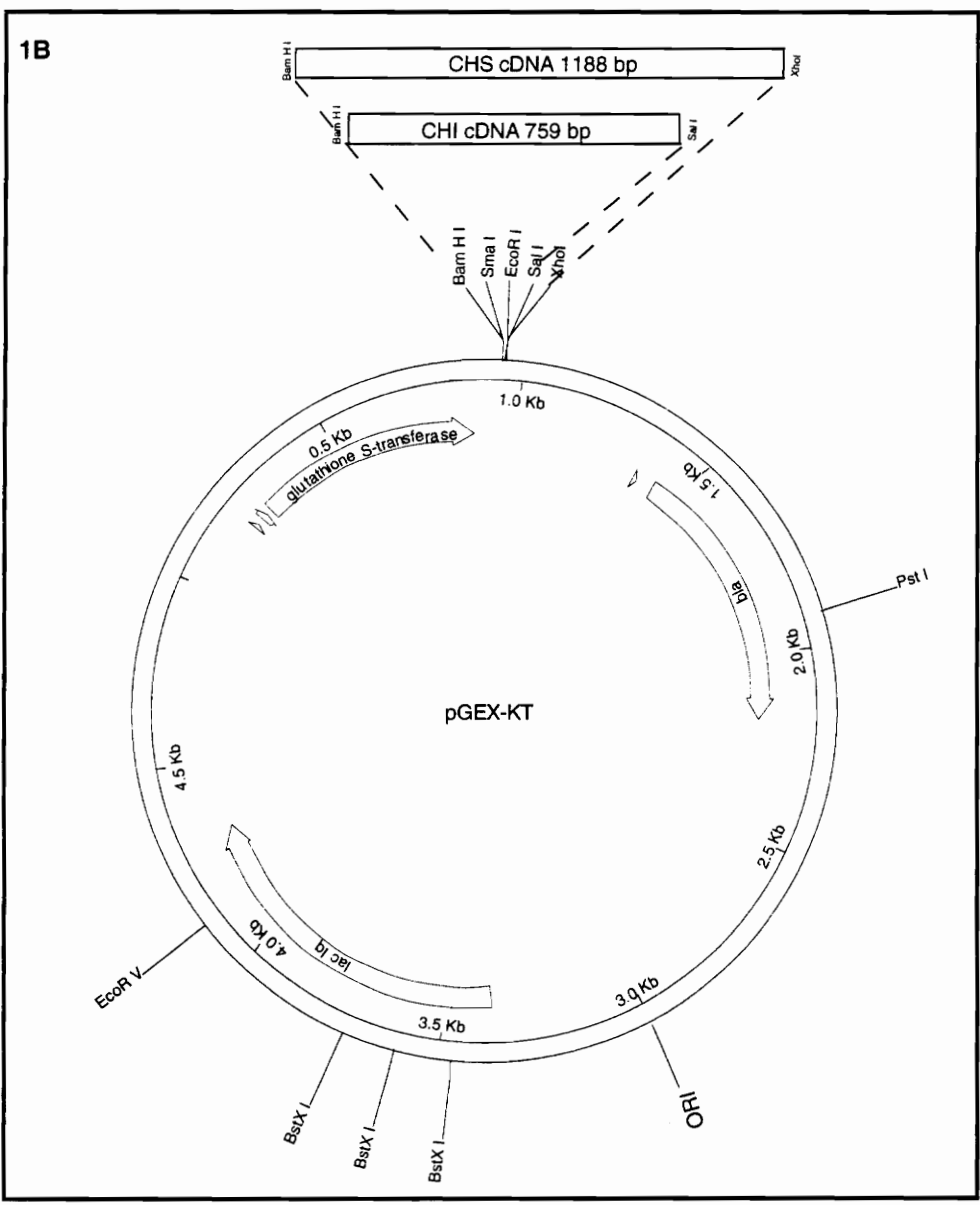
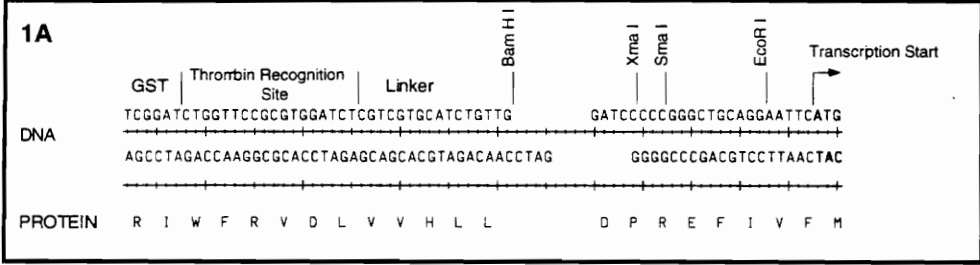
Over-expression and purification of enzymes

cDNA coding regions of CHS and CHI from *Arabidopsis* (Burbulis, Iacobucci, and Shirley, unpublished) were sub-cloned in-frame into the multiple coding site of the pGEX-KG vector (Pharmacia) (Figure 1) using directional cloning techniques (Sambrook et al., 1989). CHS was sub-cloned from a pBluescript vector as a BamHI-Sall fragment 1188 bp in length. CHI was sub-cloned from a pBluescript vector as a BamHI-XhoI fragment of 759 bp. One liter cultures of *E. coli* strain DH5-alpha (containing either pGEX, pGEX-CHS, or pGEX-CHI constructs) were grown in 2xYT media containing 100 µg/ml ampicillin (Sigma), at 37°C, 250 rpm, until OD₅₉₅=0.6-0.8 was achieved. GST-fusion protein expression was induced with 0.2 mM IPTG (Promega) at either room temperature or 37°C, shaking at 250 rpm.

To confirm induction, 500µl aliquots were removed at hourly intervals and centrifuged at 12,000 x *g* for 15 minutes at 4°C. The cell pellets were re-suspended in 1X sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol blue R-250), boiled for 10 minutes and centrifuged at 12,000 x *g* for 15 minutes at 4°C. For each time point, 7.5µl of the supernatant was separated on a 10% SDS-polyacrylamide gel. Gel electrophoresis was performed using a Mini-Protein II electrophoresis system from Bio-Rad. 10% NaDodSO₄-

Figure 1 Molecular cloning strategy for GST-CHS and GST-CHI fusion constructs.

A) Sequence showing in-frame cloning of 5'-end of cDNA (CHS or CHI) into the BamHI restriction site of pGEX-KT and the resulting amino acids. B) Restriction map of pGEX-KT showing insertion of cDNA sequences for CHS and CHI from *Arabidopsis*. Long arrows indicate gene products. Short arrows indicate inducible promoters. Abbreviations: bla - beta-lactamase, lac Iq - lactose repressor, ORI - origin of replication.



polyacrylamide gels, 7.25 x 10 cm, 0.75 mm thick, were run in a Tris-glycine buffer for 45 minutes at 200 volts (Laemmli, 1970). The gel was stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) in 5% methanol (Fisher) and 10% glacial acetic acid (Fisher). The gel was heated in a microwave for 30 seconds and then shaken slowly at room temperature for 5 minutes. The gel was destained using 5% methanol and 10% glacial acetic acid, heating for 40 seconds in a microwave, and then shaking at room temperature for 10 minutes.

For protein purification, the remaining 1 liter cultures were centrifuged at 4000 x *g* for 15 minutes at 4°C and the pellets re-suspended in 10 ml phosphate buffered saline containing 0.2 mM PMSF (Sigma) and 5 mM benzamidine (Sigma). The re-suspended cells were lysed by two passes through a French pressure cell (SLM - Aminco Instruments Inc., Urbana, IL) at 1,200 psi. Lysates were centrifuged at 12,000 x *g* at 4°C to remove insoluble material. The supernatants were incubated with 1 ml glutathione-beaded-agarose (Sigma) in 1 ml PBS at 4°C on a rotating shaker. The agarose beads were centrifuged at 4000 x *g*, 4°C and the supernatant discarded. The beads were washed 4 times in 10 ml PBS and finally re-suspended in 1 ml PBS. Ten microliters of beads were incubated with 10 units of human thrombin (Sigma) in 20 ml of cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% β-mercaptoethanol). Fusion protein was released from the beads by incubating the remaining supernatant with 1 ml PBS containing of 20

mM reduced glutathione (Gibco Life Technologies), 0.2 mM phenyl methyl sulfonyl fluoride (PMSF), and 5 mM benzamidine for 1 hour at 4°C on a rotating shaker. The data of over-expression and purification of GST-CHS and GST-CHI represent more than 10 independent experiments.

Antibody production and purification

Antibodies to GST-CHS and GST-CHI were generated in chickens by injection of purified fusion proteins (Cocalico Biologicals). Initially, 50 µg of CHS and 300 µg of CHI were subcutaneously injected into individual chickens. Two booster immunizations were performed before the egg yolk was recovered. Purification of chicken IgY's were performed per manufacturer's instructions using the Eggstract purification kit from Promega. Purified IgY's were re-suspended in PBST containing 0.5% sodium azide, aliquoted, and stored at -80°C.

Plant material

Seeds from *Arabidopsis thaliana* were sterilized for 1 minute in 100% isopropanol, 5 minutes in 50% bleach-0.05% Tween-20, and rinsed 4 times with sterile water. Seeds were re-suspended in sterile 0.05% agarose prior to plating (Kubasek et al., 1992). 20 mg of seeds were plated on agar medium containing Murishage and Skoog salts (Gibco Life Technologies) and 20% sucrose, pH 5.7. Plates were sealed with Transpore tape (3M), and incubated in darkness at 4°C

for 4 days. The plates were transferred to a growth chamber with continuous light ($120 \mu\text{E}\cdot\text{cm}^2\cdot\text{sec}^{-1}$) at 20°C . Tissue for protein and anthocyanidin extraction was harvested into liquid nitrogen, homogenized with a mortar and pestle, and stored at -80°C .

Crude protein extraction and Western Blotting

For crude soluble protein extracts, 0.5g of tissue (wet weight) was boiled in $250\mu\text{l}$ of 1X sample buffer (as described above but without glycerol or Bromophenol Blue R-250) for 10 minutes and the supernatant was collected after centrifugation at $12,000 \times g$, for 10 minutes at 4°C . $15\mu\text{l}$ of the centrifuged extract was separated on a 10% SDS-polyacrylamide gel and then transferred to 0.2 mm nitrocellulose (Bio-Rad) at 4°C for 30 minutes in a MiniProtein II electroblotter (Bio-Rad). Membranes were stained with 1X Ponceau S (Sigma) for 10 minutes at 25°C to check transfer efficiency. The membranes were blocked for 2h in PBS containing 0.5% Tween-20 (PBST) and either 10% goat serum (Gibco Life Technologies) or 5% Blotto at 25°C , incubated with anti-GST-CHS IgY (1:10), or anti-GST-CHI IgY (1:100) for 1h at 25°C , and then incubated with rabbit anti-chicken-IgY-alkaline phosphatase (1:3000) or horse-radish-peroxidase (1:4000) (Jackson Immunoresearch Laboratories) for 1h at 25°C . Bound antibodies were visualized using NBT and BCIP (Boehringer Mannheim) as alkaline phosphatase substrates or with the ECL detection kit (Amersham) as peroxidase substrates. Blots developed with ECL substrates were exposed to Kodak Biomax film

for 15-40 seconds. Results of Western blot experiments represent data from two independent experiments.

Anthocyanidin quantification

Flavonoids were extracted using a modification of the method of Rabino and Mancinelli, 1986. *Arabidopsis* tissue was homogenized in a mortar and pestle with liquid nitrogen, incubated in 1% HCl/methanol for 1 hour at 25°C, and back-extracted with chloroform to remove chlorophyll. Anthocyanidin concentrations are expressed as absorbance at 530 nm minus absorbance at 657 nm per mg wet tissue. Data from anthocyanidin spectrophotometry represent the results two independent experiments.

Results

Bacterial Over-expression

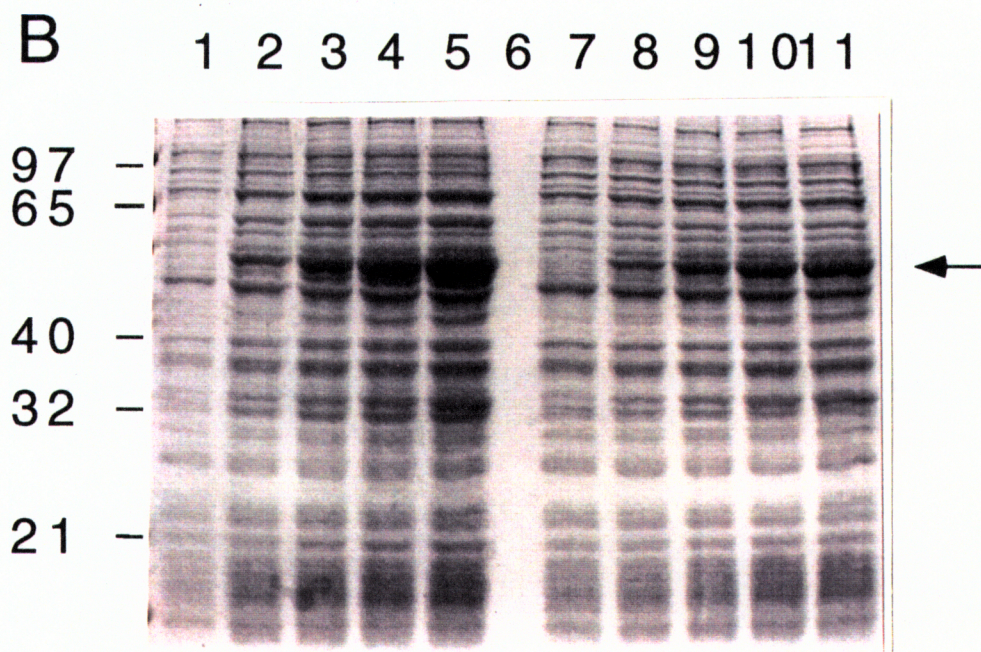
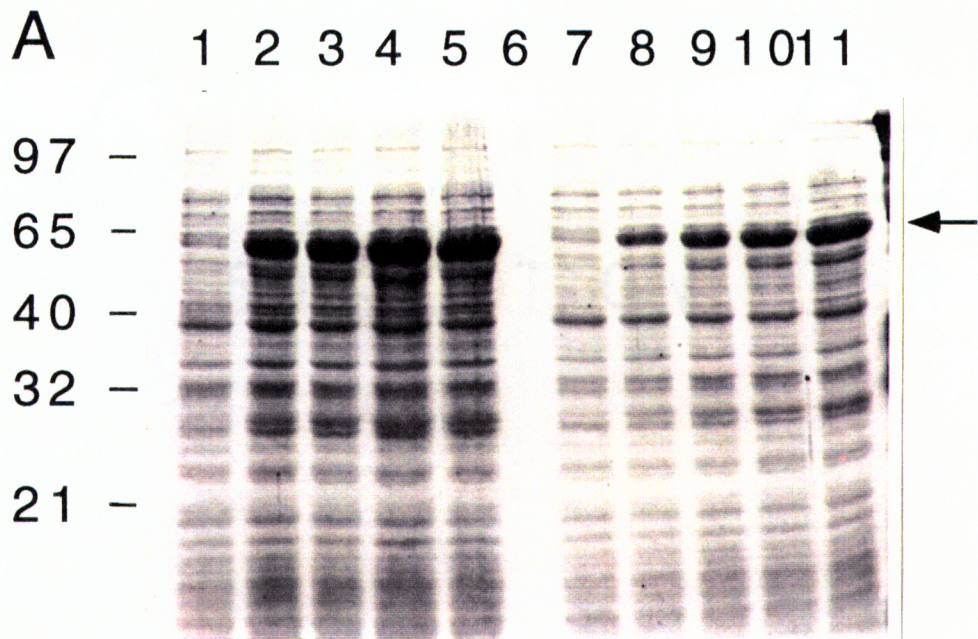
E. coli containing pGEX vectors modified to express either GST-CHS or GST-CHI were generated. Samples of crude bacterial lysates from cultures induced at 37°C or 25°C were taken at hourly time intervals and show induction of GST fusions (Figure 2) of the predicted molecular weights for GST-CHS (69 kDa) and GST-CHI (52 kDa). There is no detectable accumulation of fusion protein in cultures not treated with IPTG (data not shown). *E. coli* cells transformed with pGEX-CHS and pGEX-CHI show high levels of expression of the GST fusion proteins. At 37°C, maximum levels of expression were reached at 4 hours after inoculation (data not shown). There was significantly less fusion protein expressed at 25°C than at 37°C.

Purification of GST Fusion Proteins

GST-CHS and GST-CHI were purified to near-homogeneity as shown in Figure 2. Approximately 50 µg of GST-CHS was recovered from a liter of culture, while GST-CHI yielded approximately a milligram from the same volume of culture. Following cleavage by thrombin, no CHS peptide was detected, whereas GST-CHI yielded a 26 kDa band which corresponds to the molecular weights of both GST and CHI (data not shown).

Figure 2 GST-CHS and GST-CHI expression in *E. coli* at 37° and 25°C.

SDS-PAGE of crude lysates from *E. coli* (DH5-alpha) containing pGEX-KG fusion constructs of GST-CHS (A) and GST-CHI (B). (A) Arrow shows position of GST-CHS, which is of the predicted molecular weight (69 kDa) deduced from sequence. (B) Arrow indicates position of GST-CHI (54 kDa), which is also of the predicted molecular weight deduced from sequence. Lanes 1-5 contain extracts from cells induced at 37°C. Lanes 7-11 contain extracts obtained at 25°C. Lanes: Pre-induction (1 and 7); 1 hour post induction (PI) (2 and 8); 2 hours PI (3 and 9); 3 hours PI (4 and 10); 4 hours PI (5 and 11); lane 6 is blank.



Antibody Specificity

IgY's purified from immunized chickens showed antibody production against the fusion proteins (Figure 3), which cross-reacted with endogenous *Arabidopsis* proteins of molecular weights consistent with those predicted by deduced amino acid sequence for CHS and CHI. Null mutants for CHS (2YY6) and CHI (W86) were used as negative controls (aiding in identification of the correct proteins). Antibody titers were significantly lower for GST-CHS than for GST-CHI. Working concentration for anti-GST-CHS were 1:10, and anti-GST-CHI dilutions were optimal at 1:100. These mutants have been well characterized at the DNA level (Shirley et al., 1992; Burbulis et al., In preparation), and have been identified as having little or no correctly spliced mRNA in *tt4*, and reduced levels in *tt5* (Shirley et al., 1992). Consistent with this, no proteins of the correct molecular weight for CHS or CHI were detected in the CHS *tt4* (2YY6), or the CHI *tt5* (W86) mutants respectively. The anti-CHS and anti-CHI also cross-react with purified GST that was over-expressed and purified in *E. coli*, or purchased from Sigma (data not shown), confirming the specificity of the polyclonal antibody for the GST-fusion proteins.

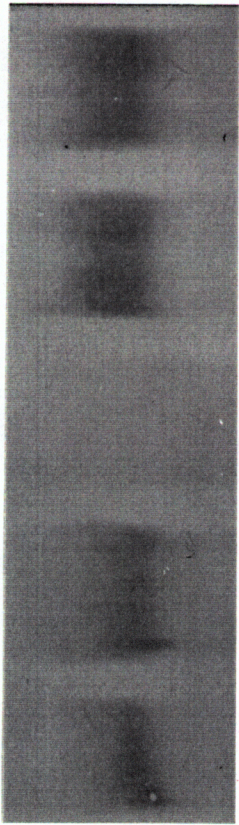
CHS, CHI, and Anthocyanidin Levels During Development

In order to measure anthocyanidin concentrations and CHS and CHI protein levels, duplicate samples of seedlings were grown under identical conditions for 8 days. Anthocyanidins were extracted and

Figure 3 Characterization of endogenous CHS and CHI proteins using polyclonal IgYs.

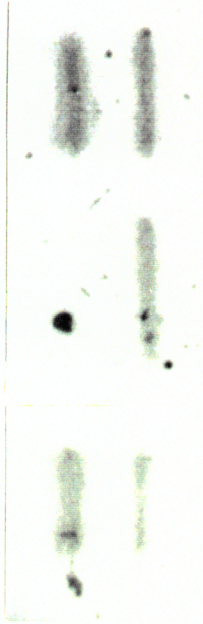
Western blots of crude extracts from 3 and 4-day-old *Arabidopsis thaliana* seedlings probed with polyclonal IgY against either CHS or CHI fusion protein were detected using BCIP/NBT alkaline phosphatase. Abbreviations: La - Landsberg (wild-type), 2YY6 and W85- CHS mutants, W86 - CHI mutant, Co - Columbia (wild-type). Arrow indicates position of CHS.

tt4 tt5 tt4 tt4
 La W85 W86 Co 2YY6



CHI

tt4 tt5
 La 2YY6 W86



CHS

quantified according to Rabino (Rabino and Mancinelli, 1986). Anthocyanidins reached maximal levels between day 5 and 6 (Figure 4) consistent with previously published data (Kubasek et al., 1992). As a control, anthocyanidins were extracted from 3-day-old Landsberg (wild-type) and *tt4* (W85) plants (Figure 5) to show differences between the detectable anthocyanidin concentrations in wild-type and flavonoid deficient mutants. As predicted, little or no anthocyanidins were observed in *tt4* (W85), while the Landsberg sample correlated with previous experiments.

Relative levels of CHS and CHI protein were determined using the polyclonal antibodies. Crude proteins extracts were prepared from frozen tissue stored at -80°C. Approximately 10 µg of crude total protein was separated by 10% SDS-PAGE. CHS and CHI protein levels are undetectable at day 2 (Figure 6), however, accumulation increased after day 3, peaking at day 5. Protein concentrations per milligram of tissue were estimated at 1 and 5 ng/mg of total protein for CHS and CHI, respectively, using the concentration of fusion protein loaded as a standard.

Figure 4 Quantitation of anthocyanidin levels in germinating *Arabidopsis* seedlings.

Anthocyanidins were extracted from seedlings as described in Methods. The data is representative of the results of two independent experiments. Relative levels of these pigments are given as A_{530} minus A_{657} .

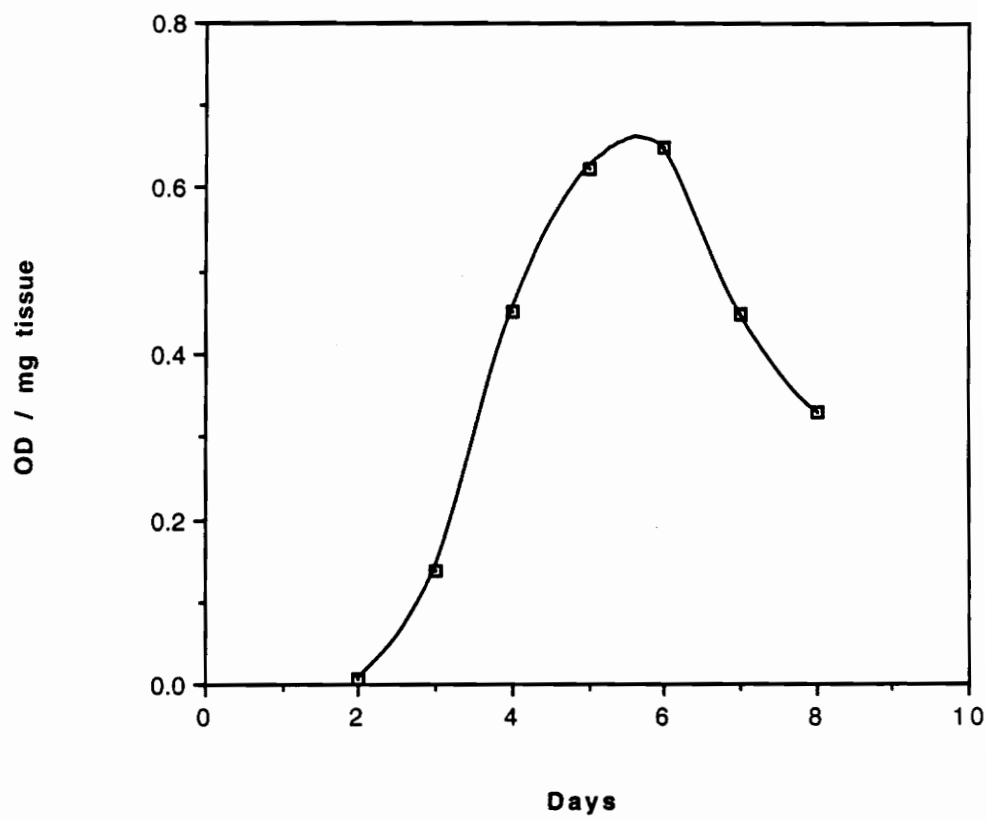


Figure 5 Anthocyanidin levels in wild type and *tt4* (W85) *Arabidopsis* seedlings.

Dark gray bars represent absorbances at 530 nm. Short black bars represent absorbances at 657. Light gray bars are differences between first two bars, representing levels of anthocyanidins extracted from 3-day-old seedlings.

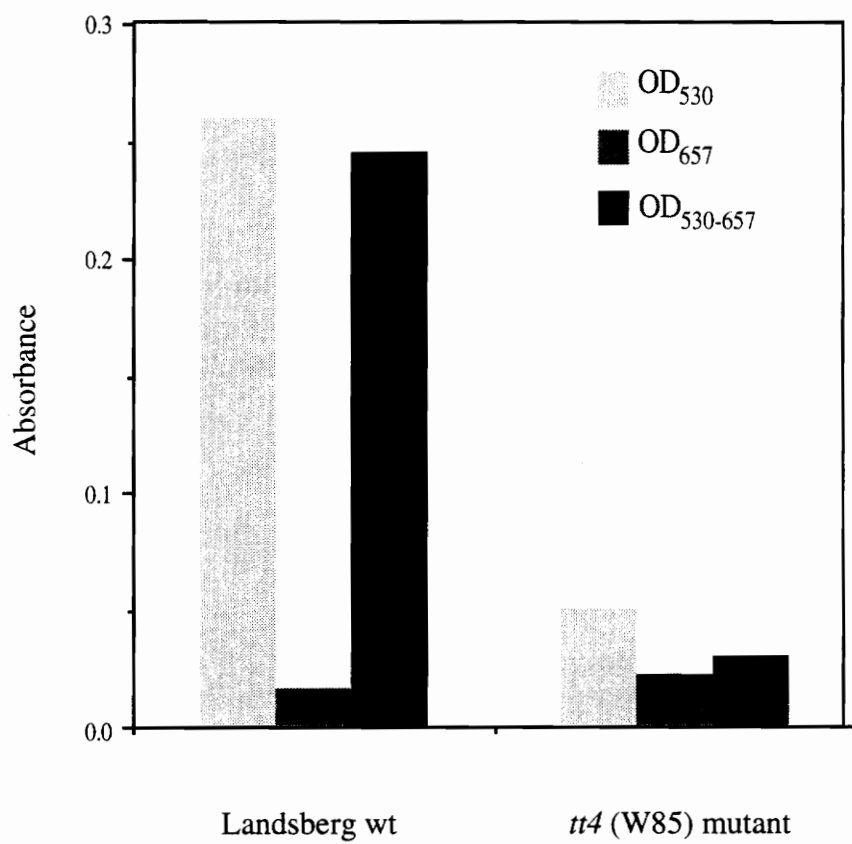
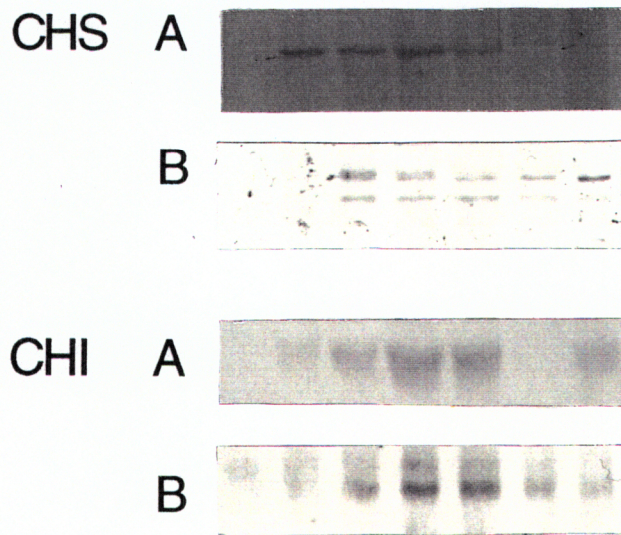


Figure 6 Levels of CHS and CHI protein during germination of *Arabidopsis* seedlings.

CHS (43 kDa) and CHI (26 kDa) proteins were detected in independent experiments (A and B) using the ECL detection kit (Amersham) on Western blots of crude extracts of *Arabidopsis thaliana* ecotype Landsberg erecta . 5 μ g of total protein were loaded in each lane. Molecular weight standards (Bio-Rad) were blotted for size estimations.

Days of Germination

2 3 4 5 6 7 8



Discussion

We have examined the accumulation of endogenous CHS and CHI proteins in developing *Arabidopsis* seedlings using antibodies generated against GST-CHS and GST-CHI fusion proteins. This analysis has shown that: (1) CHS and CHI proteins accumulate coordinately in *Arabidopsis*, (2) maximal protein accumulation does not coincide with maximal RNA or anthocyanidin levels, and (3) mutants missing one of the proteins have no accumulative effect on the other protein. Characterizing the CHS and CHI antibodies has also provided the first direct evidence that CHS and CHI proteins are absent in the *tt4* and *tt5* mutants of *Arabidopsis*, 2YY6 and W86, respectively.

The accumulation pattern of CHS and CHI proteins suggests that they are coordinately expressed in developing *Arabidopsis* and have maximal levels between days 5-6 under continuous light. This correlates with the findings that CHS activity is at its peak at day 4 in rye (Jähne et al., 1993), day 5 in primary oat leaves (Peter et al., 1991), and day 6 in maize (Jähne et al., 1993). In *Petunia hybrida* CHS protein accumulation patterns in stigmas and anthers are similar to those observed in *Arabidopsis* (Pollak 1993).

Protein accumulation does not directly correlate with the expression patterns seen at the RNA or anthocyanidin levels. Previous studies of RNA levels for CHS and CHI in developing *Arabidopsis* show they are coordinately expressed, reaching maximal levels by day 3, but are absent after day 4 (Kubasek et al., 1992),

while anthocyanidins accumulate maximally by day 5. This shows that there is a delay between transcription of the messenger RNAs and accumulation of the proteins.

The *transparent testa* mutations of *Arabidopsis* have been extensively studied at the DNA, RNA, intermediate- and end-product levels (Shirley et al., 1995), but nothing is known about the protein levels in these plants. It is known that *tt4* (2YY6) is incorrectly spliced, with low mRNA levels, and would result in a 9 kDa protein if translated, as compared to 43 kDa in wild-type CHS (Burbulis et al., In preparation). *tt4* (W85) has wild-type levels of mRNA and protein, which suggests that the glycine to serine transition has only affected function and not protein stability. W86 has low levels of mRNA, and the mature protein would be truncated by 40 amino acids (Shirley et al., 1992). Our work has demonstrated for the first time the complete absence of CHS and CHI protein from *tt4* (2YY6) and *tt5* (W86) respectively. We have also shown that *tt4* (W85) contains wild-type levels of immunoreactive CHS, although it is known that this mutant produces no flavonoids (Shirley et al., 1995).

The results presented here represent our current knowledge of the levels of expression of the flavonoid enzymes CHS and CHI in *Arabidopsis*. The polyclonal antibodies against CHS and CHI proteins described here provide us with specific probes for the elucidation of the possible feedback regulation of CHS on phenylalanine ammonia lyase (Loake et al., 1991) in *Arabidopsis*. In addition, they may provide the necessary immunocytochemical tools for future studies

on the effects of *transparent testa* mutations on sub-cellular localization and possible co-localization of these enzymes.

References

- Bernasconi, P., Walters, E.W., Woodworth, A.R., Siehl, D.L., Stone, T.E., and Subramanian, M.V. (1994)** Functional Expression of *Arabidopsis thaliana* Anthranilate Synthase Subunit I in *Escherichia coli*. *Plant Phys.*, **106**, 353-358.
- Blyden, E.R., Doerner, P.W., Lamb, C.J., and Dixon, R.A. (1991)** Sequence analysis of a chalcone isomerase cDNA of *Phaseolus vulgaris* L. *Plant Mol. Biol.*, **16**, 167-169.
- Burbulis, I.E., Iacobucci, M., and Shirley, B.W. (In preparation)** Molecular analysis of the *Arabidopsis tt4 (2YY6)* allele: evidence for flavonoid-independent male fertility. *Plant Cell*.
- Dörmann, P., Voelker, T.A., and Ohlrogge, J.B. (1995)** Cloning and Expression in *Escherichia coli* of a Novel Thioesterase from *Arabidopsis thaliana* Specific for Long-Chain Acyl-Acyl Carrier Proteins. *Arch. Biochem. Biophys.*, **3116**, 612-618.
- Feinbaum, R.L., and Ausubel, F.M. (1988)** Transcriptional Regulation of The *Arabidopsis thaliana* Chalcone Synthase Gene. *Mol. Cell. Biol.*, **8**, 1985-1992.

Feinbaum, R.L., Storz, G., and 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.

Hahlbrock, K. (1981) Flavonoids. In *The Biochemistry of Plants*, 7, pp. 425-456.

Hahlbrock, K., and Scheel, D. (1989) Physiology and Molecular Biology of Phenylpropanoid Metabolism. *Ann. Rev. Plant Phys. Plant Mol. Biol.*, **40**, 347-369.

Harker, C.L., Ellis, T.H.N., and Coen, E.S. (1990) Identification and Genetic Regulation of the Chalcone Synthase Multigene Family in Pea. *Plant Cell*, **2**, 185-194.

Hrazdina, G., Lifson, E., and Weeden, N.F. (1986) Isolation and Characterization of Buckwheat (*Fagopyrum esculentum* M.) Chalcone Synthase and Its Polyclonal Antibodies. *Arch. Biochem. Biophys.*, **247**, 414-419.

Jähne, A., Fritzen, C., and Weissenböck, G. (1993) Chalcone synthase and flavonoid products in primary-leaf tissues of rye and maize. *Planta*, **189**, 39-46.

Koes, R.E., Quattrocchio, F., and Mol, J.N.M. (1994) The Flavonoid Biosynthetic Pathway in Plants: Function and Evolution. *Bioessays*, **16**, 123-132.

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

Kubasek, W.L., Shirley, B.W., Mckillop, A., Goodman, H.M., Briggs, W., and Ausubel, F.M. (1992) Regulation of Flavonoid Biosynthetic Genes in Germinating *Arabidopsis* Seedlings. *Plant Cell*, **4**, 1229-1236.

Laemmli, U.K. (1970) Polyacrylamide gel electrophoresis. *Nature*, **227**, 680-685.

Loake, G.J., Choudhary, A.D., Harrison, M.J., Mavandad, M., Lamb, C.J., and Dixon, R.A. (1991) Phenylpropanoid Pathway Intermediates Regulate Transient Expression of a Chalcone Synthase Gene Promoter. *Plant Cell*, **3**, 829-840.

Mehdy, M.C., and Lamb, C.J. (1987) Chalcone isomerase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. *EMBO*, **6**, 1527-1533.

Mo, Y., Nagel, C., and Taylor, L.P. (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc. Natl. Acad. Sci. USA*, **89**, 7213-7217.

Peter, H.-J., Kruger-Alef, C., Knogge, W., Brinkmann, K., and Weissenböck, G. (1991) Diurnal periodicity of chalcone-synthase activity during the development of oat primary leaves. *Planta*, **183**, 409-415.

Peters, A., Schneider-Poetsch, H.A.W., Schwarz, H., and Weissenböck, G. (1988) Biochemical and Immunological Characterization of Chalcone Synthase from Rye Leaves. *J. Plant Phys.*, **133**, 178-182.

Pollak, P.E., Vogt, T., Mo, Y., and Taylor, L.P. (1993) Chalcone Synthase and Flavonol Accumulation in Stigmas and Anthers of *Petunia hybrida*. *Plant Phys.*, **102**, 925-932.

Rabino, I., and Mancinelli, A.L. (1986) Light, Temperature, and Anthocyanin Production. *Plant Phys.*, **81**, 922-924.

Rall, S., and Hemleben, V. (1984) Characterization and expression of chalcone synthase in different genotypes of *Matthiola incana* R. Br. during flower development. *Plant Mol. Biol.*, **3**, 137-145.

Robbins, M.P., and Dixon, R.A. (1984) Induction of chalcone isomerase in elicitor-treated bean cells. Comparison of rates of synthesis and appearance of immunodetectable enzyme. *Eur. J. Biochem.*, **145**, 195-202.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor: Cold Spring Harbor Laboratory.

Shirley, B.W. (In preparation) Flavonoid biosynthesis: "new" functions for an "old" pathway. *Trends Biochem. Sci.*

Shirley, B.W., Hanley, S., and Goodman, H.M. (1992) Effects of ionizing radiation on a plant genome: analysis of two *Arabidopsis transparent testa* mutations. *Plant Cell*, **4**, 333-347.

Shirley, B.W., Kubasek, W.L., Storz, G., Bruggemann, E., Koorneef, M., Ausubel, F.M., and Goodman, H.M. (1995) Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J.*, **8**, 659-671.

Singh, B., Schmitt, G., Lillis, M., Hand, J.M., and Misra, R. (1991) Overexpression of Acetohydroxyacid Synthase from *Arabidopsis* as an Inducible Fusion Protein in *Escherichia coli*. *Plant Phys.*, **97**, 657-662.

Stafford, H.A. (1990) *Flavonoid Metabolism*, Boca Raton: CRC Press.

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

Chapter 3

Phage Display Antibody Technology

Abstract

An alternative to conventional routes of immunization has recently been developed that takes advantage of the ability of filamentous bacteriophage to display recombinant antibody fragments as fusions with outer coat proteins. Genes encoding the antibodies can be recovered and subcloned into prokaryotic expression vectors, providing unlimited supplies of highly specific, high-affinity antibodies. A phage-display library with a diversity of 10^8 Fab' recombinants was screened using chalcone synthase (CHS) and chalcone isomerase (CHI) that had been purified from a bacterial overexpression system as glutathione *S*-transferase (GST) fusion proteins. Two rounds of panning using an ELISA-based identification method showed increased number of positive binders against the fusion proteins. Once isolated, these antibodies will be utilized for Western blots and immunolocalization experiments. The corresponding genes will also be expressed in transgenic *Arabidopsis* plants in an effort to disrupt the flavonoid biosynthetic pathway.

Introduction

A helpful tool for the study of proteins *in vitro* is the antibody. Until recently, all sources of antibodies have been animal immune systems. Now, a new antibody-generating technology has been developed that by-passes immunizations. This technique expresses the hyper-variable region (F'ab) of the antibody as a fusion with an outer coat protein (gene III) of single-stranded bacteriophage (fd) (McCafferty et al., 1990). Initial interest in phage display was cloning of genes when antibodies against the gene product were already available (Smith, 1985). However, the true capacity was realized when widespread use of the polymerase chain reaction (PCR) made it economically feasible to clone and duplicate the mammalian immune system.

In 1990, McCafferty et al., set about the task of creating antibodies from genes. They showed that by cloning the V_H and V_L chain domains of the anti-lysozyme antibody D1.3 into their fd phage vector (fdCAT1), phage displaying the antibody variable region as a fusion with the gene III coat protein could be produced. Gene III protein is expressed at one end of fd phage at about four copies per virion, and is responsible for attachment of the phage to the bacterial F pilus (Sambrook et al., 1989). This phage-expressed anti-lysozyme antibody (D 1.3) cross-reacted with lysozyme from hen and turkey egg-white and also with human lysozyme.

The mimicking of the immune system was accomplished using standard molecular techniques (Marks et al., 1992). Light chain variable regions (V_{λ} and V_{κ}) were amplified using PCR with degenerate primers using a cDNA library from a lymphocyte population as template (Hoogenboom and Winter, 1992). V_H and V_L segments were randomly combined through infection of *Escherichia coli* hosts that contain a specific V_H repertoire, with phage fd containing the V_L regions (Winter et al., 1994). The phage library can then be amplified and purified to yield 10^8 - 10^9 individual phage (antibodies), which mimics the murine and human immunodiversity (Marks et al., 1991). By binding antigen to the wells of ELISA plates, the library of phage can be "panned" to pull out the antibodies that recognize epitopes on the antigenic surface (Hoogenboom et al., 1992). These binders can be easily isolated and amplified using standard microbiological techniques (Hawkins et al., 1992). Once isolated, the genes encoding the antibody are amplified and sub-cloned into an expression vector that utilizes an amber mutation to express only the antibody gene products (rather than as a fusion with gene III) in a non-suppressor strain of *E. coli* (Crosby and Schorr, 1995). The F'ab antibody fragments expressed are secreted by the bacterium and purified using a monoclonal antibody (9E10) column that recognizes a partial c-myc epitope engineered into the new gene product.

Possibilities for this new technology are endless. By-passing immunization completely dissolves the need for animal immune

system factories. There is no longer the need to inject potentially harmful antigens into the blood streams of animals for the purpose of generating antibodies. Also, antibodies are fragile proteins that have short half lives inside and outside living bodies. Phage display antibody technology provides an unlimited supply of antibody, by providing the gene for the antibody. Other experimental possibilities can now be realized with the isolation of antibody genes specific for particular proteins. Once only possible with monoclonals (Carlson, *et al.*, 1988), phage display technology provides the genes for introduction directly into host cells. Studies of the effects of possible metabolic interdiction can be achieved if the gene product can be stably expressed in transgenic lines, potentially interfering with protein function and generating a specific null background for the protein of interest. Whether this technology is used for applicative medicines or general knowledge, its potential has yet to be achieved.

Materials and Methods

(adapted from Crosby, 1995)

Panning

Five ml Immuntubes (Nunc) were incubated with 100 μ g/ml fusion protein (GST-CHS or GST-CHI) in 4 ml of sterile phosphate-buffered saline (PBS) (Figure 1). The tubes were then drained, and washed 3 times in quick succession with PBS from a wash bottle, emptying vigorously. Five ml of 2% (w/v) Carnation instant milk in sterile PBS (2% MPBS) was used to block the tube, which was incubated at 37°C for 2 hours.

and λ were incubated at room temperature in the tubes, after sealing the tubes with parafilm. The tubes were gently tumbled on a rotating shaker for 30 minutes, and then left standing on a benchtop for 1.5 hours. The library was then decanted, and tubes washed 5X in quick succession with PBS-Tween 20 and 5X with PBS. Tubes were drained well, but not allowed to dry completely. 1 ml of fresh 100 mM triethylamine (TEA) (Fisher) was incubated inside the tube at room temperature with gentle tumbling on the shaker. After 10 minutes, the solution was neutralized by adding it to pre-aliquoted 0.5 ml solutions of 1M Tris-HCl pH 7.5.

The neutralized phage solution was then used to infect 10 ml of a freshly prepared mid-log culture ($OD_{595}=0.6 - 0.9$) of

Figure 1 Schematic of panning procedure for isolation and enrichment of phage-displayed antibodies.

Immunotubes are coated with antigen, blocked with blotto, and incubated with the library. Bound phage (binders) are eluted and used to infect cultures in order to amplify and recover the binders. The binders recovered are used in another round of panning against the same antigen. Generally, three to five rounds of panning are required to achieve the desired specificity and epitope-recognition diversity. More rounds of panning would enrich for fast growers and tight binders.



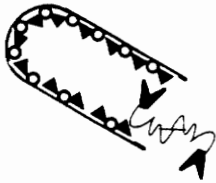
1. Coat Immunotube with antigen



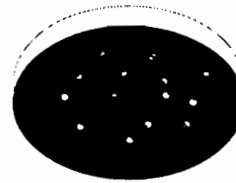
2. Block w/ Biotin



3. Incubate with Library



4. Elute Binders with pH shock (TEA)



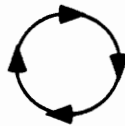
5. Infect bacteria with binders (lysogenic) at 37°C and collect lawn of cells after overnight incubation



6. Induce lytic cycle
Incubation @ 30°C



7. Centrifuge cellular debris and recover supernatant containing phage



8. Use recovered phage to pan against antigen (step 3).

Escherichia coli (F⁺) (strain TG1) in a 15 ml disposable culture tube (Fisher). The phage culture was placed in a 37°C water bath with out agitation for 30 minutes prior to plating to induce the phage to become lysogenic. After incubation and infection, the cells were plated on 2xYT medium (Maniatis et al., 1982) containing 15 µg/ml tetracycline (2xYT-Tet) (Sigma). Dilutions of 10⁻², 10⁻⁴, and 10⁻⁶ were plated and incubated at 37°C overnight to determine phage titer. The remainder of the 10 ml culture was centrifuged at 4000xg, re-suspended in 1 ml of fresh 2xYT, and plated on a 243 mm x 243 mm square Petri dish (Bioassay Dish, Nunc). Plates were inverted and incubated overnight at 37°C.

Amplification

To collect the phage, colonies from the Bioassay plate were harvested by scraping with a bent glass rod and re-suspending into 10 ml 2xYT-Tet. The cells were then transferred to 200 ml of fresh 2xYT-Tet in a 1 Liter flask and incubated overnight at 30°C (lytic). Following incubation, the culture was centrifuged at 5000xg for 10 minutes at 4°C. The phage-containing supernatant was decanted into a sterile 1 Liter flask, and phage was precipitated by adding 1/4 volume of 2.5 M NaCl + 20% (w/v) PEG-6000 (VWR) and letting stand on ice for 30 minutes. The aggregated phage were pelleted by centrifugation at 12,000 x g for 20 minutes at 4°C. The pellet was re-suspended in 40 ml of sterile PBS and re-precipitated with 8 ml of PEG/NaCl solution, incubating on ice for 15 minutes. Following a

second centrifugation at 12,000 x g for 20 minutes, the phage pellet was dried thoroughly and re-suspended in 1.5 ml sterile PBS. This phage solution was stored at -80°C until needed for subsequent rounds of panning. Only 500µl of this solution was necessary to continue panning against fresh fusion proteins.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

96-well microtiter dishes (Falcon) with 200µl 2xYT-Tet/well, were inoculated with 94 individual colonies from the dilution plates, leaving two wells as uninoculated blanks. The sealed-microtiter plates were partially covered, placed into a sealed Tupperware container, and then cultured with shaking at 37°C. An ELISA plate was coated with fusion protein at 10 ng/ml and incubated at room temperature overnight. The fusion protein was removed from the wells by beating the plate across the bench top. The ELISA plate was then submerged in PBS and then beaten across the benchtop to wash the wells. Washing 3X in PBS-Tween 20, and 3X in PBS. Wells were blocked with 2% MPBS (200µl/well) at 37°C for 2 hours.

After blocking, the plates were washed as described above, and 50µl of 4% MPBS was added to each well. The 94 individual cultures from the microtiter plate were centrifuged at 3000x g for 15 minutes at room temperature. 50 µl of each culture was added to individual wells of the ELISA assay plate and allowed to incubate for 1 hour at room temperature. After incubation/binding, the contents

of the wells were decanted, and washed, and incubated with a 1:24,000 dilution of rabbit anti-phage fd, or anti-M13 polyclonal antisera in 2% MPBS for 30 minutes at room temperature.

After 30 minutes with the anti-fd antibody, the plates were washed, and incubated in a 1:10,000 dilution of goat-anti-rabbit-horse-radish-peroxidase (HRP) conjugate (Sigma) in 2% MPBS for 30 minutes at room temperature. The plates were then washed and 100 μ l of fresh HRP development solution (10 ml of 0.1 M Na-Acetate, pH 6.0 (Fisher), 100 μ l of TMB substrate solution [10 mg/ml 3,3',5,5'-tetramethylbenzadine-HCl (Sigma) in 50% (v/v) dimethylsulfoxide (DMSO)], and 4 μ l of 30% H₂O₂ (Fisher)). Reactions were allowed to proceed for 15 minutes and then neutralized with 25 μ l/well of 2N H₂SO₄. Plates were read in a microtiter plate reader at OD₄₅₀ and OD₆₅₀. Results were reported as OD₄₅₀ minus OD₆₅₀.

Results and Discussion

Initial efforts to screen the library were carried out by myself and Peter Schorr, during December of 1994, at the Plant Biotechnology Institute at the National Research Council of Canada, in Saskatoon, Saskatchewan. These efforts were hampered due to loss of library titer resulting from storage at -80°C , instead of the usual storage at -170°C , under liquid nitrogen. Fusion protein was left behind awaiting construction of a new library, so that panning could be carried out.

Subsequent panning against GST-CHS and GST-CHI was carried out by Peter Schorr at PBI-NRCC, due to conflicts with proprietary interests in shipping the phage display library. Second round phage isolated by Peter Schorr was shipped here overnight, and two more rounds of panning (2nd and 3rd) have been successfully carried out. Phage titers after the first round were 2.0×10^5 cfu for CHS and 6.0×10^4 cfu for CHI (Table 1). Subsequent rounds of panning have apparently enriched the population of specific binders. ELISA data suggest that 3rd round phage have increased binding affinity for the fusion proteins (Figure 2).

Subsequent attempts to utilize the phage-displaying antibodies in Western analysis of fusion proteins were unsuccessful. Optimization of rabbit-anti-fd, goat-anti-rabbit-Horse radish peroxidase secondary antibody working dilutions has been performed, but either denatured conformations or insufficient binding affinity towards the fusion protein have hampered efforts to

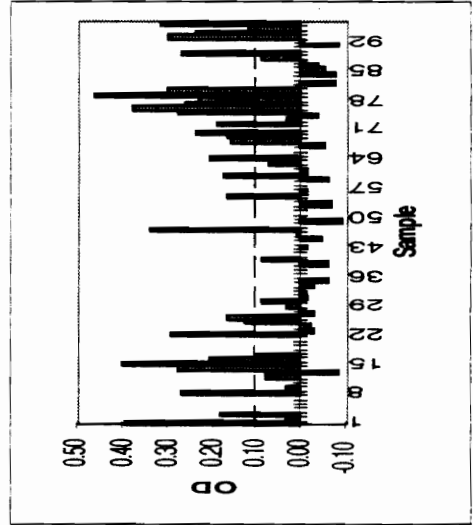
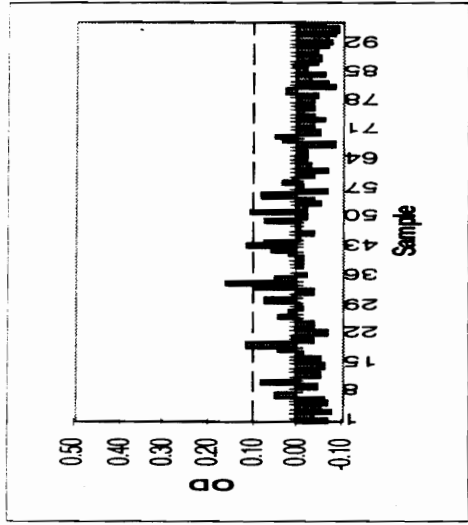
Table 1 Phage titers after first and second rounds of panning.

Antigen	2nd Round Titer	3rd Round Titer
GST-CHS	2.0 x 10 ⁵	1.4 x 10 ⁷
GST-CHI	6.0 x 10 ⁴	5.0 x 10 ⁴

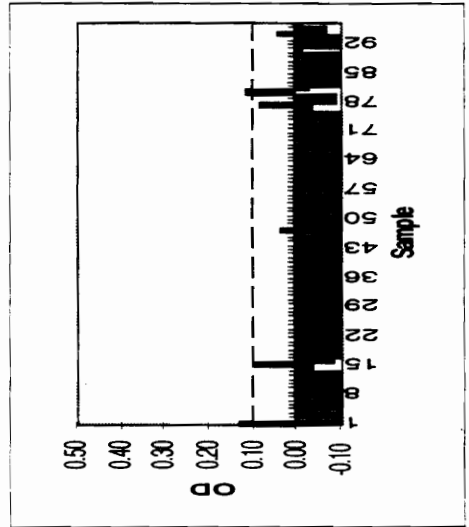
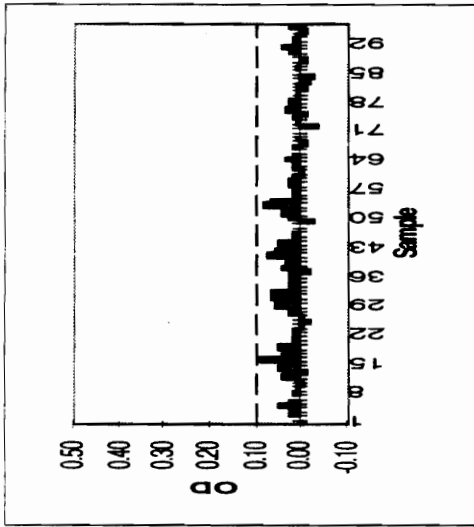
Figure 2 Identification of positive binders using ELISA and spectrophotometry.

ELISAs were used to determine the number of recovered phage that bind GST-CHS and GST-CHI fusion proteins. 94 individual phage were amplified and incubated with corresponding fusion protein. Phage were detected using anti-phage fd antibody and goat-anti-rabbit horse-radish peroxidase secondary antibody using TMB as substrate. Signal-to-noise ratio must be higher than 0.1 to indicate a positive binder (dashed line) (Bill Crosby, personal communication). Signals are expressed as A_{450} minus A_{657} .

CHI



CHS



2nd Round

3rd Round

determine if the phage-displayed-antibodies recognize endogenous CHS or CHI from *Arabidopsis*.

More rounds of panning and ELISA assays will be carried out by our laboratory. PCR cloning of the antibody genes into *E. coli* and subsequent expression of the high affinity/high specificity antibody fragments will be utilized in Western and immunolocalization studies. Introduction of the antibody genes into transgenic *Arabidopsis* plants will aid in the determination of epitope specificity, identifying putative protein-interaction domains required for assembly and localization of enzyme complexes.

References

Crosby, W.L., and Schorr, P. (1995). Principles and Applications of Recombinant Antibody Technology to Plant Biology No. Molecular Genetics Group, Plant Biotechnology Institute, National Research Council of Canada.

Hawkins, R.E., Russell, S.J., and Winter, G. (1992) Selection of Phage Antibodies by Binding Affinity: Mimicking Affinity Maturation. *J. Mol. Biol.*, **226**, 889-896.

Hoogenboom, H.R., Marks, J.D., Griffiths, A.D., and Winter, G. (1992) Building Antibodies from their Genes. *Immun. Rev.*, **130**, 41-68.

Hoogenboom, H.R., and Winter, G. (1992) By-passing Immunisation: Human Antibodies from Synthetic Repertoires of Germline V_H Segments Rearranged *in Vitro*. *J. Mol. Biol.*, **227**, 381-388.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor: Cold Spring Harbor Laboratory.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., and Winter, G. (1991) By-passing Immunization: Human Antibodies from V-gene Libraries Displayed on Phage. J. Mol. Biol., 222, 581-597.

Marks, J.D., Hoogenboom, H.R., Griffiths, A.D., and Winter, G. (1992) Molecular Evolution of Proteins on Filamentous Phage. J. Biol. Chem., 267, 16007-16010.

McCafferty, J., Griffiths, A.D., Winter, G., and Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature, 348, 552-554.

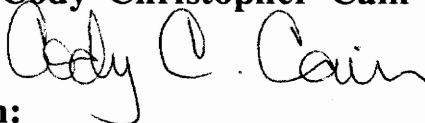
Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor: Cold Spring Harbor Laboratory.

Smith, G.P. (1985) Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface. Science, 228, 1315-1317.

Winter, G., Griffiths, A.D., Hawkins, R.E., and Hoogenboom, H.R. (1994) Making Antibodies By Phage Display Technology. Ann. Rev. Immun., 12, 433-455.

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Johnson, CS, Burbulis, IE, Pelletier, MK **Cain, CC**, and Shirley, BW. Protein-Protein interactions between the first two enzymes of flavonoid biosynthesis. Fifth International Conference on Arabidopsis Research, 8/19-22/93, Columbus, OH.

Pelletier, MK, Burbulis, IE, **Cain, CC**, Iacobucci, M, and Shirley, BW. Protein-protein interactions between chalcone synthase and chalcone isomerase from *Arabidopsis*. Fourth International Congress of Plant Molecular Biology, June 19-24, 1994, Amsterdam, The Netherlands.

Cain, CC, and Shirley, BW. Overexpression and Western Analysis of *Arabidopsis* Flavonoid Enzymes. Sixth International Conference on Arabidopsis Research, June 7-11, 1995, Madison, WI.

Grant Proposals:

Use of epitope tagging to examine the interactions between chalcone synthase and chalcone isomerase, and the subcellular localization of these proteins. CC Cain, principal investigator. Sigma Xi, Grant in aid of Research. \$500, Departmental Match \$500. Date submitted: 10-25-93 Funded: Spring 1994

Subcellular localization of chalcone synthase and chalcone isomerase, as it pertains to the wine and grape industry. CC Cain, principle investigator. American Society for Enology and Viticulture. Date submitted: 3-1-94

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