Macromolecular Organization and Cell Function: A multi-system analysis

Kevin Clarke Crosby

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Brenda S.J. Winkel, Ph.D., Chair

Jill C. Sible, Ph.D., Member

Zhijian Tu., Ph.D., Member

John M. McDowell., Ph.D., Member

Dorothea Tholl., Ph.D., Member

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Abstract

The interior of the cell is a densely crowded and complex arena, full of a vast and diverse array of molecules and macromolecules. A fundamental understanding of cellular physiology will depend not only upon a reductionist analysis of the chemistry, structure, and function of individual components and subsystems, but also on a sagacious exegesis of the dynamic and emergent properties that characterize the higher-level system of living cells. Here, we present work on two aspects of the supramolecular organization of the cell: the controlled assembly of the mitotic spindle during cell division and the regulation of cellular metabolism through the formation of multienzyme complexes.

During division, the cell undergoes a profound morphological and molecular reorganization that includes the creation of the mitotic spindle, a process that must be highly controlled in order to ensure that accurate segregation of hereditary material. Chapter 2 describes results that implicate the kinase, Zeste-white3/Shaggy (Zw3/Sgg), as having a role in regulating spindle morphology.

The congregation of metabolic enzymes into macromolecular complexes is a key feature of cellular physiology. Given the apparent pervasiveness of these assemblies, it seems likely that some of the mechanisms involved in their organization and regulation might be conserved across a range of biosynthetic pathways in diverse organisms. The Winkel laboratory makes use of the flavonoid biosynthetic pathway in *Arabidopsis* as an experimental model for studying the architecture, dynamics, and functional roles of metabolic complexes. Over the past several years, we have accumulated substantive and compelling evidence indicating that a number of these enzymes directly interact, perhaps as part of a dynamic globular complex involving multiple points of contact between proteins. Chapter 3 describes the functional analysis of a predicted *flavonol synthase* gene family in *Arabidopsis*. The first evidence for the interaction of flavonoid enzymes

in living cells, using fluorescent lifetime imaging microscopy fluorescent resonance energy transfer analysis (FLIM-FRET), is presented in Chapter 4.

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Special thanks to the Flying Spaghetti Monster for creating the universe, and then staying out of the way; Frank's mom for free food and haircuts, and, last but not least, friends and family everywhere who somehow still find a way to tolerate my nonsense.

Attributions

Several colleagues and coworkers aided in the writing and research behind several of the chapters of this dissertation. A brief description of their background and their contributions are included here.

Prof. Brenda S.J. Winkel- Ph.D. (Department of Biological Sciences, Virginia Tech) is the primary Advisor and Committee Chair. As the Principle Investigator (PI) of the laboratory, Prof. Winkel provided the material resources required for this research. Her scientific consultations and mentorship helped to focus the direction of the research. Furthermore, Prof. Winkel provided invaluable editing for Chapters 1, 4, and 5 (see details below for Chapter 3), reigning in some of my more extreme Faulknerian and sesquipedalian tendencies.

Chapter 2: A role for the kinase Zeste-white3/Shaggy in regulating spindle morphology

Edward J. Wojcik- Ph.D. (Department of Biological Sciences, Virginia Tech) currently an Assistant Professor in the Department of Biochemistry and Molecular Biology, Louisiana State University Health Science Center. Dr. Wojcik was the primary Advisor and Committee Chair at the time this work was done. As the PI of the laboratory, Dr Wojcik provided material resources for this work as well as scientific guidance. Dr. Wojcik also provided editorial assistance in the preparation of the text of this chapter. Furthermore, Dr. Wojcik collaborated in the collection of images and preparation of Figure 2.

Thomas E. Wall- (Department of Biological Sciences, Virginia Tech). Thomas was an undergraduate researcher in the Wojcik lab who assisted with the GFP protein trap screen.

Chapter 3: Functional analysis of a predicted flavonol synthase gene family in Arabidopsis

Daniel K. Owens-Ph.D. (Department of Biological Sciences, Virginia Tech), currently a postdoctoral fellow in the Department of Biological Sciences, East Tennessee State

University. Daniel was a graduate student in the Winkel lab and is a co-first author on this manuscript. He performed the *in vitro* activity assays, structural modeling, yeast two-hybrid screen, and assisted in the analysis of FLS phylogeny (Figures 3-1, 3-5, 3-6, and Table 3-1). He also prepared portions of the text.

Anne B. Alerding- Ph.D. (Department of Biological Sciences, Virginia Tech), presently an Assistant Professor in the Department of Biology, Virginia Military Institute. Anne was a postdoctoral researcher in the Winkel group and is a co-first author on this manuscript. She performed the expression analyses involving semi-quantitative RT-PCR and promoter-*GUS* experiments (Figures 3-3 and 3-4). She also assisted in screening some of the FLS T-DNA knock-out lines and prepared a portion of the text.

Aloka B. Bandara- Ph.D. (Department of Biological Sciences, Virginia Tech), current affiliation, Department of Biomedical Sciences and Pathobiology, Virginia Tech, was a postdoctoral researcher in the Winkel group who isolated the first full-length cDNA clones for FLS2, 3, and 5 by RT-PCR and made progress towards isolating cDNA clones for FLS4 and 6, as well.

James H. Westwood- Ph.D. (Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech) is a collaborating Associate Professor who supervised the execution and interpretation of the experiments involving parasitic plant infection (portions of Figure 3-4).

Prof. Brenda S.J. Winkel- (Department of Biological Sciences, Virginia Tech). Prof. Winkel was the primary PI for this work and provided invaluable assistance and scientific guidance. She also prepared samples for MS analysis (Figure 3-S2) and contributed significantly to the writing and to the preparation of all figures and tables.

Chapter 4: Interaction of flavonoid enzymes in living cells: evidence for competition of key branch point enzymes for association with the entry-point enzyme, chalcone synthase

Prof.dr Theodorus W.J. Gadella Jr- (Swammerdam Institute for Life Sciences, Section of Molecular Cytology, Centre for Advanced Microscopy, University of Amsterdam).

Prof. Gadella is a collaborator and the PI at the Centre for Advanced Microscopy in Amsterdam. He is a leading expert in the FLIM-FRET techniques and his lab has the specialized instrumentation required for the experiments central to this chapter.

Anna Pietraszewska- M.Sc. (Swammerdam Institute for Life Sciences, Section of Molecular Cytology, Centre for Advanced Microscopy, University of Amsterdam). Anna is a Ph.D. student in Prof. Gadella's lab who was responsible for directly training the author on the FLIM equipment and introducing him to the laboratory facilities. She also participated in a number of helpful discussions of the data.

Dr. Ir Joechim Goedhart- Ph.D. (Swammerdam Institute for Life Sciences, Section of Molecular Cytology, Centre for Advanced Microscopy, University of Amsterdam). Dr. Goedhart is an Assistant Professor in Prof. Gadella's group and provided technical assistance with the instrumentation at a number of points as well as engaging in a number of fruitful scientific discussions.

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Chapter 1

Perspectives and Background

I. Preface

The cell is the fundamental unit of life; all living organisms are made up of one or more cells. The interior of the cell, even that of the simplest bacterium, is incredibly complex and our knowledge of how physiological processes actually occur in this environment is strikingly limited. Although we are well into what future generations of scientists might refer to as the "'omics era," in which an impressive amount of data on the molecular composition of cells has been accumulated through genomic, proteomic, and metabolomic approaches, in the absence of other information, these advances amount to little more than the compilation of parts lists. The next frontier in cell biology is represented by our collective efforts to gain a real understanding of how all these parts work together. Through these efforts, one thing that is becoming clear is that there is an underlying, pervasive macromolecular organization that serves as the basis for cell function and that a crucial aspect of cellular physiology is the assembly of proteins into multi-component complexes (Barabasi and Oltvai, 2004; Krogan et al., 2006; Srere, 2000; Winkel, 2004).

II. Perspectives and Philosophical Context

The concept that sub-cellular components interact physically in ways that are essential to their function is not a new one. Indeed, the roots of this idea can be traced to the Aristotelian notion that there is priority of the whole over the parts (Hall, 1969). This was in direct contrast to the ideas of Democritus who thought natural processes could be explained by analyzing them in terms of the individual parts. This debate has been couched since in the dichotomy of atomism-mechanism versus organicism (or reductionism versus holism) (Hall, 1969; Hall, 1975).

Both of these tenants are based on the axiomatic assumption that biological systems consist of multiple levels that are arranged hierarchically, from the atomic to the organismal (Bruggeman et al., 2002). The reductionist viewpoint, at its most severe, might be reflected in Francis Crick's statement that "An organism is essentially nothing but a collection of atoms and molecules" (Crick, 1967) or, as put more abrasively by his erstwhile colleague, James Watson, "There is just one science, physics: the rest is just social work" (Rose, 2003). It seems unlikely that many

practicing researchers seriously subscribe to such immoderate notions, nevertheless there may be in some circles the tacit assumption that an understanding of the function of isolated components *in vitro*, such as individual enzymes or complexes, will be robustly predictive of their behavior when there are integrated into the higher level system of the living cell. The holistic or anti-reductionist stance, which at its most extreme is a characteristic element of fringe theories such as some of the more strident forms of the Gaian hypothesis (Lovelock, 2000; Lovelock and Margulis, 1974). A more rationalist perspective would be one that recognizes that there are some emergent properties in higher level systems that cannot always be anticipated by an analysis of the discrete characteristics of the individual constituents (Bruggeman et al., 2002; Shepherd and Gerald, 2006).

It can be argued that biochemistry has operated according to the reductionist paradigm: cells are broken open, components such as enzymes are separated from one another (purification), and then the characteristics and activities of the individual components are studied in isolation. Genetics has also operated, in some sense, according to a reductionist strategy; since the beginnings of genetics as a discipline the push has been to isolate the effects of a single locus. Genomics, proteomics, and other high-throughput techniques, which are essentially extensions of traditional genetics and biochemistry, continue in the reductionist vein. Even though some might oppugn this assertion, many of the recent efforts to define the "interactomes" of various model organisms can be argued to lie within the continuum of reductionism, as the resulting schema of protein-protein interaction networks is only a static representation that does not take into account the dynamic and emergent properties that exist within the higher-level systems of living cells. The most holistic approach to studying cellular physiology is computational systems biology. Endeavors in this area have the potential to model how dynamic processes, involving complex networks of molecules, unfold within the cell. However, if these models are to be accurate representations of cell function, they need to based on more then just the interactions that occur within those networks, but must be grounded in experimental data detailing biophysical and structural-level aspects of the interactions as well as the behavior of the macromolecules in the crowded environment of living cells.

This is by no means meant to devalue the merit of these methods and disciplines; research in these areas continues to contribute enormously to our knowledge of cellular biology. Instead, it is meant to emphasize that, in order to move towards a perspicacious understanding of the inner workings of the cell, an integration of a range of approaches will be necessary. To achieve this, the reductionist *in vitro* techniques and holistic computational system approaches need to be complimented by technologies that allow for the non-invasive analysis of cellular processes.

A portion of the work described here involves studies into aspects of mitotic spindle formation relies upon immunofluorescent cytology and live cell imaging, but also makes use of reagents developed through a mix of classical and molecular *Drosophila* genetics. A more comprehensive approach is represented in our efforts to understand the architecture, dynamics, and function of multi-enzyme complexes, specifically, the flavonoid biosynthetic complex in *Arabidopsis*.

III. Shaggy and the Spindle

One of the most evident manifestations of macromolecular organization within the cell occurs during mitosis, when an intricate scaffold is assembled to drive the accurate and controlled segregation of hereditary material during cell division. The mitotic spindle, which serves as the basis for this scaffolding, is a highly dynamic structure that needs to be tightly regulated by a complex ensemble of proteins to ensure its proper function. One aspect of this regulation involves the formation of precise spindle morphology and centrosome positioning, which relies upon the interaction of a number of cytoskeletal and associated elements.

Glycogen synthase kinase-3 was first described as functioning to phosphorylate and inactivate glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984). In *Drosophila*, Zeste-white 3/Shaggy (Zw3/Sgg) was identified as the homologue of the ß isoform of GSK and was found to function in the regulation of the Wnt signaling pathway (Rosenblatt et al., 2004; Ruel et al., 1993; Siegfried et al., 1992); circadian rhythms (McCartney et al., 2001); and phosphorylation of p53 (Basu et al., 1999; Williams and Goldberg, 1994), and of cyclins D and E (Scaerou et al., 1999; Wojcik et al., 2001). Recent studies have suggested multiple roles for GSK-3β in cell

division in animals (Cheng et al., 2008; Izumi et al., 2008; Saurin et al., 2008; Wakefield et al., 2003; Zumbrunn et al., 2001). A growing body of evidence indicates that Zw3/Sgg also has a function in regulating centrosome migration, which in turn has a profound impact on spindle position and morphology in *Drosophila* (Buttrick et al., 2008; Cliffe et al., 2004; McCartney et al., 2001; Rusan et al., 2008). The current model posits that a protein complex consisting, in part, of Armadillo (Arm, the *Drosophila* homologue of β-catenin), Adenomatous polyposis coli2 (APC2) and End-binding protein 1 (EB1) regulate the attachments between microtubules and cortical actin that are necessary for the completion of centrosome separation (Buttrick et al., 2008; Cliffe et al., 2004; McCartney et al., 2001). It is proposed that Zw3 regulates this complex by phosphorylating APC2 and possibly Arm, as well (Cliffe et al., 2004; McCartney et al., 2001). Zw3, in turn, is regulated by the protein kinase, Akt (Buttrick et al., 2008; Wakefield et al., 2003).

The disruption of elements both upstream (Akt) and downstream (APC2) of Zw3/Sgg has been shown to result in mitotic defects involving abnormalities in centrosome separation and spindle structure (Buttrick et al., 2008; Rusan et al., 2008). Chapter 2 describes studies in which we utilized loss of function alleles of *zw3* to directly assess the role of this enzyme in somatic cell mitosis in late larval *Drosophila* neuroblasts. We show that in the absence of Zw3/Sgg, dividing cells showed marked effects on centrosome migration, often resulting in bent mitotic spindles. We also observed a perturbation in cell cycle progression manifested by an increase in the length of metaphase, which is suggestive of an activation of the spindle assembly checkpoint (Basu et al., 1999). These results provide convincing evidence that Zw3/Sgg functions as part of a larger network of protein complexes that regulates the migration and positioning of the centrosomes.

IV. The Macromolecular Organization of Metabolism

While the organization of metabolism and biosynthetic enzymes within the cell is not as immediately evident as the elegant assembly of macromolecular components that takes place during cell division, the implications of these multienzyme systems for cellular physiology are just as profound. Metabolic pathways are frequently depicted as linear arrangements of enzymes, substrates, intermediates, and products, which, while informative, provides no real

information on how these components actually function and interact within the sub-cellular environment. During the last several decades, however, there has been a growing focus on the functional significance of macro-molecular interactions. Yet, as Paul Srere has pointed out, a number of experiments dating to the first half of the 20th century were already illustrating the sub-cellular organization of various metabolic processes (Srere, 2000). In the 1940s, the enzymes of the tricarboxylic acid (TCA) cycle were isolated in an aggregate by David Green's lab. Green felt that the association of these enzymes was functionally important and coined the term "multi-enzyme complex" to describe this system (Green, 1949). In experiments done in the 1960s, where *Neurospora* and *Euglena* were subjected to ultracentrifugation and cellular components became stratified into layers, it was found that that the aqueous, cytosolic fraction was devoid of any measurable enzymatic activity (Kempner and Miller, 1968; Zalokar, 1960). This suggested that *in situ* very few enzymes are not in some way associated with larger cellular structures or complexes.

In the decades since these early experiments, a great deal of supporting evidence for the existence of such multi-enzyme complexes - sometimes referred to in the literature as metabolons (Srere, 1985) - has been accumulated. Well-established models for metabolons include the TCA cycle, trytophan synthase, and the fatty acid synthase complex (Beeckmans et al., 1990; Jenni et al., 2006; Maier et al., 2006; Maier et al., 2008; Srere, 1987; Srere, 2000). In plants, metabolic systems that have served as models for research into multi-enzyme complexes include the Calvin cycle, the cysteine synthase complex, dhurrin biosynthesis, and the phenylpropsanoid/flavonoid biosynthetic pathway (Achnine et al., 2004; Jørgensen et al., 2005; Nielsen et al., 2008; Ralston and Yu, 2006; Winkel, 2004).

A number of functional and regulatory roles are facilitated by the organization of metabolism into macromolecular complexes. Instead of diffusing freely thought the cytoplasm, metabolites could be channeled between the active sites of multiple enzymes, both maintaining a high local concentration of substrates and sequestering potentially toxic or labile intermediates. This mechanism could also direct flux between branch pathways that compete for common metabolites. Differential subcellular localization of these enzyme complexes might also serve to

deliver specific endproducts with a high degree of spatiotemporal control. Additionally, the assembly and disassembly of metabolic complexes could provide a means for the cell to respond rapidly to a range of environmental or developmental stimuli (Mathews, 1993; Ovádi, 1991; Srere, 1985; Winkel, 2004).

V. The Flavonoid Multienzyme Complex

Our laboratory makes use of the flavonoid biosynthetic pathway **(figure 1-1)** in *Arabidopsis* as a model for studying the architecture and dynamics of enzyme complexes. Flavonoids, which are derived from the larger phenylpropanoid pathway, are involved in a variety of essential functions including protection from ultraviolet light, the regulation of auxin transport (gravitropic response), defense against herbivores and pathogens, and in reproduction as fruit and flower pigments (Fritsch and Grisebach, 1975; Hrazdina et al., 1978). The biosynthesis of these compounds is one of the most intensively-studied areas of plant metabolism, and therefore the genetics and biochemistry of the enzymes that comprise the core flavonoid pathway are relatively well understood. Furthermore, it has long been believed that the flavonoid biosynthetic enzymes might function as a larger metabolic complex (Wagner and Hrazdina, 1984). Experiments utilizing labeled precursors and intermediates not only served to elucidate the basic steps of phenylpropanoid and flavonoid biosynthesis, but also provided early evidence for the direct transfer of metabolites between the biosynthetic enzymes (Hrazdina et al., 1987). Cell fractionation studies led to the suggestion that this biosynthesis might occur along the endoplasmic reticulum (ER) (Hrazdina et al., 1978). This notion was buttressed by later immuno-EM analyses that showed that chalcone synthase (CHS), the first committed enzyme in flavonoid biosynthesis, was localized along the cytoplasmic face of the rough ER (Saslowsky and Winkel-Shirley, 2001). These data led initially to a model in which the phenylpropanoid and flavonoid enzymes were proposed to form a loosely-associated, linear array along the ER, anchored by the cytochrome P450 hydroxylases, cinnamate 4-hydroxylase (C4H) and flavonoid-3'-hydroxylase (F3'H) (Burbulis and Winkel-Shirley, 1999; Winkel-Shirley, 1999).

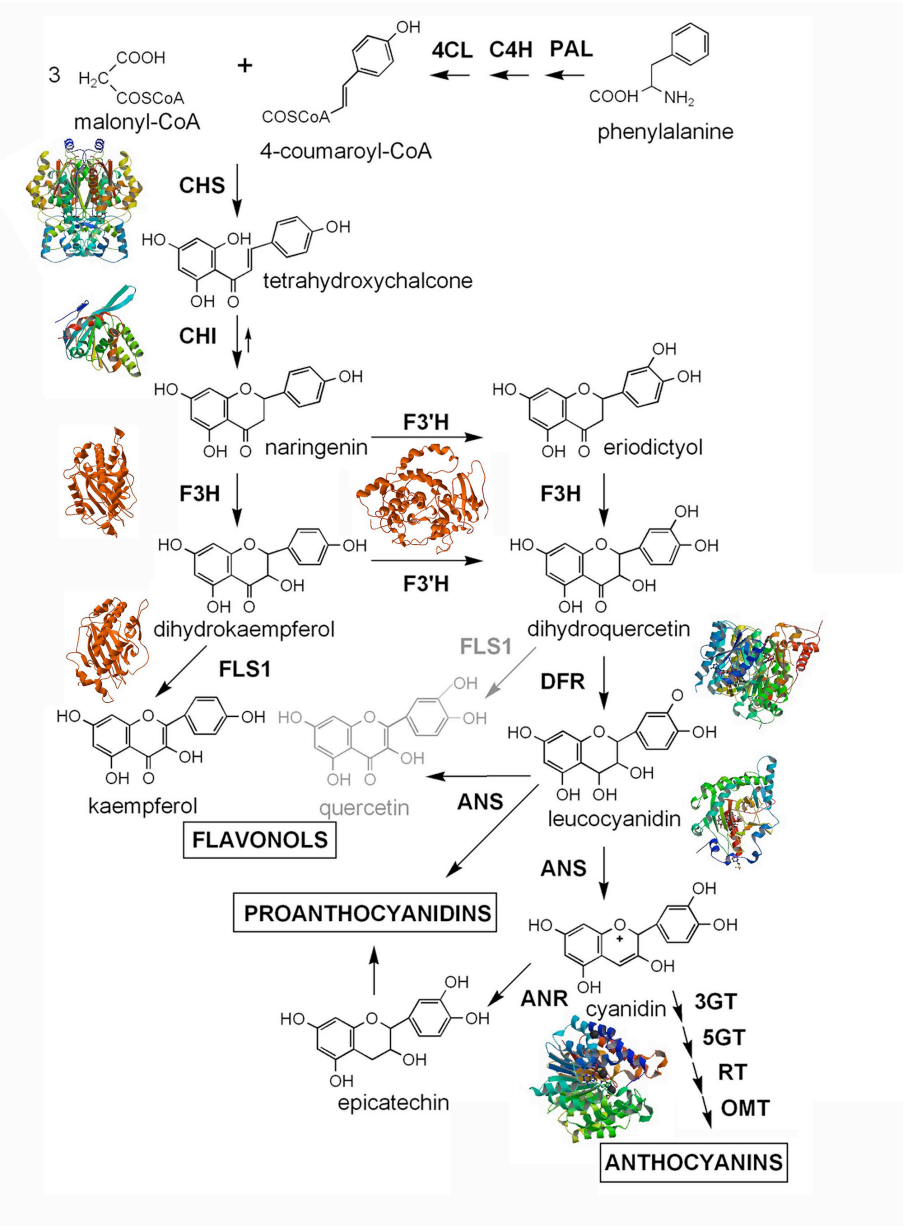

Figure 1-1. Schematic of flavonoid metabolism. Protein structures in mixed colors represent the three enzymes in the pathway for which crystal structures have been solved: chalcone synthase (CHS) and chalcone isomerase (CHI; from Medicago sativa, pdb ids 1CGK and 1EYP), dihydroflavonol reductase (DFR; from grape, 3C1T) and anthocyanidin synthase (ANS; from *Arabidopsis*, 1GP6). Structures in orange are homology models for, flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) (Winkel lab, unpublished) and flavonoid 3' hydroxylase (F3'H) (Schuler lab, Rupasinghe et al., 2003) Other abbreviations: anthocyanidin reductase (ANR), cinnamate 4-hydroxylase (C4H), p-coumarate:CoA ligase (4CL), leucoanthocyanidin reductase (LAR), and phenylalanine ammonia-lyase (PAL). The step shown in gray is not favored by the *Arabidopsis* enzyme *in vitro*

A variety of experiments subsequently provided evidence for the ability of many of the enzymes of the core flavonoid pathway to undergo direct protein-protein interactions. Specifically, yeast two-hybrid experiments showed that CHS could bind to chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), and flavonol synthase 1 (FLS1) in an orientation-dependent manner. Additionally, interactions were detected amongst the downstream enzymes, CHI, F3H, FLS1, and DFR in these assays. Corroborating evidence for the associations among CHS, CHI, and F3H were provided by affinity chromatography and co-immunoprecipitation experiments utilizing plant extracts, while immunofluorescence and immuno-electron microscopy showed that CHS and CHI colocalize in epidermal and cortex cells of the *Arabidopsis* root.

These results led us to revise the original linear model of flavonoid enzyme organization, proposing instead a dynamic globular complex involving multiple points of contact between proteins. Preliminary evidence from molecular modeling and surface plasmon resonance studies indicate that electrostatic interactions may play a role in the assembly of the flavonoid metabolon (Bowerman, Dana, Watkinson, and Winkel, unpublished data). Nevertheless, as with most such systems, the precise nature of how these proteins interact to form a metabolic complex and regulate flux into competing branch pathways has yet to be determined, especially within the context of living cells.

In Chapter 3 we describe the functional analysis of a predicted flavonol synthase (FLS) gene family. The relative levels and ratios of the two primary classes of flavonols that are found in *Arabidopsis*, kaempferol and quercetin, vary depending upon tissue type and developmental stage. In *Arabidopsis*, unlike other higher plants, the enzymes of the central flavonoid pathway are all encoded by a single gene copy. The one apparently exception is FLS, which has six homologs in the *Arabidopsis* genome. This discovery raised the possibility that the various isoforms might have distinct substrate specificities and that differential expression of these genes might mediate the production of a diverse range of flavonol profiles. However, we discovered that the only protein with detectable enzymatic activity was produced by the *FLS1* gene, although several members of the putative gene family are expressed in a tissue and cell-type

specific manner. Furthermore, yeast two-hybrid assays show that multiple FLS proteins are able to interact with other flavonoid enzymes. This opens up the suggestion that the non FLS1 proteins may play a noncatalytic role in flavonoid biosynthesis. Perhaps one that is structural in nature, whereby through the association of these non-active proteins with the flavonoid enzyme complex, FLS1 or perhaps other flavonoid enzymes are selectively displaced.

As was noted above, our group has accumulated substantial evidence suggesting that a number of the flavonoid biosynthetic proteins may interact in a dynamic multienzyme complex. However much of the data have come from *in vitro* experiments. In Chapter 4, we report on our study utilizing fluorescent resonance energy transfer (FRET), imaged by means of fluorescent lifetime imaging microscopy (FLIM), to extend our analysis of these interactions in living cells. Using this technique, we were able to detect associations between CHS and both FLS1and DFR, providing us with the first evidence for the interaction of flavonoid enzymes in living cells. We also obtained results that suggest that FLS1 competes with DFR for binding to CHS. FLS1 and DFR are key branch point enzymes in flavonoid biosynthesis, so this finding supports a model in which flux through divarications in the pathway is, at least in part, controlled by the regulated association and dissociation of different biosynthetic enzymes with a core metabolic complex.

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Chapter 2

A role for the kinase Zeste-white3/Shaggy in regulating spindle morphology

Kevin C. Crosby and Edward J. Wojcik[1]

[1]Present address: Department of Biochemistry and Molecular Biology; LSU School of Medicine, New Orleans, LA 70112

I. Introduction

Glycogen synthase kinase-3 was first described as functioning to phosphorylate and inactivate glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984). In flies, Zw3/Sgg was identified as the homologue of the ß isoform of GSK and was found to function in the regulation of the Wnt signaling pathway (Rosenblatt et al., 2004; Ruel et al., 1993; Siegfried et al., 1992); circadian rhythms (McCartney et al., 2001); and phosphorylation of p53 (Basu et al., 1999; Williams and Goldberg, 1994), and of cyclins D and E (Scaerou et al., 1999; Wojcik et al., 2001). Recent studies have suggested a role for GSK-3β in mitosis in animals. The *Drosophila* GSK3β, Zw3, was found to mediate the anchoring of the mitotic spindle to the cortex of embryos during early syncytial divisions (Fodde et al., 2001). Moreover, application of chemical inhibitors of GSK-3 to HeLa cells can induce some generalized defects in mitotic spindle formation (Zumbrunn et al., 2001). Here we utilize loss of function alleles of *zw3* to directly assess the role of this enzyme in somatic cell mitosis. *zw3* mutant larval neuroblasts produce lengthy, curved metaphase spindles that do not appear to be under tension. Furthermore, the spindle assembly checkpoint is compromised in *zw3* mutants, resulting in episodes of premature sister chromatid separation. The dynamic localization of a fully functional GFP-Zw3 fusion protein during mitosis correlates with the observed defects. Therefore, Zw3 is required for spindle assembly in larval neuroblasts, possibly by mediating spindle dynamics and tension.

II. Results and Discussion

i. Zw3 is Required for Mitotic Spindle Morphogenesis in Larval Neuroblasts

A well establish system for the study of somatic mitotic phenotypes are the *Drosophila* larval neuroblasts (Gatti and Baker, 1989; Gatti and Goldberg, 1991). This is due to a characteristic of *Drosophila* development whereby most the cells in the larval animal are produced during the early divisions of embryogenesis, when the components of mitosis are primarily maternally derived. The majority of these larval cells will not undergo any additional divisions, with the exception of those cells in the imaginal discs and central nervous system, which divide most prominently in the late larval stage, prior to the beginning of metamorphosis (Glover, 1989; Glover et al., 1989; Ripoll et al., 1992). A further advantage of studying larval neuroblasts is

that these cells are large and very amenable to cytological examination. We examined the *zw3* allele, $zw3^{b12}$, a mutation caused by a small, x-ray induced inversion within the major transcription units of *zw3* (Bourouis et al., 1990; Siegfried et al., 1990). Some minor tissue and stage specific protein products are still produced, although neither is sufficient for viability beyond the larval stages (Ruel et al., 1993).

Immunofluorescent analysis of dividing neuroblasts taken from larval brains of *zw3* zygotic mutants revealed marked defects in spindle architecture during metaphase that were never seen in wildtype animals **(figure 2-1).** Most commonly, these abnormal spindles seemed to be "loose" and often crescent shaped **(figure 2-1 B, E)**; in other, less frequently observed cases, the metaphase chromosomes were appeared far "off-center," although the spindle did not take on the characteristic crescent shape **(figure 2-1 c, d)**. This phenotype could be eliminated by the genetic addition of a heat-shock driven *zw3* cDNA transgene (Siegfried et al., 1992). Results are quantified in **(table 2-1, figure 2-2)**.

A mitotic phenotype similar to what we observe in *zw3* mutant neuroblasts was recently reported in a study that analyzed the impact on mitosis of applying actin and myosin II inhibitors on mammalian cells (Rosenblatt et al., 2004). The authors found that disruption myosin II function frequently results in "lopsided" spindles, where chromosomes fail to align between the spindle poles, and instead remain on one side or another of the cell. The conclusion drawn from this result and others is that myosin II mediated attachment to cortical actin in these cells is responsible for the correct positioning of centrosomes. Zw3 has already been shown to mediate spindle attachment to the cortical Actin caps in syncytial *Drosophila* embryos (McCartney et al., 2001) and our own unpublished data suggests that Zw3 might play a role in actin organization in syncytial embryos as well. This raises the possibility that Zw3 might help mediate the contacts between the spindle poles and the cell cortex, which in turn contributes to proper spindle structure and orientation.

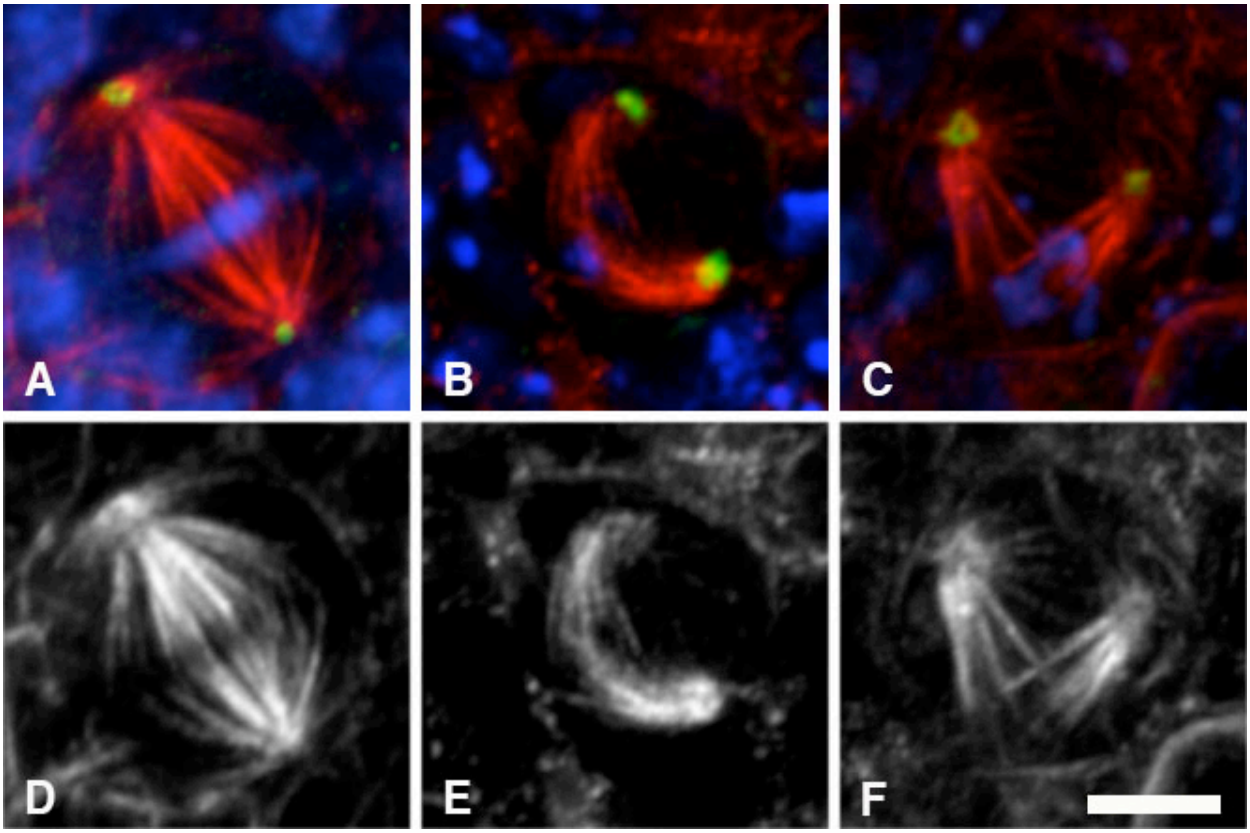

Figure 2-1. *zw3* zygotic mutants exhibit marked defects in spindle architecture during metaphase. Confocal images of metaphase third-instar neuroblasts immunostained to show DNA (blue), α-tubulin (red), and γ-tubulin (green), with the individual α-tubulin channel shown in ***D-F***. ***A***, ***F*)** Wild-type; ***B-C***, ***E-F*)** *Zw3*^{1}*/Y*. Scale bar: 5 μm

Table 2-1 Spindle angle measurements

Genotype	*n*	Mean	SD
Oregon R	23 spindles 6 brains	167.59°	9.49
$Zw3^{1}/Y$	33 spindles 13 brains	136.26°	35.29
$Zw3^{1}/Y$; hsZw3A	27 spindles 8 brains	168.44°	9.95

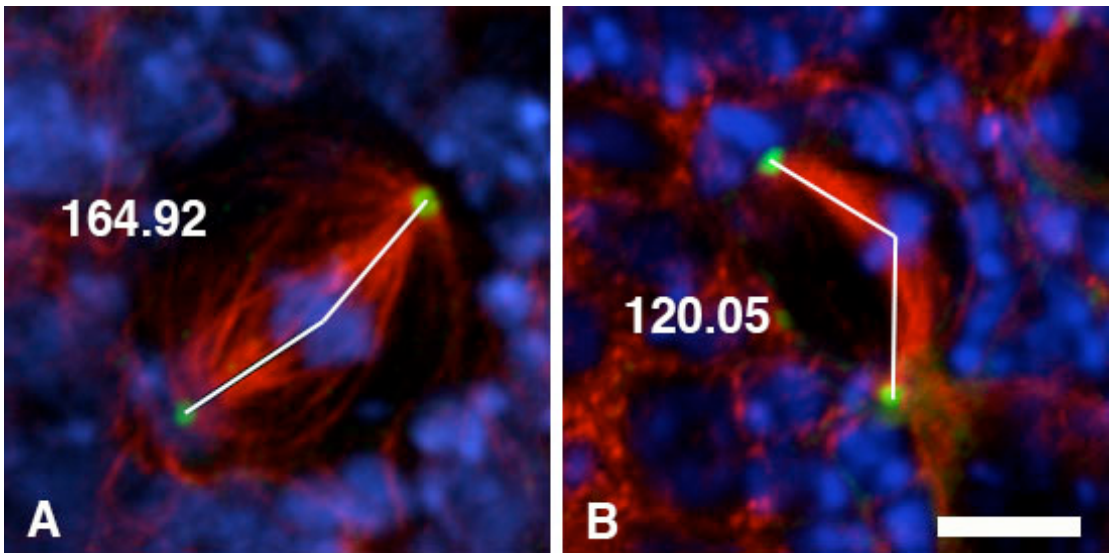

Figure 2-2. Confocal images of metaphase third-instar neuroblasts. DNA (blue), α-tubulin (red), and γ-tubulin (green). **A)** Wild-type; **B)** *$Zw3^{1}/Y$*

ii. Zw3 Mutations Impair the Spindle Assembly Checkpoint

We would expect such spindle defects as are observed in *zw3* mutants to activate the spindle assembly checkpoint. Activation of this checkpoint causes an arrest in mitosis at metaphase and a corresponding increase in the metaphase to anaphase ratio. The ratio of metaphase to anaphase cells is elevated in mutant brains compared to wild-type (**table 2-2**), consistent with a delay in mitosis at metaphase. However, in an assay to determine the robustness of the spindle assembly checkpoint, both mutant and wild-type brains were treated with colchicine. Wild-type neuroblasts will arrest at metaphases in response to this treatment, with each pair of sister chromosomes remaining attached. Typically, neuroblasts with mutations that compromise normal checkpoint function exhibit high levels of premature sister chromatid separation (PSCS) in the range of 20-60% of all mitotic figures (Basu et al., 1999; Scaerou et al., 1999; Williams and Goldberg, 1994). In the case of the *zw3* mutation, we found that the increase in incidence of PSCS versus wildtype was relatively low, but still significant **(table 2-2)**. This suggests that Zw3 does not play a central role in the spindle assembly checkpoint and that the effect on the spindle assembly checkpoint may be an indirect consequence of its loss of function. Alternatively, Zw3 function in the checkpoint may be partially redundant with other players. This weakening of the spindle assembly checkpoint might account for the moderately elevated metaphase/anaphase ratio and the low frequency of polyploidy figures, both of which are characteristically observed in mutations which affect spindle assembly (for example see (Wojcik et al., 2001)).

Table 2-2: Metaphase to anaphase ratio and PSCS rates

Genotype	metaphase/anaphase	PSCS (%)
Oregon R	3.75	1.1
zw3/Y	7.3	11.5

For metaphase/anaphase ratio 4 wt brains and 16 mutant brains were analyzed. For PSCS analysis, 6 wt brains and 47 mutant brains were processed

A known substrate of Zw3/GSK-3ß is the Adenomatous polyposis coli protein (APC) (Zumbrunn et al., 2001). APC has been found to localize to the kinetochore in mammalian cells in association with the microtubule end binding protein EB1 (Fodde et al., 2001). GSK3ß phosphorylation has been shown to regulate the stability of APC binding to microtubule ends (Zumbrunn et al., 2001) and studies in HeLa cells has shown that APC localizes to microtubule ends during metaphase, forming a complex with Bub1 and Bub3 (Kaplan et al., 2001). *In vitro* evidence from this study suggested that GSK3ß phosphorylation of APC facilitates a subsequent phosphorylation by the Bub kinases. This suggests a possibility that Zw3 may contribute to a hypothetical role for APC, perhaps acting through other intermediaries (for example see (Banks and Heald, 2004)), in regulating microtubule-kinetochore attachments and spindle tension. A breakdown in this system could contribute to the weakening of the spindle assembly checkpoint as well as contributing to the disorganized and relaxed spindle shapes observed in *zw3* mutants.

iii. GFP-Zw3 Undergoes a Dynamic Pattern of Localization to both Centrosomes and Nuclei During Mitosis

During the course of a protein trap screen, utilizing an artificial exon containing the coding sequence for GFP contained in a transposable element (Morin et al., 2001) we isolated a line in which the GFP containing transposon was inserted in the locus of *zw3*. This line could be maintained as a homozygous stock, suggesting that Zw3-GFP retained all the functions of the native protein required for viability. Additionally, our Zw3-GFP chromosome was able to rescue the $zw3^{b12}$ mutation to full viability (data not shown). Timelapse observations of living neuroblasts reveal a dynamic localization during mitosis (**figure 2-3)**. There is a general cytoplasmic presence, with prominent localization at the centrosomes. Additionally, Zw3 localizes to the nucleus during two distinct points during mitosis, first at the onset of metaphase and then again during telophase. This observation is intriguing as the nuclear envelope is not thought to be intact at either of these points. When we examine syncytial embryos, we witness very similar centrosome localization and nuclear shuttling during mitosis (data not shown). This localization pattern is suggestive of a functional role for Zw3 during mitosis. The significance of the nuclear shuttling has yet to be discerned and is the subject of on-going investigation.

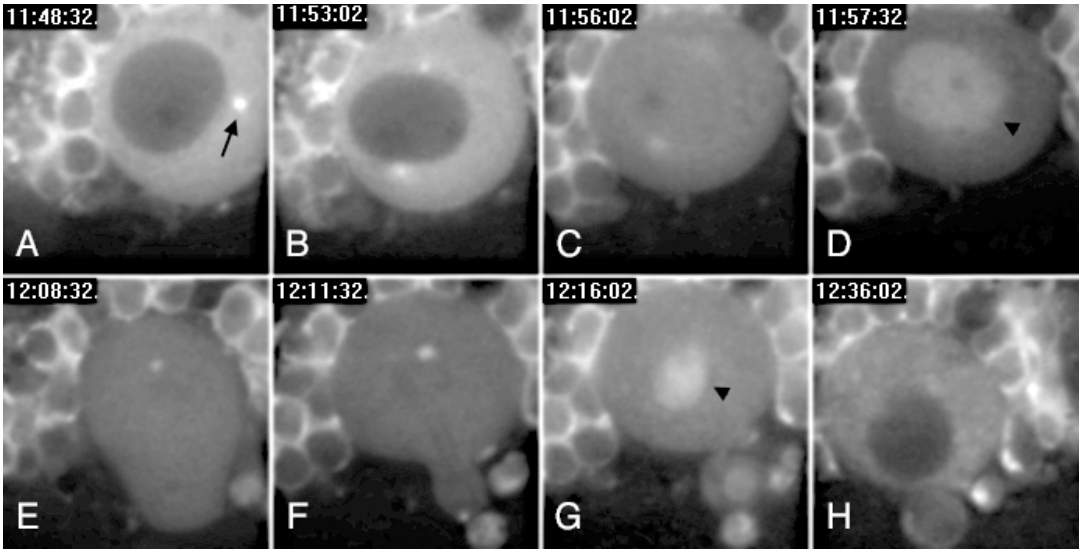

Figure 2-3: Localization of Zw3-GFP in giant larval neuroblasts**.** Time-lapse series showing Zw3-GFP mitotic localization in a short term primary cell culture of larval neuroblasts. ***A-B)*** cytoplasmic and centrosomal (arrow) localization during pro-metaphase and metaphase. ***C-D)*** influx into the nucleus at the onset of metaphase. (arrowhead) ***E-F)*** late anaphase and telophase. ***G)*** concentration in the nucleus in late-telophase (arrowhead). ***H)*** post cytokinesis.

III. Summary

The results presented here suggest a novel role for Zw3-GSK3ß in the formation of the mitotic spindle in *Drosophila* neuroblasts. Additionally, loss of Zw3 function was shown to weaken the spindle assembly checkpoint. While, it seems possible that Zw3 is operating through independent pathways to influence each of these events, we have hypothesized that Zw3-GSK-3ß may be acting upon APC to mediate microtubule tension and dynamics, and possibly microtubule-kinetochore attachments. Zw3 may also contribute to spindle structure and positioning in neuroblasts by mediating the attachment and movement of the poles along the cell cortex. In order to determine the validity of our proposed models, future work will be directed towards evaluating the impact of loss of Zw3 function on some of the specific components of our hypothetic pathways, such as APC, Bub1, and Eb1, as well as identifying the specific mitotic phosphorylation targets of Zw3-Gsk3ß.

IV. Materials and Methods

Fly lines

All fly stocks were maintained according to standard protocols (Ashburner, 1989). Genes and alleles are described at Flybase.org. The ZW3-GFP line was generated utilizing a GFP protein-trap screen utilizing techniques and fly lines described in (Morin et al., 2001) and site of insertion was mapped using inverse PCR (http://www.fruitfly.org/about/methods/inverse.pcr.html). *Zw3* allelic fly lines and the heat shock rescue line *HsZw3A* were all kindly provided by Dr. Esther Siegfried of Penn State Univeristy (Siegfried et al., 1992; Siegfried et al., 1990; Steitz et al., 1998).

Preparation of Whole-Mount Larval Brains for Immunofluorescence

*Drosophila t*hird-instar *Oregon R* wild-type, $Zw3^{1}/Y$, Zw^{1}/Y; *hsZw3A*, and $Zw3^{1}/Zw3^{M11\text{-}1}$ brains were dissected in 0.7% saline and prepared for immunofluorescence as described previously (Wojcik et al., 2001). Microtubules were labeled utilizing a rat monoclonal anti-α tubulin (YOL1/34; Accurate Chemical) diluted 1:200 and Alexa 647 conjugated goat anti-rat secondary antibody (Molecular Probes) at a 1:200 dilution. Centrosomes were visualized with a mouse monoclonal anti-γ tubulin (GTU-88; Sigma) at a dilution of 1:200 and Alexa 488 goat anti-mouse secondary antibody (Molecular Probes). DNA was stained with DAPI (Sigma) at a concentration of 0.5μg/ml. Confocal images were collected using a Zeiss LSM 510 laser scanning confocal microscope on a Zeiss Axiovert 100M. Digital files were processed using ImageJ. Figures were then assembled using Adobe Photoshop.

Determination of mitotic index and PSCS analysis

To determine the mitotic index and for PSCS analysis, third-instar larval brains were dissected in 0.7% NaCl, cultured for 1 h in 10^{-5} M colchicine in 0.7% NaCl, and then transferred individually to 1% sodium citrate hypotonic solution for exactly 5 min. Squashing, fixation and aceto-orcein staining were carried out as described (Karess and Glover, 1989). Observations were made in phase-contrast and at least 20 fields were scored per brain. To determine the mitotic index of *zw3* lines were observed by aceto-orcein staining without prior treatment in colchicine or hypotonic solution.

Time-lapse Recording of Zw3-GFP in Primary Cultures of Larval Neuroblasts

The *Drosophila* line expressing Zw3-GFP was generated using the protein-trap screen as described (Morin et al., 2001). Briefly, GFP expressing lines were established from larvae that had been screened for fluorescence. was determined using inverse PCR, as described by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/about/methods/inverse.pcr.html). These lines were then screened for insertions that had tagged proteins showing localizations suggestive of a role in mitosis. Primary cultures of larval neuroblasts were prepared as described

(Savoian and Rieder, 2002). Time-lapses were captured on a Zeiss Axiovert 200 at intervals of *x* minutes using Metaview (Universal Imaging).

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Chapter 3

Functional analysis of a predicted flavonol synthase gene family in Arabidopsis[1]

Daniel K. Owens[2,3], Anne B. Alerding[2,4], Kevin C. Crosby[2],

Aloka B. Bandara[5], James H. Westwood, and Brenda S.J. Winkel*

Departments of Biological Sciences (D.K.O., A.B.A., K.C.C., A.B.B., and B.S.J.W.) and Plant Pathology, Physiology, and Weed Science (J.H.W.), Virginia Tech, Blacksburg, VA 24061-0390

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[2] These authors contributed equally to the work presented in this manuscript.

[3] Present address: Department of Biological Sciences, East Tennessee State University, Johnson City, TN 37614.

[4] Present address: Department of Biology, Virginia Military Institute, Lexington, VA 24450.

[5] Present address: Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, VA 24061.

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Author's Contributions:

KCC screened the FLS T-DNA knockout lines: INRA_AJ599907 (FLS1), GABI_429B10 (FLS2); GABI_317E12 (FLS5), and SALK_003879 (FLS6). With BSJW conceived, designed and performed *in planta* analyses (as represented by figures 3-7, 3-8, and supplemental figure 3-1). BSJW, DKO, and ABA wrote the paper.

I. Abstract

The genome of *Arabidopsis thaliana* contains five sequences with high similarity to *AtFLS1,* a previously-characterized flavonol synthase gene that plays a central role in flavonoid metabolism. This apparent redundancy suggests the possibility that Arabidopsis uses multiple isoforms of FLS with different substrate specificities to mediate the production of the flavonols, quercetin and kaempferol, in a tissue-specific and inducible manner. However, biochemical and genetic analysis of the six *AtFLS* sequences indicates that, although several of the members are expressed, only *AtFLS1* encodes a catalytically-competent protein. *AtFLS1* also appears to be the only member of this group that influences flavonoid levels and the root gravitropic response in seedlings under non-stressed conditions. This study showed that the other expressed *AtFLS* sequences have tissue- and cell-type specific promoter activities that overlap with those of *AtFLS1* and encode proteins that interact with other flavonoid enzymes in yeast two-hybrid assays. Thus it is possible that these "pseudogenes" have alternative, non-catalytic functions that have not yet been uncovered.

II. Introduction

Flavonoids are well-known plant natural products that have a wide array of physiological functions in plants, while also contributing significant health-promoting properties to plant foods. Many of the roles in plants, including UV protection, regulation of auxin transport, modulation of flower color, and signaling, have been attributed to a subclass of flavonoids known as flavonols, that are among the most abundant flavonoids (Bohm et al., 1998; Harborne and Williams, 2000). These same compounds have also been identified with the antioxidant, antiproliferative, antiangiogenic, and neuropharmacological properties of flavonoids (Lee et al., 2005; Kim et al., 2006; Kim and Lee, 2007). Although concerns have been raised about the potential deleterious effects of high levels of dietary or supplemental flavonols, these appear to be largely unfounded (Havsteen, 2002; Okamoto, 2005). As a result, understanding the synthesis of flavonols is of particular interest from the perspective of metabolic engineering, as illustrated by recent efforts to up-regulate flavonol biosynthesis in tomato (*Lycopersicon esculentum*) fruit (Schijlen et al., 2006) and rice (Reddy et al., 2007) and overproduce flavonols in *E. coli* (Leonard et al., 2006; Katsuyama et al., 2007).

Most plants synthesize derivatives of one or more of the three major flavonols, quercetin, kaempferol, and myricetin, which differ by only a single hydroxyl group on the flavonoid B ring, and yet can specify quite different biological activities. The ratio of these flavonols varies substantially among different tissues and can be altered in response to environmental cues (Winkel-Shirley, 2002). For example, UV-B light has been shown to specifically induce the accumulation of quercetin derivatives in Petunia, which have a higher antioxidant potential and are therefore deemed more effective sunscreens than other flavonols (Ryan et al., 2002). Quercetin has also been shown to be most effective at inhibiting the auxin efflux carrier (Jacobs and Rubery, 1988), and quercetin and kaempferol exhibit different spatial and temporal distribution patterns in Arabidopsis roots that are consistent with roles in controlling auxin movement (Peer et al., 2001; Peer et al., 2004). Interestingly, quercetin is also frequently identified as a primary bioactive compound in medicinal and food plants (Havsteen, 2002; Kim et al., 2006; Nichenametla et al., 2006).

Synthesis of flavonol aglycones has long been attributed to a single enzyme, flavonol synthase (FLS), which competes with several other enzymes for dihydroflavonol substrates. Among these are flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H), which mediate the addition of hydroxyl groups to the B ring of flavanones, flavones, dihydroflavonols, and flavonols (Hagmann et al., 1983; Kaltenbach et al., 1999), and dihydroflavonol reductase (DFR), which drives flux away from flavonols into anthocyanin and proanthocyanidin biosynthesis (Davies et al., 2003). More recently, anthocyanidin synthase (ANS) has been shown to use both dihydroflavonols and leucoanthocyanidins *in vitro* for the synthesis of flavonols, the latter suggesting an alternative route to quercetin using a substrate normally associated with anthocyanin and proanthocyanidin biosynthesis (Turnbull et al., 2004; Wellmann et al., 2006; Lillo et al., 2008). Some of the competition for common substrates appears to be mediated by differential expression of genes required for upstream (flavonol) versus downstream (anthocyanin and proanthocyanidin) pathways (Pelletier et al., 1997; Mehrtens et al., 2005). Yet how these enzymes cooperate to control the metabolic balance among the branch pathways of flavonoid biosynthesis, possibly through participation in one or more enzyme complexes, remains to be fully determined. In fact, efforts to use enzymes such as FLS and ANS to engineer

altered flavonoid profiles have had consistently unpredictable outcomes (for example, Schijlen et al., 2006; Wellmann et al., 2006; Reddy et al., 2007).

Flavonoid biosynthesis in Arabidopsis is relatively simple compared to many other higher plants, involving the production of only three major classes of compounds, flavonols, anthocyanins and proanthocyanidins. With only one apparent exception, the enzymes of the central flavonoid pathway, including chalcone synthase (CHS), chalcone isomerase (CHI), DFR, flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), anthocyanidin synthase (ANS), and anthocyanidin reductase (ANR/BAN), are encoded by single genes. The exception is FLS, for which we have identified six homologs in the Arabidopsis genome. This raises the possibility that gene duplication has led to a group of differentially regulated genes encoding isoforms with varying substrate specificities, facilitating the synthesis of different flavonols to meet the dynamic physiological needs of the plant. Here we describe an effort to test this hypothesis by examining the expression patterns and biochemical characteristics of the six Arabidopsis FLS isoforms, as well as the impact of knockout mutations on phenotypes association with flavonoid metabolism. The results of these experiments provide new insights into the mechanisms controlling flavonol accumulation *in vivo*.

III. Results

i. Identification of a *FLS* Gene Family in Arabidopsis

The first Arabidopsis gene with high homology to *FLS* genes from other plant species, *AtFLS1* (At5g08640), was originally identified in the EST database a number of years ago (Pelletier et al., 1997). Analysis of flavonols in an *En*-induced mutant line and activity assays with recombinant protein confirmed that the gene encoded a protein with FLS activity (Wisman et al., 1998; Prescott et al., 2002). Five additional sequences with high homology to *FLS* genes were subsequently uncovered during sequencing of the Arabidopsis genome, which we have designated *AtFLS2* (At5g63580), *AtFLS3* (At5g63590), *AtFLS4* (At5g63595), *AtFLS5* (At5g63600), and *AtFLS6* (At5g43935) (Arabidopsis Genome Initiative, 2000). These sequences cluster more closely with *FLS* genes from other plants than with other plant flavonoid 2-oxoglutarate dioxygenases (2-ODDs), both at the nucleotide (data not shown) and predicted

amino acid (**Figure 3-1**) levels. The six genes are all located on chromosome 5, with *AtFLS2, 3, 4,* and *5* arranged in a 7.5 kb tandem array (**Figure 3-2**). The four clustered genes are no more closely related to each other than to the other two genes, with *AtFLS2* the most distantly related at the nucleotide level (48-51% identity) and the others exhibiting 62 to 73% identity. This suggests that the duplications leading to the amplification of this gene family, including the *AtFLS2-5* tandem array, are ancient events.

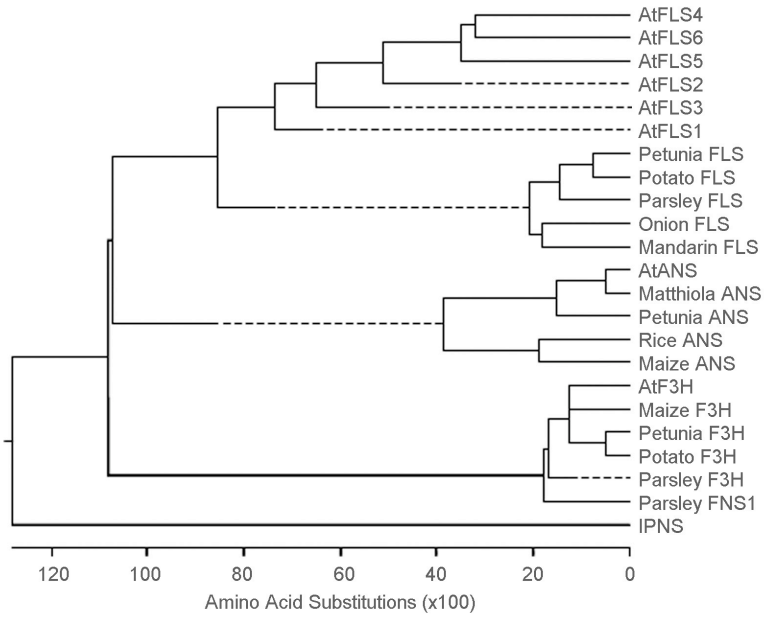

Figure 3-1. Phylogeny of the AtFLS isoforms and other dioxygenases of the flavonoid pathway based on predicted amino acid sequences

AtFLS1, *3*, and *5* appear to encode full-length proteins and all contain two introns at identical positions, corresponding to two of the five intron sites that are conserved among plant *2-ODD* genes (Prescott and John, 1996). The *AtFLS2* gene contains a large second intron and the predicted coding sequence encodes a truncated protein lacking key C-terminal residues required for Fe^{2+} coordination (H220, D222, and H276 in *AtFLS1*) and α-ketoglutarate binding (R286 and S288 in *AtFLS1*) (Lukacin and Britsch, 1997; Wilmouth et al., 2002). The situation is more complex for *AtFLS4* and *AtFLS6,* both of which are predicted to contain an additional intron relative to the other four *AtFLS* genes, in what is otherwise the second exon (TAIR 7.0

genome sequence, released 4-23-07; Swarbreck et al., 2008). To date, no full-length cDNA sequences have been reported for *AtFLS4*. Of the four *AtFLS4* EST sequences available in GenBank (Alexandrov et al., 2006) and RIKEN Genomic Sciences Center (Seki et al., 2004), only one spans the region containing the predicted additional intron and these sequences are not spliced out, severely truncating the coding region. As described in further detail below, RT-PCR analysis of Ler roots identified multiple transcripts for *AtFLS4* that apparently arise from a complex differential splicing scheme. Sequence analysis of four of these cDNAs showed erroneous splicing at the 3' ends of exons 1 or 2, resulting in premature stop codons (**Figure 3-2**); all also retained the additional predicted intron sequences. In the case of *AtFLS6,* no cDNA or EST sequences have been reported to date and efforts to amplify transcripts from root RNA by RT-PCR were unsuccessful (data not shown). If this gene is expressed at all, the transcript is likely to be processed in a manner similar to *AtFLS4*. Thus it appears that *AtFLS2, 4* and *6* are pseudogenes that are unlikely to contribute to flavonol synthase activity in Arabidopsis.

ii. *AtFLS* Gene Expression Patterns

To test the possibility that the *AtFLS* genes have acquired differential patterns of expression, transcript abundance and promoter activities were examined over the course of Arabidopsis plant growth and development. Plants growing in soil under a 16 h photoperiod were sampled at regular intervals over a 7-week period. Semi-quantitative RT-PCR was used to compare the abundance of the transcripts in whole seedlings and in plant organs known to accumulate high levels of flavonols (Shirley et al., 1995). *AtFLS1* displayed the broadest pattern of expression (**Figure 3-3A**). The highest *AtFLS1* transcript levels were detected during the reproductive stage, in the developing inflorescence, floral buds, flowers, and siliques. Lower, but still substantial, levels were detected in the roots and shoots of young seedlings and in leaves of later vegetative stages. This pattern is consistent with the publicly available microarray data for different stages of Arabidopsis development (Genevestigator, Fig. 3B and C; AtGenExpress, data not shown). Interestingly, the *AtFLS2* pseudogene appears to be expressed at high levels in the shoot apex and lower stem, tissues where *AtFLS1* transcripts were not detected, and at low levels in flowers and siliques (**Figure 3-3A**). These patterns are also reflected in the microarray data (Fig. 3B and C). *AtFLS5* appears to be expressed at much lower levels, with transcripts detected primarily in seedling roots, while *AtFLS3* expression was undetectable or extremely low in all

samples examined; these findings are again consistent with the microarray data (**Figure 3-3B-C**). Expression of the *AtFLS4* and *6* pseudogenes was not examined, as these genes appear to have little, if any expression based on the EST databases; they are also not represented on either the 8K or 22K array used to generate the data compiled in Genevestigator. It is interesting to note that the microarray data indicates that expression of *AtFLS1*, but not *AtFLS2, 3* or *5*, parallels that of the other "early" flavonoid genes during development, in the response to light, and several other external cues (**Figure 3-3B-D**). This is also reflected in the ATTED-II database, where *AtFLS1* expression has a 0.83-0.84 correlation score with other "early" flavonoid genes, while *AtFLS3* and *AtFLS5* are correlated with each other (score of 0.70) but not with any other flavonoid genes (Obayashi et al., 2007).

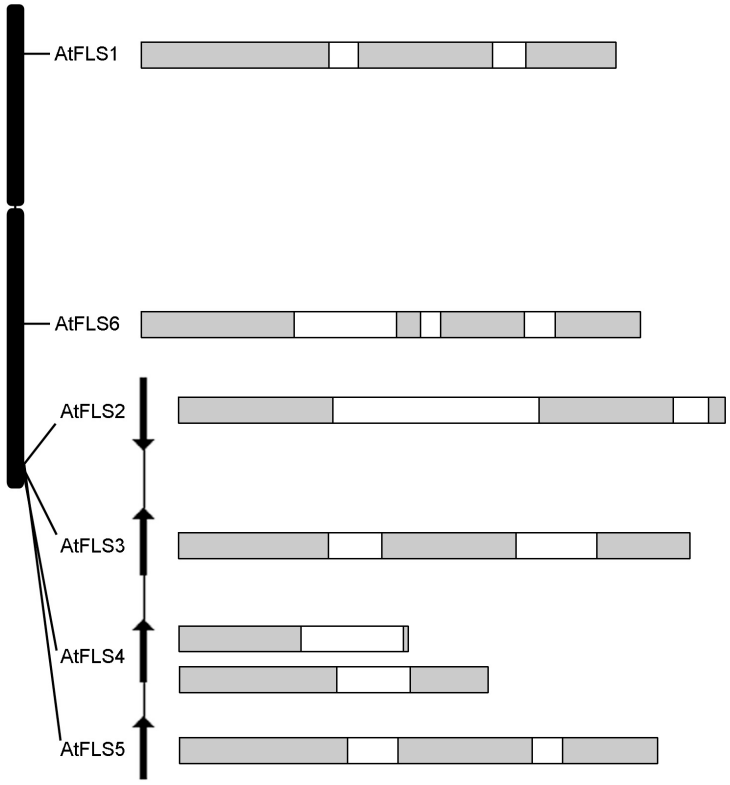

Figure 3-2. Arrangement of the *AtFLS* genes in the Arabidopsis genome. All six genes are located on chromosome 5. *AtFLS2-5* are clustered in a 7.5 kb region. *AtFLS1, 3,* and *5* appear to comprise full or near full-length coding sequences, while the *AtFLS2, 4,* and *6* coding regions are truncated, with the *AtFLS4* gene giving rise to multiple forms, apparently due to alternative/aberrant splicing. Exons are shown in gray, introns in white

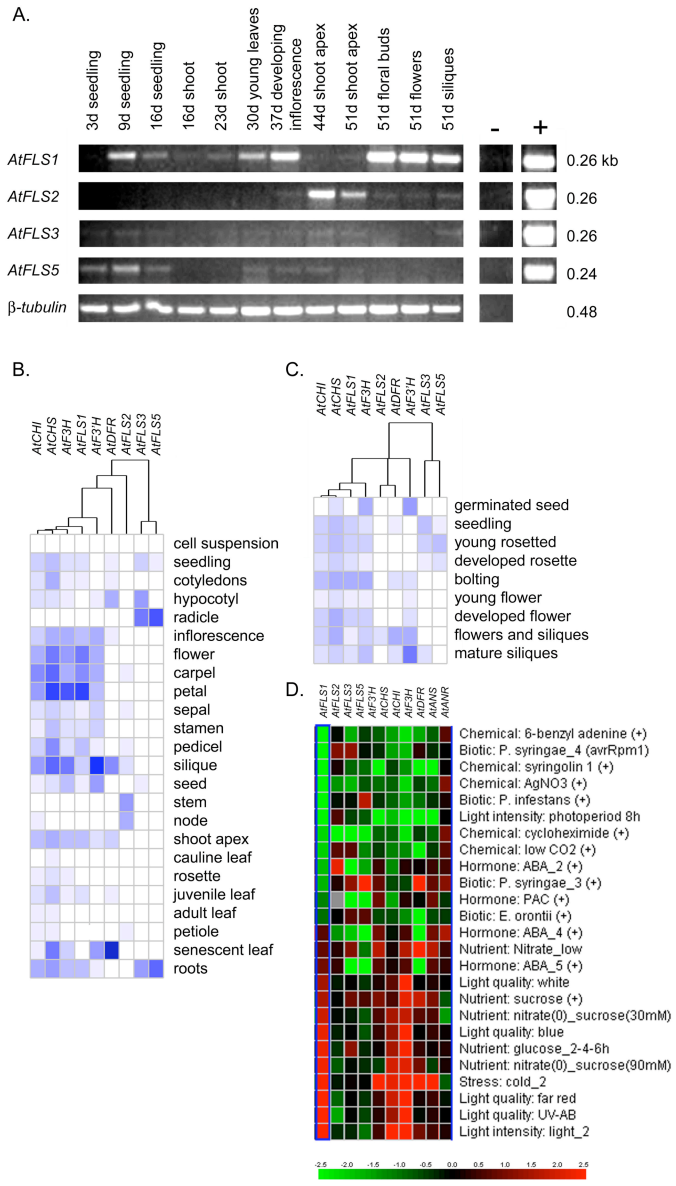

Figure 3-3. Analysis of *AtFLS* gene expression. (A) Semi-quantitative RT-PCR analysis of plants and plant tissues at various developmental stages. Positive controls (+) contained cDNA clones for each gene, negative controls (-) contained no template. RNA samples from whole seedlings and shoots of 16-day-old plants were analyzed and used to infer gene expression in roots. (B-D) Data from the public microarray databases for the *AtFLS* genes and other select flavonoid genes obtained using Genevestigator (Zimmermann et al., 2004).

Figure 3-4. Developmental, organ-specific, and parasite-induced expression of the *AtFLS* genes. Promoter-*GUS* fusions were analyzed in multiple independent transgenic lines by histochemical staining with X-GLUC. Staining was observed primarily in 3-day-old seedlings (A-C), 9-day-old seedlings (D-I), initiating lateral roots in plants of various ages (J-L), trichomes on 30-day-old plants (M-O), and reproductive structures of 51-day-old plants (P-U). Arrows identify the root-shoot transition zone in A-C, G-I and lateral roots in J-L. Unlike the *AtCHS* promoter (V), expression of the *AtFLS1-5* promoters was not induced by infection with the plant parasite, *O. aegyptiaca* (W-AA).

Developmental gene expression patterns were further investigated by analyzing transgenic plants

containing *AtFLS1, 2, 3,* and *5* promoter sequences fused to the ß-glucuronidase (GUS) gene. *AtFLS1, 3,* and 5 were expressed in the root-shoot transition zone of 3-day-old seedlings and along the length of the roots at 9 days (**Figure 3-4A-C and G-I**). In 9-day-old seedlings, *AtFLS3* promoter activity was strongest in the vascular bundle, while the *AtFLS5* promoter was active from the vascular bundle up to, but not including, the epidermis, although it was not possible to resolve staining differences between the endodermis and cortex. Compared to *AtFLS3* and *5* in 9-day-old roots, *AtFLS1* expression appeared more sporadically, with no consistent expression pattern emerging in roots at this stage of development. *AtFLS1* and *3* root expression decreased in later vegetative stages, but *AtFLS5* was sporadically detected in various positions of older roots (data not shown). All three isoforms showed expression in initiating lateral roots, especially in young plants (**Figure3-4J-L**).

In vegetative shoots, *AtFLS1* promoter activity was consistently detected in young leaves, appearing in the upper epidermal tissues and especially concentrated in the youngest initiating leaves (near the shoot apical meristem), including the trichomes (**Figure 3-4M**). The *AtFLS3* and *5* promoters were also active in young leaves, but limited to trichomes for *AtFLS3* and the meristem for *AtFLS5* (**Figure 3-4N and O**). While *AtFLS1* expression was visible in leaf tissues for all transgenic lines that we investigated, this was not the case for *AtFLS3* and *5*, where expression was limited to a few lines each, consistent with the overall lower gene expression levels for these isoforms as assessed by RT-PCR. High levels of *AtFLS1* promoter activity were also detected in reproductive tissues, specifically in petals, stamens (filament and anther), carpels (stigma), and siliques (pedicel/valve junction), and sporadically through the perianth of young bud clusters and mature flowers (**Figure 3-4P-R**), consistent with the results of RT-PCR analysis. No *AtFLS3* or *AtFLS5* promoter activity was detected in any of these tissues. However, this is the one stage where *AtFLS2* promoter was observed, with the highest GUS activity occurring in the shoot apex at the base of the inflorescence bolt and in the pedicel/valve junction (**Figure 3-4S-U**), consistent with the RT-PCR experiments and the Genevestigator microarray database.

A hallmark of flavonoid genes such as CHS, CHI, and DFR is that their expression is strongly induced by a variety of environmental factors, including both biotic and abiotic factors that cause mechanical damage to the plant (for example, McKhann and Hirsch, 1994; Djordjevic et al.,

1997; Reymond et al., 2000; Richard et al., 2000; Peters and Constabel, 2002; Pang et al., 2005). One example is the induction of *CHS* gene expression in diverse plant species by *Orobanche,* a plant parasite that forms a physical connection with host roots and activates a variety of wound- and JA-inducible genes (Griffitts et al., 2004; J.A. Westwood, unpublished data). To test whether the *AtFLS* genes were also induced by infection with this parasite, the promoter-GUS plants were grown for 3 weeks in a semi-hydroponic system and then infected with *O. aegyptiaca* as described in Westwood (2000). Unlike the *AtCHS* promoter, which was strongly induced upon invasion of the *Orobanche* haustorium, the *AtFLS1-5* promoters did not exhibit any detectable activity in this assay (**Figure 3-4V-AA**). The *AtFLS1-5* promoters were also not induced when roots were accidentally damaged during handling, unlike the *CHS* promoter, which showed strong activation at sites of breakage (data not shown). A similar lack of wound inducibility of *FLS* genes relative to other flavonoid genes has recently been reported in *Populus*, and was suggested to reflect the lack of participation of FLS in the synthesis of condensed tannin defense molecules (Tsai et al., 2006). Therefore, although *AtFLS1* is coordinately expressed with other flavonoid genes during development (Pelletier et al., 1997) (**Figure 3-3**), it is also subject to distinct regulation in response to environmental factors.

iii. *In Vitro* Enzyme Activity of AtFLS1, 3, and 5.

In Arabidopsis, as in other plant species, the relative levels of quercetin and kaempferol vary substantially depending on the tissue and cell type (Peer et al., 2001; Tohge et al., 2005; Kerhoas et al., 2006; Stracke et al., 2007). To test the possibility that differential expression of AtFLS isoforms with distinct substrate specificities could determine the relative ratios of these two flavonols, AtFLS1, 3, and 5 enzymes were produced in *E. coli* as thioredoxin fusion proteins and assayed using a variety of substrates. Consistent with previous reports, AtFLS1 was very effective at converting dihydrokaempferol (DHK) to kaempferol (**Figure 3-5A**) (Wisman et al., 1998; Lukacin et al., 2003), while only a portion of the supplied dihydroquercetin (DHQ) was converted to quercetin by this enzyme under the same conditions (**Figure 3-5B**) (Turnbull et al., 2004). In addition, a portion of naringenin, normally the substrate for F3H, was converted to DHK by AtFLS1, some of which was subsequently converted to kaempferol (**Figure 3-5C**) (Prescott et al., 2002). Thus AtFLS1 exhibited a clear preference for DHK in these assays while, surprisingly, DHQ was used less well than even naringenin. However, neither AtFLS3 nor

AtFLS5 appeared to have enzyme activity with any of the substrates under a variety of conditions that included variations in pH, temperature, enzyme and substrate concentration, and enzyme enrichment and cleavage procedures (**Figure 4-5A-C** and data not shown). Close inspection of the primary sequences of the AtFLS proteins identified a region spanning approximately 30 amino acids that is present in AtFLS1 and all other plant flavonoid dioxygenases, but that is altered or absent in AtFLS2-6 (**Figure 3-6A**). Included in this region are arginine and glutamic acid residues (Arg25 and Glu29 in AtFLS1) that are invariant in all other plant dioxygenases, as well as numerous other residues that are strictly conserved among the flavonoid 2-ODD enzymes, FLS, F3H, ANS, and FNS1. To analyze this region on a structural level, homology models were constructed based on the crystal structure of Arabidopsis ANS (pdb id: 1GP4, Wilmouth et al., 2002), with which AtFLS1, 3, and 5 exhibit 37.8, 33.9, and 31.4% amino acid identity, respectively (**Figure 3-6B**). The root mean square deviation (RMSD) values for the homology models of AtFLS3 and AtFLS5 when compared to AtFLS1 were 1.23 and 1.48Å, respectively, indicating that the structures are quite similar overall, including the architecture of the jellyroll core. However, there appeared to be substantial differences in the positions of several key active site residues among these proteins. In particular, the Fe^{2+} coordinating residue Asp222 differed by 3.28Å between AtFLS 1 and 3, while the adjacent His220 varied by 2.55Å between AtFLS1 and 5. The largest apparent differences are near the N-terminus and in the largely unstructured C-terminus. The conserved 30 amino acids that are altered or absent in the AtFLS2-6 proteins comprise a region in the AtFLS1 model that contains a seven-residue α-helix (residues 26-31) near the mouth of the jellyroll motif and is otherwise largely unstructured. The absolutely conserved Arg25 is adjacent to this helix, while the conserved Glu29 is in the center of the helix. This structural element appears to be missing in AtFLS3 and AtFLS5. An additional N-terminal α-helix in the homology model (AtFLS1 residues 5-8) is also absent in AtFLS3 and is present, but appears to be positioned differently, in AtFLS5. Further evidence for the functional importance of this amino terminal region comes from analysis of expression constructs derived from AtFLS1 clone EST 153O10T7, which lacks the coding sequences for the 21 N-terminal amino acids (Pelletier et al., 1999). The truncation completely eliminates the first α-helix and the first seven residues in an unstructured region of the conserved 30 amino acid fragment. Protein produced from this construct had no activity with any of the tested substrates when assayed under the same

conditions as the full-length AtFLS1 (data not shown).

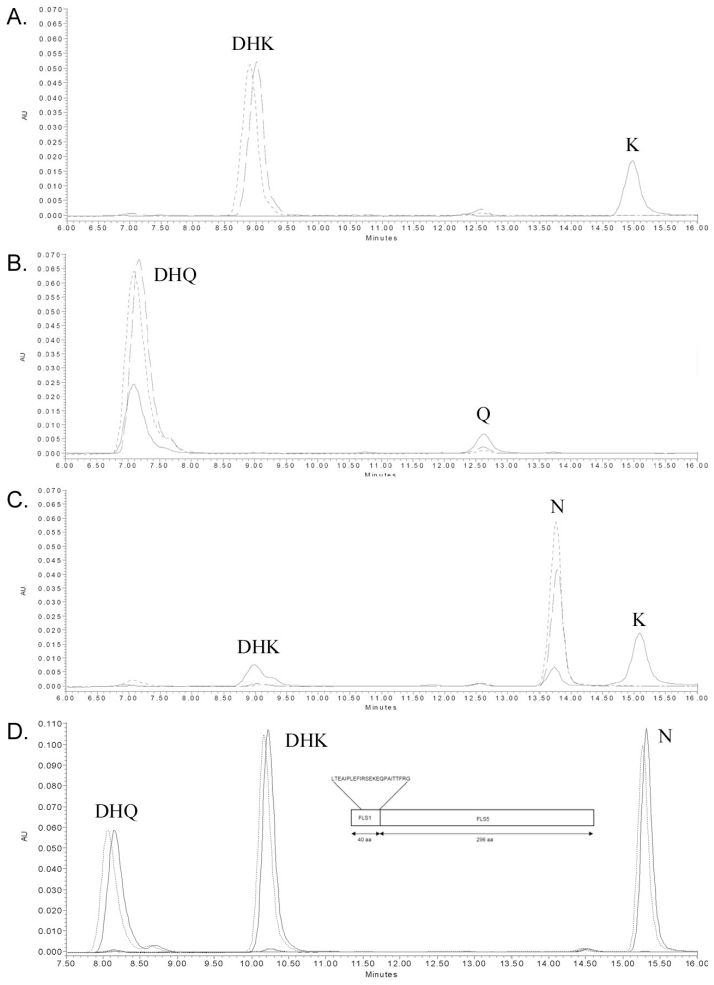

Figure 3-5. AtFLS1, AtFLS3, and AtFLS5 enzyme activity. Recombinant AtFLS1(—), AtFLS3 (----), and AtFLS5(····) proteins were assayed with the substrates DHK (A), DHQ (B), and naringenin (C). HPLC chromatograms extracted at 289 nm are shown, with peaks labeled as DHK, DHQ, K (kaempferol), Q (quercetin), and N (naringenin). D. Analysis of AtFLS1/AtFLS5 chimera. HPLC chromatograms of assays the AtFLS1/AtFLS5 chimera (—) and a thioredoxin negative control (----) with DHQ, DHK, and N or without substrate. Inset shows structure of the AtFLS1/AtFLS5 chimera formed from the 40 N-terminal amino acids of AtFLS1 and the 296 C-terminal amino acids of AtFLS5. Introduced amino acids are shown above the structure.

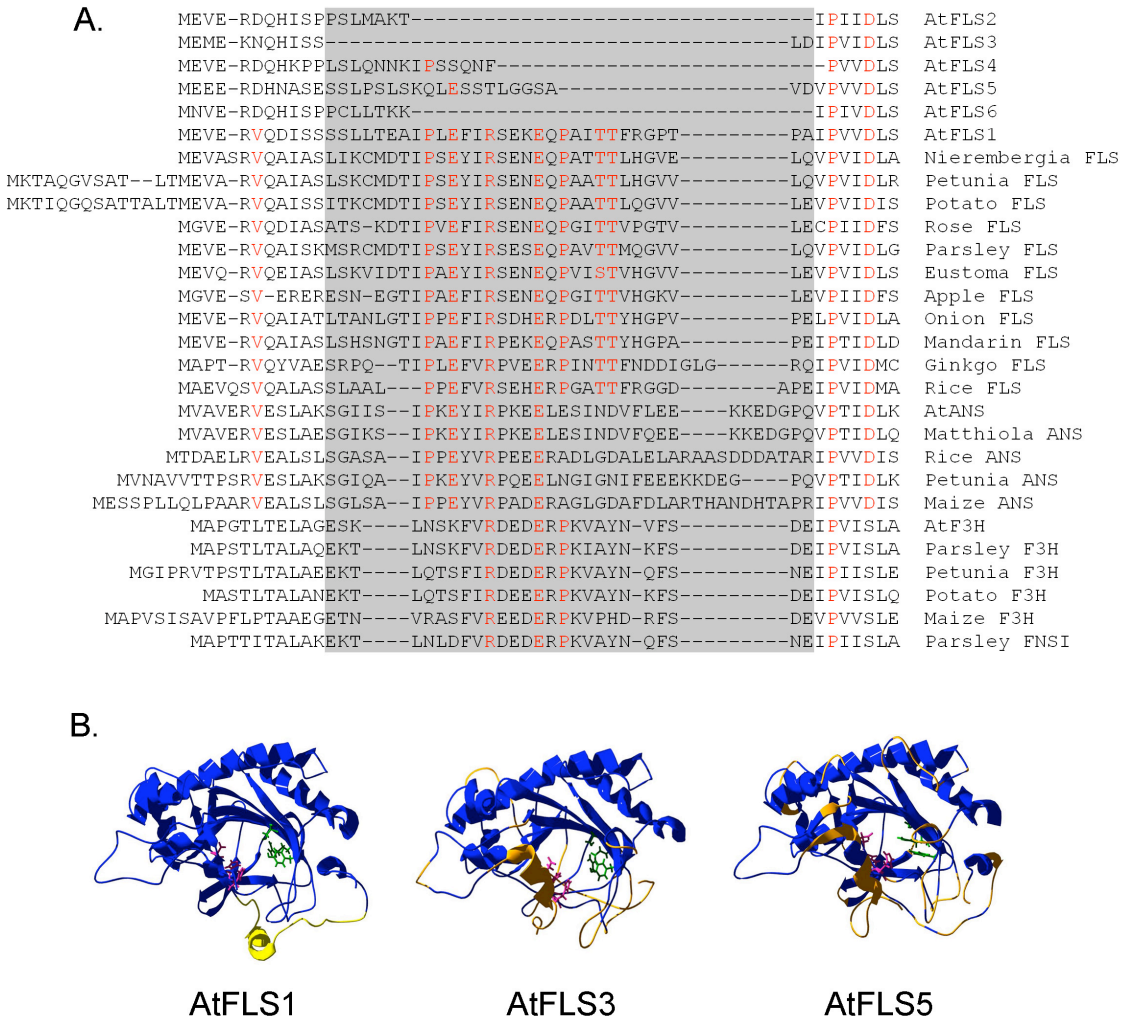

Figure 3-6. Structural analysis of the AtFLS1, 3, and 5 proteins. (A) N-terminal sequence alignment showing the highly conserved region that is altered or missing in AtFLS2-6 (highlighted in gray), including residues that are strictly conserved in the various enzyme subclasses (shown in red). (B) Homology models generated based on the crystal structure of AtANS (At4g22880) are shown looking into the core of the jelly-roll motif. The predicted Fe^{2+} coordinating residues are shown in pink, the α-ketoglutarate binding residues in green. The yellow region in AtFLS1 identifies the N-terminal fragment missing in all other AtFLS isoforms. Regions colored orange in AtFLS3 and 5 are those with RMSD values greater than 2.75Å relative to AtFLS1.

To test the possibility that the N-terminal region of AtFLS1 could restore the activity of the inactive AtFLS isoforms, a chimeric construct was generated in which the N-terminal 30 amino

acids of AtFLS5 were replaced with the first 40 amino acids of AtFLS1 (Figure 3-5D). However, the chimeric protein also had no detectable activity with any of the tested substrates. This indicates that the 21 N-terminal amino acids of AtFLS1 are required for activity in that enzyme, but are not sufficient to restore activity to AtFLS5. This suggests that the structural integrity of the remaining AtFLS5 gene product underwent further decay following loss of the critical N-terminal residues.

iv. Two-hybrid Analysis of Interactions with Other Flavonoid Enzymes

Table 3-1. Yeast 2-hybrid analysis of interactions between AtFLS1, 3, and 5 with other flavonoid enzymes.

	AtFLS1	AtFLS3	AtFLS5
CHS	+/-[1]	+/-	+/-
CHI	-/-	-/-	-/-
F3H	+/+	ND[2]	ND
DFR	+/+	-/-	-/+

[1] AtFLS fused to activation domain/AtFLS fused to binding domain.

[2] not determined.

The possibility that the FLS proteins may serve non-enzymatic roles as part of a flavonoid biosynthetic metabolon was investigated by yeast 2-hybrid analysis of potential interactions of AtFLS1, 3, and 5 with AtCHS, AtCHI, AtF3H, and AtDFR. The proteins were analyzed in all possible pairwise combinations, fused to either the activation or binding domains of GAL4 (Chevray and Nathans, 1992; Kohalmi et al., 1998). The observed interactions are summarized in Table 1. AtFLS1, 3, and 5 interacted with AtCHS when they were fused to the GAL4 activation domain, but not when fused to the bait domain. AtFLS1 also interacted with AtF3H and AtDFR in both configurations. The only other positive result was for AtFLS5 fused to the GAL4 bait domain with AtDFR. These findings are reminiscent of those reported previously for AtCHS, AtCHI, and AtDFR (Burbulis and Winkel-Shirley, 1999) and suggest that AtFLS1 may function as yet another component of a flavonoid multienzyme complex. Moreover, although AtFLS3 and AtFLS5 do not have measurable enzyme activity, these proteins appear to have retained the ability to physically interact with other members of the central flavonoid

biosynthetic pathway and could conceivably play structural and/or regulatory roles in flavonoid metabolism.

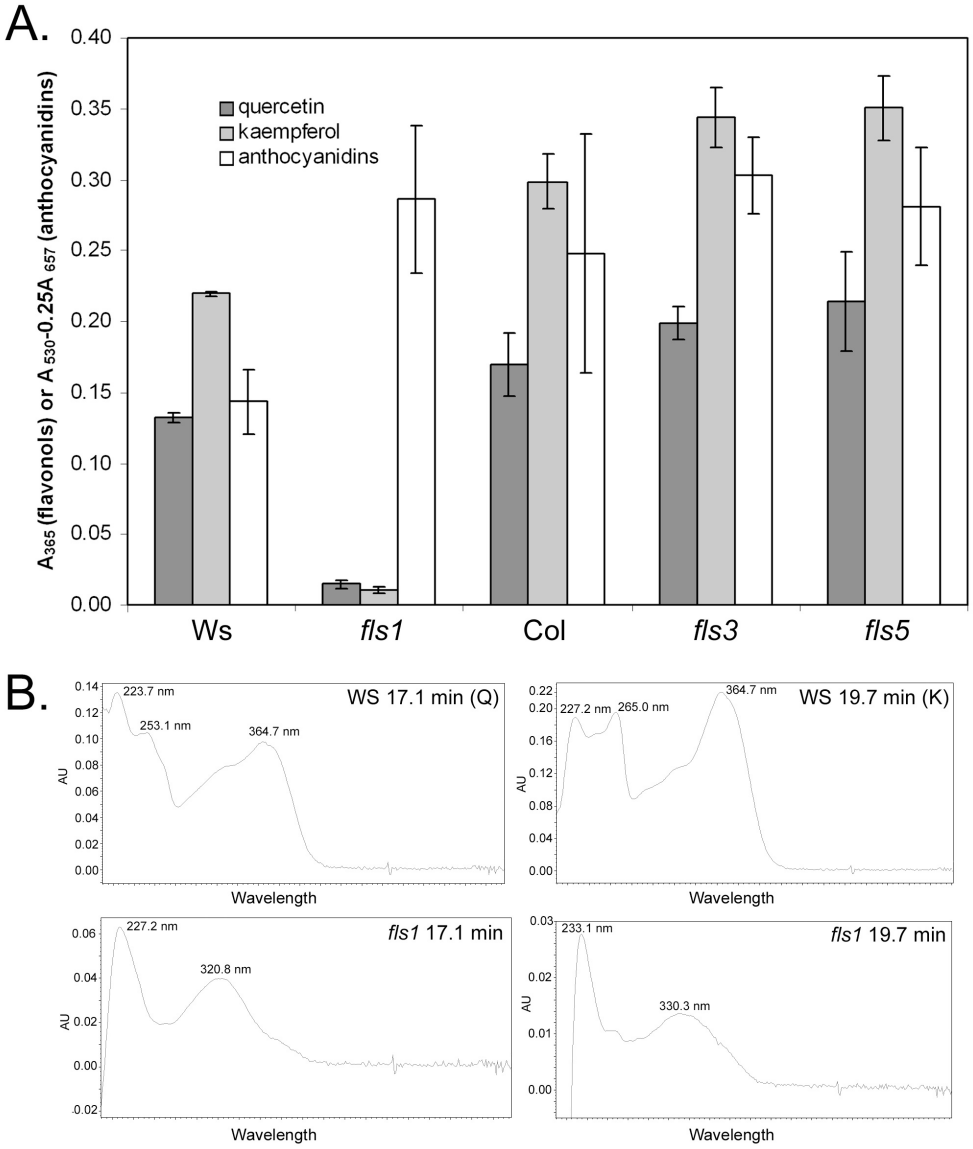

Figure 3-7. Effects of *fls1, fls3,* and *fls5* mutations on flavonol and anthocyanidin levels in 4-day-old seedlings. (A) Quercetin and kaempferol levels were quantified by HPLC by extracting chromatograms at 365 nm and integrating peaks corresponding to authentic standards. Anthocyanidin levels were determined spectrophotometrically. (B) Representative UV-Vis spectra for peaks in WS and *fls1* with retention times corresponding to (Q) and kaempferol (K) standards.

v. *AtFLS1-6* In Planta Gene Function

To further investigate the possibility that *AtFLS* genes play unanticipated roles in flavonoid biosynthesis *in planta*, knockout lines were identified for each of the genes in the SALK and GABI-KAT T-DNA collections (Alonso et al., 2003; Rosso et al., 2003). Homozygous lines were obtained in the Columbia background for *AtFLS2* (GABI 429B10), *AtFLS3* (SALK_050041), *AtFLS4* (SALK_002309), *AtFLS5* (GABI 317E12), and *AtFLS6* (SALK_003879) as described in the Methods. The only knockout candidate for *AtFLS1,* SALK_076420, was found to be embryo lethal in the homozygous state (data not shown). However, this insertion lies in the intergenic region shared by *AtFLS1* and a divergently-transcribed gene (At5g08630) that encodes a DDT domain-containing protein of unknown function. Two other T-DNA insertions, in the coding region of At5g08630 (SALK 004358 and 039219), were also homozygous lethal (data not shown), indicating that this phenotype in the SALK_076420 line was due to disruption of the adjacent gene, not *AtFLS1*. A line homozygous for an insertion in the 5'UTR of *AtFLS1*, AJ588535, was subsequently recovered in the Ws background from the INRA collection (Ortega et al., 2002).

Extracts were prepared from whole seedlings and from flowers, in which these genes were found to be expressed at high levels in the experiments described above. The insertion in *AtFLS1* resulted in a substantial reduction in peaks with retention times corresponding to those of quercetin and kaempferol and these peaks had different UV-vis absorption spectra than did those from wild type ws and the authentic standards (Fig. 7 and supplementary material). This suggested that these compounds in *fls1* were sinapate esters, not flavonols, similar to what is observed in Arabidopsis *CHS* and *F3H* null mutants (Li et al., 1993; Owens et al. 2008; Figure 3-7B). To examine this possibility further, seedling extracts were analyzed by LC/MS; surprisingly, small quantities of both quercetin and kaempferol were detected in *fls1* (Figure 3-S2). The *fls1* plants also exhibited a much more intense red coloration of the hypocotyl and cotyledons during germination and at the base of the stalk of mature plants compared to wild-type (data not shown). Analysis of anthocyanidin levels in seedlings showed that *fls1* seedlings accumulated approximately twice as much of these pigments per g of dry weight as the wild-type WS counterpart (Figure 3-7A). This apparent diversion of flux into neighboring branch pathways is similar to what has been reported for other flavonoid mutants, such as banyuls, which is deficient in ANS (Devic et al., 1999). In contrast, neither the *fls3* and *fls5* lines, nor any

of the other *FLS* mutant lines exhibited a detectable effect on flavonol or anthocyanidin accumulation, either in flowers or seedlings (**Figure 3-7A**, supplementary material, and data not shown). This suggests that only *AtFLS1* contributes to flavonol synthesis in Arabidopsis.

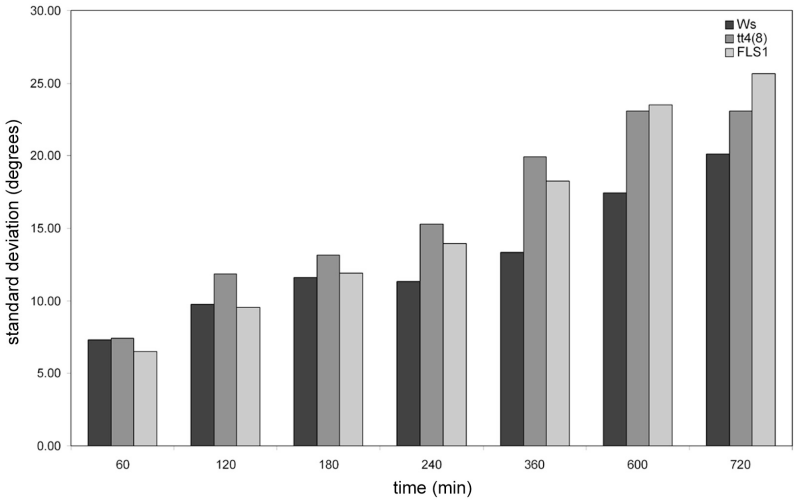

Figure 3-8. Effects of the *fls1* and *tt4*(8) mutations on root gravitropism. MS-sucrose agar plates containing 4-day-old wild-type and mutant seedlings were rotated 90º and root angles were measured relative to the original direction of growth. Bar graph shows the standard deviation in bending angle for the *tt4*(8) and *fls1* mutants versus the corresponding Ws wild type

The *fls1* mutant also provides a new genetic tool for exploring the role of flavonols in root gravitropism. Extensive work over the past several years with Arabidopsis *tt4* mutants has provided strong support for a model in which flavonoids, and flavonols in particular, function to slow auxin transport in specific cell files in order to cause root curvature (Brown et al., 2001; Buer and Muday, 2004; Lewis et al., 2007; Peer and Murphy, 2007). To provide further support for the specific role of flavonols, as opposed to other flavonoids, in this process, the gravity response of *fls1* roots was compared to that of *tt4*(8), an allele in the WS background, and the wild-type WS. Surprisingly, neither *tt4*(8) nor *fls1* exhibited a substantial difference in the response of roots to gravity relative to WS wild type (data not shown). This could reflect differences in the ecotype that was used (Ws versus Col) compared to previously-published experiments. However, both *fls1* and *tt4*(8) showed a distinct difference from WS in the amount of variation in the response among seedlings (Figure 3-8). This suggests that, although the overall response is similar in WS, with or without flavonols present, the precision of the response is much higher in the presence of these compounds. The similarity between *fls1* and *tt4*(8)

provides further evidence that it is flavonols, and not other flavonoids, that mediate root bending in response to gravity.

IV. Discussion

Gene families are common features of the genomes of complex organisms, including plants (Jander and Barth, 2007). Still, the finding that Arabidopsis genome contains six sequences with high homology to *FLS* was surprising in that all other flavonoid enzymes in this species appear to be encoded by single copy genes (Winkel, 2006). The presence of multiple *AtFLS* genes suggested the possibility of differentially-expressed isoforms with different substrate specificities. This could then explain the different relative levels of kaempferol and quercetin that are present in various tissues and under different environmental conditions in Arabidopsis (Peer et al., 2001; Ryan et al., 2002; Tohge et al., 2005; Kerhoas et al., 2006; Lea et al., 2007; Stracke et al., 2007). It was also consistent with the report by Wisman et al. (1998) that *En*-induced disruption of the *AtFLS1* gene abolished quercetin accumulation, but had no effect on kaempferol levels, suggesting another source of FLS activity was present.

We therefore carried out a thorough biochemical and genetic analysis of the six predicted *AtFLS* genes. Unlike the situation for the putative Arabidopsis cinnamyl alcohol dehydrogenase (CAD) multigene family, where the products of six genes had high CAD activity and three had low activity (and eight additional genes were simply misannotated) (Kim et al., 2004), in this case only one of six genes was found to encode a catalytically-competent protein. The products of the other five genes encode products that appear to lack critical functional residues, either as a result of premature stop codons (*AtFLS2* and *6*), alternative and mis-splicing (*AtFLS4*), or loss of a small region near the 5' end of the gene that may have resulted in further functional degeneration of the downstream sequences (*AtFLS3* and *5*). Thus, the theory that Arabidopsis uses different *FLS* genes to mediate differential synthesis of quercetin and kaempferol in different tissue or cell types appears to be incorrect. One possibility is that the FLS activity of the ANS enzyme may contribute to the differential accumulation of kaempferol and quercetin, as suggested by Lillo et al. (2008). Differential expression of the F3'H enzyme could also mediate these ratios, as illustrated by the large increases in kaempferol levels observed in petunia flowers expressing an antisense construct for *F3'H* (Lewis et al., 2006). In addition, recent work on the PAP1 and PFG1-3 R2R3-MYB factors indicates that this variation is regulated, at least in part, at the level

of gene expression, with a network of many different transcription factors interacting with the various flavonoid gene promoters to orchestrate differential biosynthesis of flavonoid products (Tohge et al., 2005; Stracke et al., 2007).

This network of transcriptional control also explains how *AtCHS* and *AtFLS1* may be coordinately regulated during development, but differentially expressed in response to parasitization by *Orobanche*. Even though flavonoids are not required for the parasitization process, in that the CHS mutant, *tt4*(2YY6) is just as efficiently parasitized as wild-type, CHS may still contribute to the localized production of flavonoids as part of the plant stress response system as parasitized *tt4* plants accumulated a lower root mass than wild-type controls (Westwood, 2000). The lack of induction of the *AtFLS1* promoter by the parasite suggests that this does not involve FLS activity, which is surprising since flavonols are known to have potent free-radical scavenging activity (e.g., Braca et al., 2003).

It also remains to be explained how the *fls1* T-DNA knockout line produced small quantities of quercetin and kaempferol at the seedling stage (**Figure 3-7 and 3-S2**), while *fls1 En* mutants accumulate quercetin, both in UV-treated mature plants and in seeds (Wisman et al., 1998; Routaboul et al., 2006). Although it appears that none of the other *AtFLS* genes can contribute FLS activity, it is possible that AtANS is able to do so (Turnbull et al., 2004; Lillo et al., 2008). Like AtFLS1, AtANS can produce flavonols at high efficiency *in vitro* from naringenin, DHK, and DHQ. The fact that AtANS is not able to fully substitute for AtFLS1 *in vivo* suggests that the intracellular organization and/or localization of the flavonoid pathway could restrict access of ANS to these intermediates. Interestingly, AtANS also produces quercetin via an alternative route, from its "natural" substrate, leucocyanidin (Turnbull et al., 2000; Turnbull et al., 2004). Because quercetin is the preferred product of this reaction *in vitro*, it has been suggested that the production of cyanidin glycosides involves channeling of the flav-2-en-3,4-diol intermediate directly from ANS to a flavonoid glycosyltransferase (Nakajima et al., 2001; Turnbull et al., 2003). This channel may be sufficiently "leaky" to allow some accumulation of quercetin, which is uncovered in the *fls1* mutant lines.

If the *AtFLS2-6* genes do not contribute to flavonol biosynthesis, then what drove the duplication of these genes at two sites far removed from *AtFLS1* in the Arabidopsis genome? Perhaps part

of the explanation has to do with the fact that AfFLS1 is located in a 1 Mb region exhibiting the second-highest level of evidence of recent positive selection; this region of the genome may thus have limited potential for diversification and the evolution of new gene function (Clark et al., 2007). Does the fact that the *AtFLS2-6* genes have apparently been maintained over substantial evolutionary time indicate that they once had, or still retain, functional importance? *AtFLS4* and *6* appear to be fully quiescent, non-functional pseudo-genes. However, *AtFLS2, 3* and *5* are still expressed in patterns that partially overlap with that of *AtFLS1*. Yeast two-hybrid assays suggest that AtFLS3 and 5 could compete with AtFLS1 for interactions with other proteins, perhaps during assembly and/or localization of the flavonoid enzyme complex.

The possibility also remains that all four expressed *FLS* genes have other as-yet-unknown functions. We recently reported that CHS and CHI are localized not just at the endoplasmic reticulum, but also in the nucleus (Saslowsky et al., 2005), and this now also appears to be the case for an (iso)flavone malonyltransferase from *Medicago truncatula* (Yu et al., 2008). These proteins may therefore have "moonlighting" functions similar to a growing list of enzymes in plants and other organisms with functions independent of their catalytic activities (Moore, 2004; Sriram et al., 2005). It should also be noted that the Arabidopsis genome also contains distant relatives of *CHS, CHI, F3H,* and *DFR*, although with much less similarity than for the FLS gene family (14-44% aa identity, and one exception, at 63%, for CHI; Table S3; TAIR 7.0 genome sequence, released 4-23-07; Swarbreck et al., 2008). The phenotypes of mutations in *CHS, CHI, F3H*, and *DFR* (*tt4, tt5, tt6,* and *tt3*, respectively) indicate that the distant relatives are unlikely to contribute directly to flavonoid biosynthesis. It therefore appears that the gene family model described for *FLS*, with one catalytically-active member and several "pseudogenes," may also apply to other flavonoid genes, particularly in the case of CHI. There is also a growing awareness that the "promiscuity" of metabolic enzymes such as ANS, as well as flavonoid glycosyltransferases (Lim et al., 2004) and *O*-methyltransferases (Deavours et al., 2006), is more the rule than the exception (Taglieber et al., 2007). These "alternative" functions of otherwise well-characterized proteins may well represent new paradigms that must be taken into account in efforts to develop framework models of cellular metabolism.

V. Materials and methods

Plant Material and Growth Conditions

Arabidopsis plants were grown in 7.5 x 5.5 x 5.5 cm pots containing Sunshine Mix #1 soil (Sungro Horticulture Processing, Bellevue, WA) in a climate-controlled incubator (I-66LLVL, Percival Scientific, Inc., Perry, IA) with a 16/8 hr photoperiod, 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent light, 20ºC constant temperature. The soil was amended with Osmocote controlled release fertilizer (Scotts Inc., Marysville, OH) or weekly fertilizing with 0.015 % (w/v) Miracle-Gro® 15-30-15 (Scotts Inc.). Under these conditions, inflorescence development was prominent at 6 weeks after planting. Seedlings for analysis of flavonol and anthocyanidin content were grown on the surface of MS/sucrose/agar plates under continuous light as described previously (Saslowsky and Winkel-Shirley, 2001).

Sequence Analysis of the *AtFLS* Gene Family

Sequences for the six members of the *AtFLS* gene family in Col-0 (*AtFLS1*-At5g08640; *AtFLS2*-At5g63580; *AtFLS3*-At5g63590; *AtFLS4*-At5g63595; *AtFLS5*-At5g63600; *AtFLS6*-At5g43935 were obtained from The Arabidopsis Information Resource (TAIR) and analyzed using Lasergene® (DNAStar, Inc.). Gene maps were prepared by comparing Col and L*er* sequences using published ESTs (TAIR) and cloned L*er* cDNAs (see below, *AtFLS* cloning).

Construction of *AtFLS* Promoter-*GUS* Reporter Gene Fusions, Arabidopsis Transformation, and Histochemical Localization of GUS Activity

Intergenic regions upstream of the start codon of each *AtFLS* isoform (1002 bp for *AtFLS1*; 1109 bp for *AtFLS2*; 768 bp for *AtFLS3*, 645 bp for *AtFLS4*, and1374 bp for *AtFLS5*) were amplified from L*er* genomic DNA by PCR using Elongase (Invitrogen Corp., Carlsbad, CA) or Taq polymerase, incorporating a *Sph*I site in the forward primers and *Hind*III in the reverse primers (Table S1). The fragments were first cloned into pBluescript KS+ (Stratagene) and sequences confirmed prior to subcloning into the *Bam*HI/*Hind*III sites in the binary vector, pBI121 (Clontech), replacing the CaMV 35S promoter. *AtFLSpGUS* fusion constructs and positive and negative controls (pBI121 and pBI101 vectors, respectively) were introduced into *Agrobacterium tumefaciens* (GV3101) and then used to transform L*er* plants by the floral dip method (Clough and Bent, 1998). Ten independent transgenic T_1 lines were selected for each construct and control vector on solid Murashige-Skoog growth medium with 0.005 % (w/v)

kanamycin. Transgenic lines were confirmed by PCR, using the forward primers (Table S1) with a reverse primer complementary to the 5' end of the B-glucuronidase gene, 5'-ACTTTGCCGTAATGAGTG-3'.

To test for the expression of promoter-GUS constructs, T_2 plants were grown on soil (6 plants per pot) for 3, 9, 16, 23, 30, 37, 44, and 51 days as described above or in a semi-hydroponic system for infection with *Orobanche aegyptiaca* as described in Griffitts et al. (2004). GUS activity was assayed using a histochemical procedure modified from Sieburth and Meyerowitz (1997). Plants were submerged in 90% (v/v) 4ºC acetone for 15 min, rinsed in water, and soil particles were removed from roots using forceps. Plants were then blotted on tissue paper, placed into microcentrifuge tubes, covered with staining solution [50 mM phosphate buffer pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide, cyclohexylammonium salt, Gold Biotechnology)], vacuum infiltrated three times for 30 sec at 5 to 10 Torr, and then incubated overnight at 37ºC. Chlorophyll was removed with subsequent rinses of 15%, 30%, 50%, 75%, 80%, and 100% (v/v) ethanol. GUS stained plants were then transferred to water in Petri dishes (16-day or older plants) or onto glass microscope slides and photographed using a digital camera system (3CCD, MIT) mounted on a dissecting microscope (Stemi SVII Apo, Zeiss).

Determination of *AtFLS* Gene Expression by RT-PCR

Tissues from two independent biological replicates of representative developmental stages were flash-frozen in liquid nitrogen and stored at -80ºC. Total RNA was extracted using an RNeasy Plant Mini Kit with optional DNase treatment (Qiagen Inc., Valencia, CA). cDNA was prepared from 5 µg total RNA in 33 µl final volume using the *NotI*-$d(T)_{18}$ primer and other standard components included with the First-Strand cDNA synthesis kit (Amersham Biosciences). The resulting cDNA served as a template for PCR amplification of either 0.3 kb of each *AtFLS* isoform or 0.5 kb of *β-tubulin* (At5g62690, Chen et al., 2003) as a control using the primers listed in Table S1. RT-PCR reactions contained 20 pmol of each primer, 2 mM deoxynucleoside triphosphate, 0.5 units Taq polymerase (New England BioLabs), and template cDNA (either 2.5 µl cDNA for *AtFLS* reactions or 0.5 µl cDNA for *β-tubulin* reactions). Reactions used the following parameters: 94ºC for 2 min, 26 cycles of 94ºC for 30 s, 60ºC (*AtFLS1*, *AtFLS2*, *β-tubulin*) or 56ºC (*AtFLS3*, *AtFLS5*) for 30 s, and 72ºC for 1 min. cDNA clones for *AtFLS1*,

AtFLS2, *AtFLS3*, and *AtFLS5* in pBluescript KS+ (described below) were used as a positive control for cDNA amplification at 56ºC and 60ºC (50 ng vector per reaction). These constructs were also used to confirm the specificity of the primers. A second independent (biological) replicate of this experiment was performed and produced similar results.

AtFLS Cloning, Expression, and Activity Assays

The *AtFLS1* coding region was amplified by PCR from cDNA generated using the iScript™ cDNA synthesis kit (Bio-Rad) and RNA isolated with the RNeasy plant mini-kit (Qiagen) from 15-day-old Arabidopsis Ler roots. The *AtFLS2* coding region was amplified by PCR from Arabidopsis Col EST clone SQ202h01 (Accession No. AV564339). *AtFLS3* and *AtFLS5* were amplified by RT-PCR utilizing RNA isolated from 4-day-old Arabidopsis Ler seedlings using the method described by Pelletier et al. (1996). All reactions used primers that incorporated *Eco*RI and *Xho*I sites (Table S1) to allow cloning into the corresponding sites in pET32a (Novagen).

An *AtFLS1/AtFLS5* chimeric construct was generated by amplifying a 925 bp fragment from the *Sph*I site in pET32a through the first 120 bp in *AtFLS1* using the primers shown in Table S1. The product was used to replace the corresponding fragment in pET32a-*AtFLS5*. The integrity of all clones was confirmed by DNA sequencing. It should be noted that, although the *AtFLS1, 3,* and *5* sequences were derived from the Ler ecotype, identical products are encoded by the corresponding genes in Col.

The pET-FLS constructs were used to transform BL21(DE3)pLysS cells and produce recombinant protein essentially as described in Pelletier, et al. (1999). Expression was induced by the addition of IPTG to 1 mM final concentration and incubation for 4 h at room temperature, 250 rpm. Similar levels of expression were observed for all of the constructs, as assessed by SDS-PAGE. Cells were harvested by centrifugation at 7400 x g, 4ºC, for 10 min, and stored at -80ºC. Frozen cells were resuspended in 3 ml of 0.2 M glycine (pH 8.5) and lysed by sonication on ice. The resulting cell slurry was centrifuged at 16,170 x g, 4ºC, for 40 min and the supernatant used as the source of crude enzyme in activity assays.

FLS Activity Assays

The FLS assay was based on the method of Britsch and Grisebach (1986). Each 100 µl reaction contained 10 mM α-ketoglutaric acid (disodium salt), 10 mM ascorbic acid, 0.25 mM ferrous sulfate, 50 mM glycine (pH 8.5), and 0.1 mM substrate. All flavonoid compounds were dissolved in 80% HPLC-grade methanol at a starting concentration of 10 mM. The ferrous sulfate solution was prepared in 50 mM HEPES, pH 7.5 containing 10 mM ascorbic acid to inhibit oxidation of Fe^{2+}. All other assay components were suspended in 50 mM HEPES, pH 7.5. The solutions were degassed under vacuum for 10 min, equilibrated under N_2 for 5 min, and again degassed under vacuum for 10 min immediately before use.

Activity assays were performed at 25ºC for up to 60 min using crude extracts containing similar amounts of each recombinant protein (approximately 3.5 to 100 µg, depending on the experiment, as assessed by comparison to a dilution series of bovine serum albumin on a Coomassie blue-stained SDS-PAGE gel). Reactions were initiated by the addition of substrate and terminated by extraction with ethyl acetate (1:1, v:v), performed by adding 200 µl of ethyl acetate and mixing well for 1 min. Solvent layers were separated by centrifugation at 13,000 rpm for 5 min. A 100 µl aliquot of the organic layer was then re-extracted with another 200 µl ethyl acetate and 200 µl of the organic layer combined with the initial 100 µl extract (R. Lukacin, personal communication). The solvent was evaporated in a SpeedVac under low heat. Dried samples were reconstituted in 50 µl of 80% methanol, mixed for 5 min, and spun at 13,000 rpm.

Supernatants were analyzed by HPLC using a Waters system with a 2996 photodiode array and Millenium 3.2 or Empower 2 software. Samples were kept at 4ºC prior to analysis. A 20 µl aliquot was injected and fractionated at room temperature as described in Pelletier and Shirley (1996) except that the absorbance was monitored from 200 to 600 nm. The resulting data were analyzed by extracting a single wavelength chromatogram at 289 nm; an unidentified peak that co-elutes with DHQ was subtracted from all of the samples.

Protein Structure Modeling

Homology models were generated for AtFLS1, AtFLS3, and AtFLS5 based on the crystal structure of Arabidopsis ANS (Wilmouth et al., 2002). The sequence of each protein was aligned with ANS and five models generated using MODELLER6 essentially as described in

Dana, et al. (2006). These five structures were then combined by coordinate averaging with the first structure used as the reference and overlay was on the backbone to generate a single structure. The resulting average structure was subjected to 500 steps of steepest descent minimization using the Sander module of AMBER7. The structure was solvated and the net charge of the system brought to zero by the addition of Na^+ atoms using LeaP. Equilibration was performed on the water and counter ions by molecular dynamics at constant volume for 100 ps. The solvent and counter ions as well as the entire system were each subjected to 500 steps of steepest descent minimization. All molecular dynamics calculations were performed using the AMBER 94 force-field with a time step of 2 fs and coordinates collected every 1 ps. Molecular dynamics consisted of an 80 ps heating phase to raise the temperature from 0 K to 300 K, a 100 ps constant volume equilibration, and a 1 ns constant pressure phase. All calculations were performed using up to eight processors on Virginia Tech's Laboratory for Advanced Scientific Computing and Applications Linux cluster (Anantham). Final models were generated by coordinate averaging from the last 100 ps of dynamics simulation and minimization data. The solvent and Na^+ ion coordinates were removed from the analyzed files using the Vi text editor to improve visualization of the model. Models were visualized and analyzed using DeepView/Swiss-Pdb Viewer v3.7 sp5 and rendered with POV-ray v3.5. Structural comparisons were performed by aligning the isoform homology models using the Deep View iterative magic fit function, and calculating the corresponding RMSD values.

Yeast Two-Hybrid Analysis

Coding regions for *AtFLS1*, *AtFLS3*, *AtFLS5*, and *AtDFR* were amplified from the pET32a constructs described above and for *AtF3H* from a pBluescript construct (Pelletier and Shirley, 1996) using the primers listed in Table S1. Each PCR product was then digested with either *Sal*I (*AtFLS1*, *AtFLS3*, and *AtDFR*), *Xho*I (*AtFLS5*), or *Pst*I (*AtF3H*) and *Not*I and then inserted into the corresponding sites in the yeast two-hybrid vectors, pBI880 and pBI881 (Kohalmi et al., 1998). Plasmids were transformed into *E. coli* DH10B cells by electroporation. The sequence integrity of all clones was confirmed by sequencing. HF7c yeast cells (Feilotter et al., 1994) were transformed simultaneously with bait and prey constructs essentially as described in Kohalmi et al. (1998). Several independent colonies from each transformation were used to inoculate -Leu -Trp broth and then cultured on -Leu -Trp -His solid medium at 30ºC.

Characterization of T-DNA Knockout Lines

Lines segregating for T-DNA insertions in the *AtFLS1, 2, 3, 4,* and *6* genes were obtained from the SALK and INRA collections; homozygous T-DNA knockout lines were obtained for *AtFLS2* and *AtFLS5* from GABI-KAT. These included INRA AJ588535 (insertion in 5'UTR of *AtFLS1*), SALK_076420 (*AtFLS1* promoter), GABI 429B10 (second intron of *AtFLS2*), SALK_050041 (third exon of *AtFLS3*), SALK_002309 (third exon of *AtFLS4*), GABI 317E12 (first intron of *AtFLS5*), and SALK_003879 (third intron of *AtFLS6*). Homozygous lines were identified/confirmed by PCR analysis using slight modifications of the method of Edwards et al. (1991) to extract genomic DNA from one large leaf from each plant. In the first method, extraction was in 750 µl of 50 mM Tris pH 8, 10 mM EDTA pH 8. Following incubation at 65ºC for 10 min, 200 µl of 5 M KOAc was added and the sample was incubated on ice for 20 min. The sample was then centrifuged at 13,000 rpm for 10 min, the supernatant was mixed with 750 µl isopropanol, spun at 13,000 rpm for 10 min, and the pellet rinsed twice in cold 80% ethanol. The pellet was then resuspended in 1 mM Tris, pH 7.5, and 0.1 mM EDTA for 15 min at 37ºC. In the second method, extraction was in 350 µl of 200 mM Tris pH 7.5, 25 mM EDTA pH 7.5. The samples were incubated at 65ºC for 10 min, centrifuged at 13,000 rpm for 10 min, and the supernatant mixed with an equivalent volume of isopropanol followed by 5 min incubation at room temperature. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min and then resuspended overnight in 100 µl ddH_2O. PCR was performed using 1-2 µl of each sample with the primers and annealing temperatures given in Table S2 in a total volume of 10-20 µl. PCR products were analyzed by agarose gel electrophoresis.

Anthocyanidin and Flavonol Assays

Four-day-old seedlings were collected in pre-weighed 2 ml cryo-tubes (Corning) containing two 3 mm diameter stainless steel balls, type 316 (Small Parts). Tissue was then flash-frozen in liquid nitrogen and freeze-dried for 36-48 hrs in a lyophilizer in the same tubes. For HPLC analysis of flavonols, 50 µl of 1% acetic acid in 80% methanol was added per mg of tissue dry weight. Samples were ground by agitation for 3 min in a 5G paint-mixer (IDEX) and then clarified by centrifugation at 13,000 rpm, 4ºC for 15 min. The samples were then hydrolyzed by the addition of an equal volume of 2N HCl, followed by incubation at 70ºC for 40 min. An equal

volume of 100% methanol was added to prevent precipitation of aglycones. Samples were again centrifuged at 13,000 rpm, 4ºC for 15 min and then analyzed by HPLC as described above for the FLS activity assays except that chromatograms were extracted at 365 nm. For spectrophotometric analysis of anthocyanidins, 30 µl of 1% HCl in methanol was added per mg of tissue dry weight. Samples were ground and clarified as above, except that centrifugation was at room temperature. The supernatant was mixed with 2/3 volume of ddH_2O and then back-extracted with an equivalent volume of chloroform to remove chlorophyll. Samples were centrifuged at 13,000 rpm for 10 min and the upper, aqueous phase mixed with two volumes of 60% extraction buffer/40% water. Absorbance at 530 and 657 nm was used to determine the relative levels of anthocyanidins in these samples as described by Mancinelli and Schwartz (1984). Three independent biological replicates were analyzed for each genotype.

Gravitropism Assays

Seedlings were grown on the surface of MS/2% sucrose/agar plates under continuous light at 23ºC for 4 days. Plates were rotated 90º relative to initial growth orientation and placed at room temperature under normal ambient light conditions. Seedlings were photographed every 30 min for the first 5 h, then every 60 min for another 5 h; a final photograph was taken at 12 h. Changes in the angle of root tips relative to the original orientation were measured using Photoshop and analyzed using Microsoft Excel.

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VIII. SUPPLEMENTAL MATERIAL

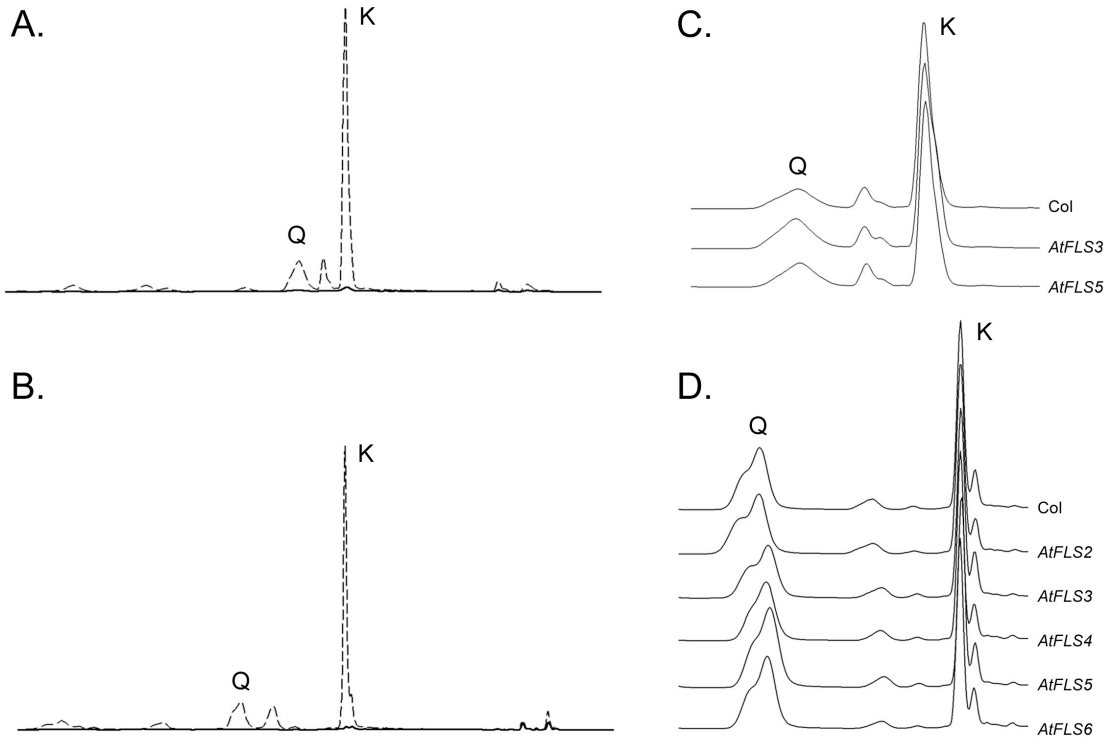


Figure 3-S1. Effects on flavonol accumulation of T-DNA insertions in the *AtFLS* genes. Extracts made from various tissues were analyzed by HPLC; chromatograms at 365 nm are shown overlaid in panels A and B and offset in panels C and D. Peaks were identified as quercetin (Q) or kaempferol (K) based on comparison with retention times and UV/vis absorbance profiles of authentic standards. **A**, Columbia (--) and *AtFLS1* (INRA AJ588535) (–) flowers; **B**, Ws (--) and *AtFLS1* (INRA AJ588535) 4-day-old seedlings; **C**, Columbia, *AtFLS3* (SALK_050041), and *AtFLS5* (GABI 317E12) flowers; **D**, Columbia and *AtFLS2* (GABI 429B10), *AtFLS3* (SALK_050041), *AtFLS4* (SALK_002309), *AtFLS5* (GABI 317E12), and *AtFLS6* (SALK_003879) roots.

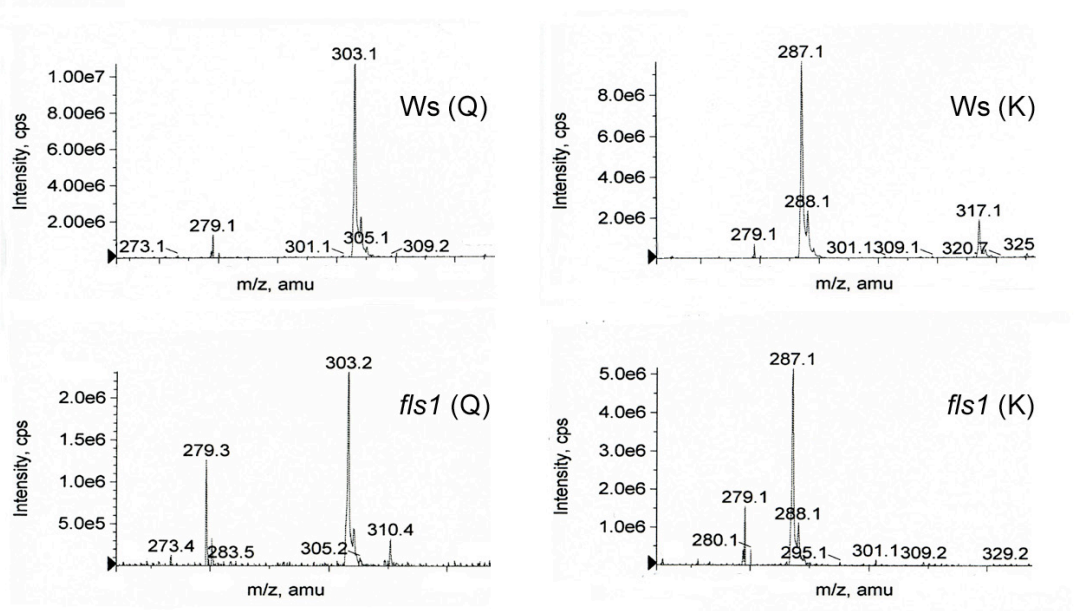


Figure 3-S2. LC-MS analysis of flavonols. Extracts from 4-day-old WS and *AtFLS1* seedlings were hydrolyzed to remove glycoside residues and then analyzed by LC-MS. Positive ion [(M+H)+] traces are shown for peaks that absorbed at 365 nm and had retention times corresponding to quercetin (m/z = 303) and kaempferol (m/z + 287).

Table 3-S1. Primers used in cloning and RT-PCR

Cloning of promoters into pBI121		
AtFLS1	sense	CGCGCATGCGAATGCGCGTCGTCGAG
	antisense	CGCGGATCCTATTTTTTTTGGTAGTTTTC
AtFLS2	sense	CGCGCATGCAATGTTCCCAACCCTGAG
	antisense	CGCGGATCCAATGGTCGCTGGAAATAATG
AtFLS3	sense	CGCGCATGCCTTCTTTGTCGTTGTATG
	antisense	CGCGGATCCGGTTTTCAGTTTCACTCG
AtFLS4	sense	CGCGCATGCGAAAACCCTCAATACCTAC
	antisense	CGCGGATCCGATTCTAATTTTTTTGTGATC
AtFLS5	sense	CGCGCATGCAGGTAAAATCGAGATCAT
	antisense	CGCGGATCCTGCTTCATAGGTGTGAG
RT-PCR		
AtFLS1	sense	AAGGATACGGAACAAAGCTTC
	antisense	CATATACTCCGCCATCTCTC
AtFLS2	sense	GGTTACACGACGAATCTCAAG
	antisense	ATCCTCATTACATACTCGGTC
AtFLS3	sense	GGATCGACGCGGAGTATAC
	antisense	GAAATTTGTAGTCTTTGTAGAC
AtFLS5	sense	TATCTAGGAGGTATAAACAATTG
	antisense	TAAGCACATACTCTGCCGTG
β-tubulin	sense	TCAAGAGGTTCTCAGCAGTAC
	antisense	CCTTCTTCATCCGCAGTTG
Amplification of coding regions for cloning into pET32a		
AtFLS1		CGCGAATTCATGGAGGTCGAAAGAGTCC
		CGCCTCGAGTCAATCCAGAGGAAGTTTATTG
AtFLS2		CCGGAATTCATGGAAGTTGAGAGAGA
		CCGCTCGAGTCACTCGATAGGAAGTT
AtFLS3		CCGGAATTCATGGAGATGGAGAAAAAC
		CCGCTCGAGTCAGTCGAGAAGAAGCT
AtFLS5		CCGGAATTCATGGAAGAAGAGAGAGA
		CCGCTCGAGTCAGTAGACAGGAAGAT
AtFLS1/5		CGGCATGCAAGGAGATGG
chimera		5’CGGTCGAC-GTTGGACCTCGGAATG3’
Amplification of coding regions for cloning into two-hybrid vectors		
AtFLS1		CGGTCGACGATGGAGG-TCGAAAG
		AGCGGCCGCTCAATCCAGAGGAAG
AtFLS3		CGGTCGACCATGGAGATGGAG
		AGCGGCCGCTCAGTCGAGAAGAAG
AtFLS5		5’CCGCTCGAGCATGGAAGAAGA-GAGAG
		AGCGGCCGCTCAGTAGACAGGAAG
AtDFR		ATGTCGACGCTGCAGGAATTCATG

	AGCGGCCGCGGCACACATCTG
AtF3H	GCTGCAGA-TGGCTCCAGGAACTTTG
	AGCGGCCGCAGCGAAGATTTGGTCGAC

Table 3-S2. Primers and annealing temperatures used to identify/confirm homozygous FLS knockout lines.

FLS1 (INRA AJ588535)		
FLS1 wt s1	ATTAGAATGGCCAACCAAAGTTTGTT	
FLS1 a1	CTTTCTTCGTCGGGATCGCTTAGATC	54.6°C
LB4 (INRA tDNA)	CGTGTGCCAGGTGCCCACGGAATAGT	
FLS1 wt s2	CTTTACAGTAATTAGAATGGCCAACC	
FLS1 a2	CGCACGCCTCACGCTTTCTT	53.8°C
Tag5 (INRA tDNA)	CTACAAATTGCCTTTTCTTATCGAC	
FLS2 (GABI-Kat 429B10)		
FLS2 s1	CAGTTTACCCTATTCAGACAAGACA	
FLS2 wt a1	CATTTTCATAGTATTAAAAGTTTCTGC	53.5°C
o08409 alt (GABI-Kat LB)	CATATTGACCATCATACTCATTGCT	
FLS2 s2	GGAACAGTTTACCCTATTCAGACA	
FLS2 wt a2	CTATTACTAAATCATTCAACTATTCCTAT	53.2°C
GABI-Kat LB2	TATAATAACGCTGCGGACATCTAC	
FLS3 (SALK 050041)		
Salk_050041_Sense	ATGGAGATGGAGAAAAACCAAC	
Salk_050041_Antisense	GTATAAAGGTTATCGTGTATCTC	60°C
LBc1	AAAAGAAAAACCACCCCAGTAC	
FLS4 (SALK 002309)		
FLS4_gene_sense	CGCGAATTCATGGAGGTCGAGAGAGACC	
FLS4_gene_antisense	CGCCTCGAGTTAAAGGCTCGAACTTGGGAG	60°C
LBc1	AAAAGAAAAACCACCCCAGTAC	
FLS5 (GABI-Kat 317E12)		
FLS5 wt s1	GAAATTGACCCTAAGCACATACTCTG	
FLS5 a1	ATTTAAATCCCCTCTAGCCTTGTT	54.3°C
o08409 alt (GABI-Kat LB)	CATATTGACCATCATACTCATTGCT	
FLS5 wt s2	GGCTCCTATGACTAATTCCGTATCTTG	
FLS5 a2	CAGTTACCCTATTTAAATCCCCTCTA	54.1°C
GABI-Kat LB2	TATAATAACGCTGCGGACATCTAC	
FLS 6 (SALK 003879)		
FLS6 s1	CATATCTCCACCGTGTCTCCTAAC	
FLS6 wt a1	GCCGTAACCCTAACCCTTTTGATA	55.8°C
Salk LB4	AGGGCCAGGCGGTGAAG	
FLS6 s2	CGGTAAGCACGAGACAACAAAAATA	
FLS6 wt a2	CCATCACCACTCCCAAATAATACT	56.6°C
Salk LB3	GGACCGCTTGCTGCAACT	

Table 3-S3. Arabidopsis flavonoid gene homologs.

Gene name	Locus name	Genpept identifier	Homology with parent gene1
AtCHS	AT5G13930	15240753	100
	AT4G34850	18418528	14.2
AtCHI	AT3G55120	15233190	100
	AT1G53520	15220807	24.1
	AT1G60290	none	n/a
	AT1G60330	none	n/a
	AT2G26310	42569342	17.4
	AT3G63170	18412649	20.5
	AT5G05270	18414838	26.8
	AT5G66220	145359723	63.2
AtF3H	AT3G51240	15230433	100
	AT5G12270	18416826	26.1
AtFLS1	AT5G08640	15242339	100
AtFLS2	AT5G63580	15242825	61.8
AtFLS3	AT5G63590	15242827	69.7
AtFLS4	AT5G63595	22328029	58.6
AtFLS5	AT5G63600	79332035	57.5
AtFLS6	AT5G43935	22327556	67.0
AtDFR	AT5G42800	15239063	100
	AT4G35420	30690351	42.6
	AT4G27250	30687527	37.9
AtANS	AT4G22880	15235853	
	AT4G22870	15235850	97.32

[1]percent amino acid identity

[2]gene fragment adjacent to AtANS

Supplementary Methods

LC-MS Analysis of Plant Extracts

Hydrolyzed extracts were prepared from 4-day-old Ws and *fls1* seedlings as described for HPLC analysis of anthocyanin levels, except that the tissue was not lyophilized. Approximately 5-fold more *fls1* tissue was extracted in the same volume as for the Ws seedlings. These samples were then analyzed on an Agilent 1200 LC system using a Waters Nova-Pak C18 4 μm, 3.9 x 150 mm HPLC column with a gradient of deionized water containing 0.01% formic acid and 0.01% ammonium acetate (solvent A) and acetonitrile (solvent B). The column effluent was passed through a flow cell (absorbance was monitored at 365 nm) and then into a Turbo-V "turboionspray" source on a AB Sciex 3200 Q-Trap mass spectrometer, which was operated in the positive ion mode. Scanning was performed with the Enhanced MS function from 100 to 500 amu at a rate of 1000 amu/second. Standard ion source parameters were employed.

Chapter 4

Interaction of flavonoid enzymes in living cells: evidence for competition of key branch point enzymes for association with the entry-point enzyme, chalcone synthase

Kevin C. Crosby[1], Theodorus W. J. Gadella, Jr[2], and Brenda S.J. Winkel[1]*

[1]Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061-0390
[2]Swammerdam Institute for Life Sciences, Section of Molecular Cytology, Centre for Advanced Microscopy, University of Amsterdam, Kruislaan 316, 1098 SM, Amsterdam, The Netherlands

I. Introduction

A substantial corpus of research indicates that many aspects of cellular metabolism depend upon the assembly of biosynthetic enzymes into macromolecular complexes, sometimes referred to as metabolons (Ovádi and Saks, 2004; Ovádi and Srere, 2000; Srere, 1985; Srere, 1987). These metabolons can function to concentrate chemical substrates, channel potentially toxic intermediates between enzymes, regulate flux between competing branches of the pathway, and deliver products to where they are needed within the cell. Numerous examples of multi-component enzyme systems have been described. These include the TCA cycle, the tryptophan synthase compex, fatty acid synthase, and dhurrin biosynthesis (Beeckmans et al., 1990; Hyde et al., 1988; Jenni et al., 2006; Maier et al., 2008; Nielsen et al., 2008).

Our laboratory makes use of the flavonoid biosynthetic pathway in *Arabidopsis* as a model for the study of the architecture and dynamics of enzyme complexes. Flavonoids, which are derived from the larger phenylpropanoid pathway, are involved in a variety of essential functions including protection from ultraviolet light, the regulation of auxin transport (gravitropic response), defense against herbivores and pathogens, and in reproduction, as fruit and flower pigments (Winkel-Shirley, 2001; Winkel, 2004). The biosynthesis of these compounds is one of the most intensively-studied areas of plant metabolism, and therefore the genetics and biochemistry of the enzymes that comprise the core flavonoid pathway are relatively well understood. Furthermore, it has long been argued that the flavonoid biosynthetic enzymes might function as a larger metabolic complex (reviewed in Hrazdina and Jensen, 1992; Stafford, 1990; Winkel-Shirley, 1999). Experiments utilizing labeled precursors and intermediates not only served to elucidate the basic steps of phenylpropanoid and flavonoid biosynthesis, but also provided early evidence for the direct transfer of metabolites between the biosynthetic enzymes (Amrhein and Zenk, 1971; Czichi and Kindl, 1977; Hrazdina and Wagner, 1985; Jacques et al., 1977; Margna and Vainjarv, 1981). Cell fractionation studies led to the suggestion that this biosynthesis might occur along the endoplasmic reticulum (Fritsch and Grisebach, 1975; Hrazdina et al., 1978; Wagner and Hrazdina, 1984) This notion was buttressed by later immuno-EM results that showed that chalcone synthase (CHS), the first committed enzyme in flavonoid

biosynthesis, was localized along the cytoplasmic face of the rough ER (Hrazdina et al., 1987). These data led initially to a model in which the phenylpropanoid and flavonoid enzymes were proposed to form a loosely-associated, linear array along the ER, anchored by the cytochrome P450 hydroxylases, cinnamate 4-hydroxylase (C4H) and flavonoid-3'-hydroxylase (F3'H) (Hrazdina and Jensen, 1992).

A variety of experiments subsequently provided evidence for the ability of many of the enzymes of the core flavonoid pathway to undergo direct protein-protein interactions. Specifically, yeast two-hybrid experiments showed that CHS could bind to chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), and flavonol synthase 1 (FLS1) in an orientation-dependent manner. Additionally, interactions were detected amongst the downstream enzymes, CHI, F3H, FLS1, and DFR in these assays (Burbulis and Winkel-Shirley, 1999; Owens et al., 2008a; Owens et al., 2008b). Corroborating evidence for the associations among CHS, CHI, and F3H were provided by affinity chromatography and co-immunoprecipitation experiments utilizing plant extracts (Burbulis and Winkel-Shirley, 1999), while immunofluorescence and immuno-electron microscopy showed that CHS and CHI colocalize in epidermal and cortex cells of the *Arabidopsis* root (Saslowsky and Winkel-Shirley, 2001).

These results led us to revise the original linear model of flavonoid enzyme organization, proposing instead a dynamic globular complex involving multiple points of contact between proteins (Burbulis and Winkel-Shirley, 1999; Winkel-Shirley, 1999). Preliminary evidence from molecular modeling and surface plasmon resonance studies indicate that electrostatic interactions may play a role in the assembly of the flavonoid enzyme complex (Dana, Watkinson, and Winkel, unpublished data). Nevertheless, the precise nature of how these proteins interact to form a metabolic complex and regulate flux into competing branch pathways remains largely unknown, especially within the context of living cells. In this study, we utilized fluorescent resonance energy transfer (FRET), imaged by means of fluorescent lifetime imaging microscopy (FLIM), to probe the interactions of these enzymes in living plant protoplasts.

II. Results and Discussion

Three enzymes from the core flavonoid biosynthetic pathway, CHS, FLS1, and DFR were selected for this study; CHS catalyzes the first committed step in flavonoid biosynthesis, while FLS1 and DFR lie at a major branch point in the pathway and share common substrates (the dihydroflavonols) (**figure 4-1**). A series of fluorescent protein (FP) fusion constructs, all under control of a constitutive 35S promoter to allow for transient expression in *Arabidopsis* mesophyll protoplasts (Sheen, 2001), were made for each enzyme utilizing the cyan and yellow variants, SCFP3A and SYFP2 (Kremers et al., 2006), and the optimized red fluorescent protein, mCherry (Goedhart et al., 2007; Shaner et al., 2004). Using this system of constructs, we were able to sequentially test, in living plant cells, three of the interactions that had been identified previously using *in vitro* approaches. Additionally, we were also able to explore the possibility that the two downstream enzymes, FLS1 and DFR, might compete for CHS binding.

FLIM analysis showed a decrease in lifetime of FLS1-scfp3a when this protein was expressed together with either syfp2-CHS or CHS-syfp2 (**figure 4-2**). Although there was a difference in the weighted mean reductions across experiments depending upon the orientation of CHS vis-à-vis syfp2 (**figure 4-2, table 4-1**), these were minor. However, scfp3a-FLS1 showed only a small level of FRET as measured by change in lifetime, in the presence of either CHS acceptor construct. Immunoblot analysis using an anti-GFP antibody showed that a full length scfp3a-FLS1 fusion protein was being produced in protoplasts transfected with this construct (data not shown) and there is some indication that comparable levels of FRET occur between scfp3a-DFR and both syfp2-FLS1 and FLS1-syfp2 **(**supplementary material). Therefore it is likely that the scfp3a-FLS1 construct produces a stable, full-length protein, but that the fluorescent protein interferes sterically with the interaction of FLS1 and CHS. Another possibility is that FLS1-CHS binding is not compromised, but that the fp-FLS1 orientation simply positions the donor fluorophore such that its the r (distance) and/or k^2 (dipole-dipole orientation) relative to the acceptor inhibit FRET efficiency.

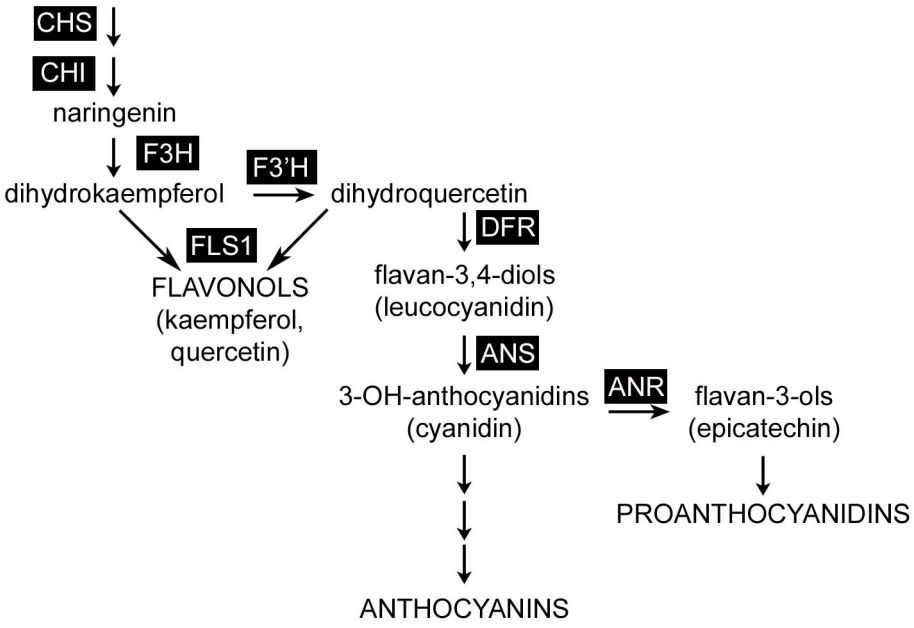
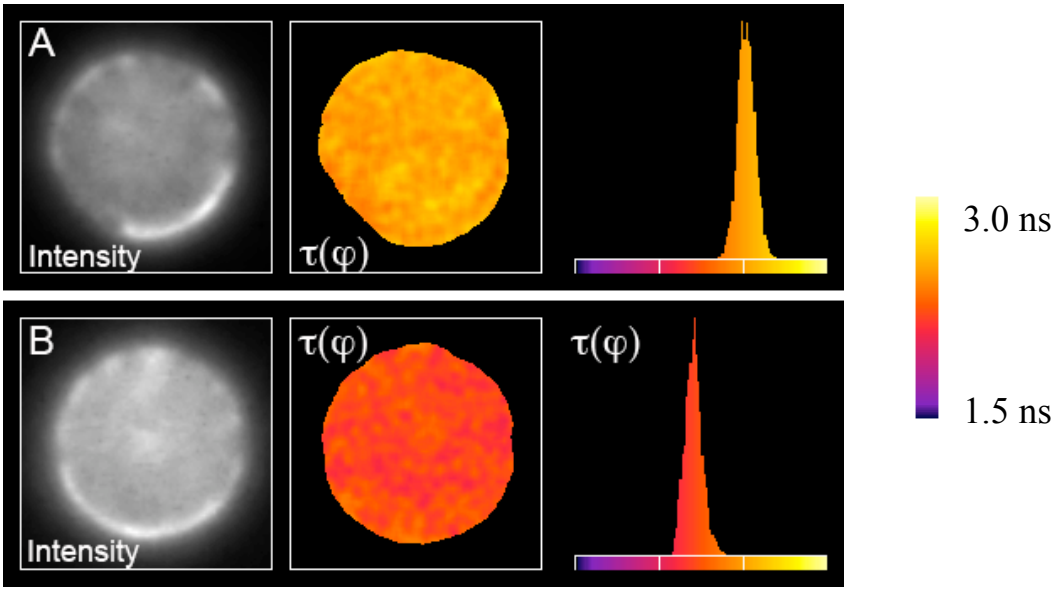


Figure 4-1: Schematic of the core flavonoid pathway in *Arabidopsis*. Enzyme names are shown in black boxes and the three major classes of flavonoid endproducts are indicated in capitals. Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase, FLS1, flavonol synthase 1; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase.

A.



B.

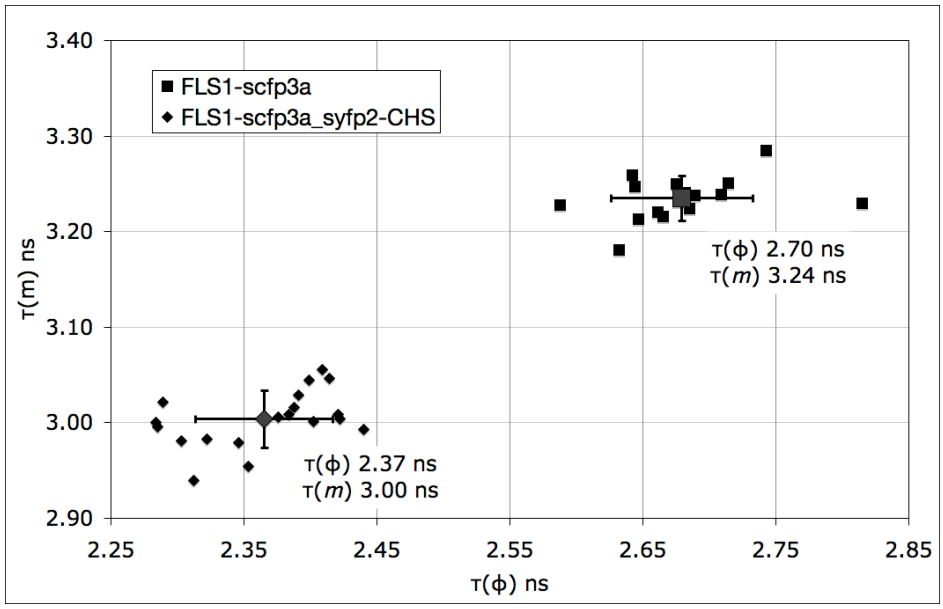


Figure 4-2: Interaction of FLS1 and CHS as shown by FLIM-FRET analysis. (A) Images from representative protoplasts showing intensity, phase lifetime map-τ(φ), and 1-D lifetime histogram for FLS1-scfp3a. Top panels (a) are images from a protoplast expressing FLS1-scfp3a alone and bottom panels (b) are from a protoplast expressing both FLS1-scf3a and syfp2-CHS. (B) Scatterplot showing distribution of phase lifetimes, τ(φ), plotted against modulation lifetimes, τ(*m*), for protoplasts expressing FLS1-scfp3a (smaller closed squares) and FLS1-scfp3a + syfp2-CHS (smaller closed diamonds). Mean lifetimes (larger square and diamond) and standard deviation for each sample set are also shown.

The co-expression of scfp3a-DFR with syfp2-CHS or CHS-syfp2 also showed a shift to shorter lifetimes from those seen for scfp3a-DFR alone, indicative of an interaction (**Table 1**). Once again, the orientation of the fluorescent protein with regard to CHS had little impact on the relative FRET levels. There were no C-terminal fusions constructs available for DFR, so it was not possible to determine if this alternative orientation would affect the efficiency of energy transfer.

Table 4-1 Tabulation of FRET data.

Donor	Acceptor	Exp	N_D	N_{DA}	$\Delta\tau(\varphi)$ ns	$\Delta\tau(m)$ ns	$E\ \tau(\varphi)$
FLS1-scfp3a	syfp2-CHS	2	40	44	0.28	0.13	10.8%
FLS1-scfp3a	CHS-syfp2	2	27	30	0.22	0.16	8.5%
scfp3a-FLS1	syfp2-CHS	1	13	8	0.16	0.05	6.6%
scfp3a-FLS1	CHS-syfp2	1	13	13	0.11	0.07	4.8%
FLS1-scfp3a	syfp2	1	17	17	-0.02	0.003	-0.7%
scfp3a-DFR	syfp2-CHS	3	59	65	0.25	0.07	10.4%
scfp3a-DFR	CHS-syfp2	2	23	19	0.24	0.03	10.3%
scfp3a-DFR	syfp2	2	43	39	0.05	0.07	2.3%

In the case of multiple experiments, the $\Delta\tau$ and E values represent the weighted means for those replicates

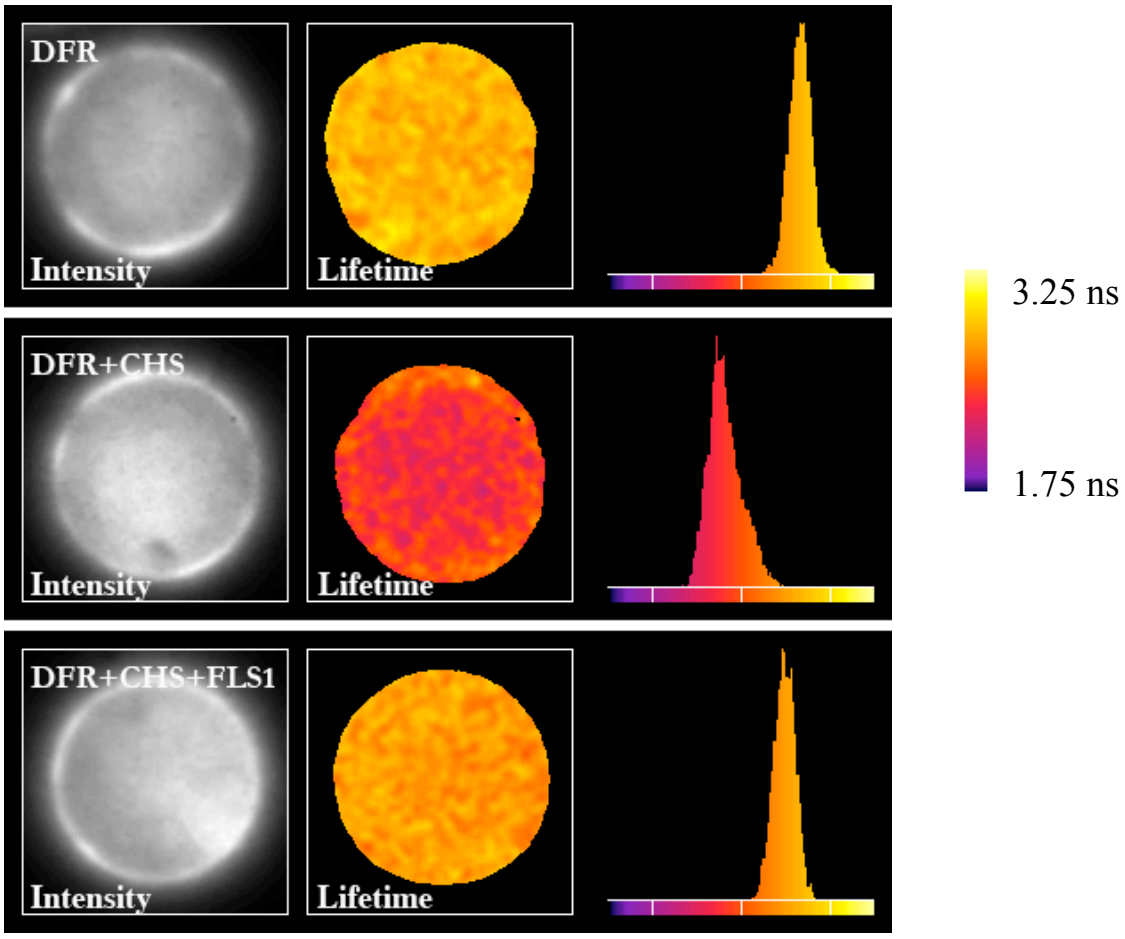
Results from FRET experiments are typically presented in terms of an efficiency (*E)* value. The formulae used to calculate this value vary depending upon the technique employed in making the FRET measurements. There is a standard calculation that is often employed to arrive at an *E* value for lifetime-based FRET measurements (**see methods**). Nevertheless, it is difficult to accurately quantify efficiency from these types of data. In intensity-based measurements, the contributions of individual components are linearly additive, making it possible to reliably calculate *E* for an ensemble of molecules. However, fluorescent decay is a non-linear process; therefore individual lifetime contributions are not additive (van Munster et al., 2005). Instead, in

cases where a fluorophore exhibits multi-exponential fluorescent decay, the lifetime calculations based on demodulation, $\tau(M)$, is more biased towards longer lifetime components than the lifetime based on phase-shift, $\tau(\varphi)$, resulting in a $\tau(\varphi) < \tau(M)$ (Gadella et al., 1993; van Munster et al., 2005). Furthermore, when there is a heterogeneous population of donor molecules, quenched (FRETing) and non-quenched (non-FRETing), non-quenched and partially quenched donors will contribute disproportionately to FLIM measurements, resulting in a trend that can be represented by $E > E(\varphi) > E(M)$ (Vermeer et al., 2004).

With these caveats in mind, FRET efficiencies based on $\tau(\varphi)$ were calculated for the FLS1/CHS and the DFR/CHS pairs (**Table 1**). While these E values (≈10%) are low compared to what is typically recorded for stable, high-affinity interactions (Kremers et al., 2006; Vermeer et al., 2004), they are comparable to what has been observed in studies of protein pairs in plant systems using time-resolved based methods (Adjobo-Hermans et al., 2006; Bayle et al., 2008). The fact that shifts in $\tau(M)$ are much less pronounced than those observed for $\tau(\varphi)$ for both the FLS1/CHS and the DFR/CHS pairs suggests that there are multiple populations of donor molecules exhibiting different levels of FRET. Such a heterogeneous population of donors is consistent with a model of the flavonoid metabolic complex that exists as a transient and dynamic entity, with a continuous association and disassociation of the component enzymes.

Having established that both FLS1 and DFR interact with CHS, we next sought to examine whether these two proteins might compete for CHS binding. For these experiments, syfp2-DFR and mCherry-CHS were used as the donor/acceptor pair, while FLS1-scfp3a was co-expressed as a competitor. The FP tag on FLS1 did not directly affect our FRET measurements (**see controls in Table 2)**, but make it possible to quantify expression of the competitor (see methods). A comparable decrease in lifetime was observed when expressing the syfp2-DFR/mCherry-DFR pair as was observer earlier with the scfp3a-DFR/syfp2-CHS pair. However, when FLS1-scfp3 was also expressed, the donor lifetime for syfp3a-DFR shifted to a value close to that of donor alone **(Fig. 3, Table 2**). This shift was not nearly as pronounced when scfp3a-FLS1, a construct

A.


DFR
Intensity
Lifetime
DFR+CHS
Intensity
Lifetime
DFR+CHS+FLS1
Intensity
Lifetime
3.25 ns
1.75 ns

B.

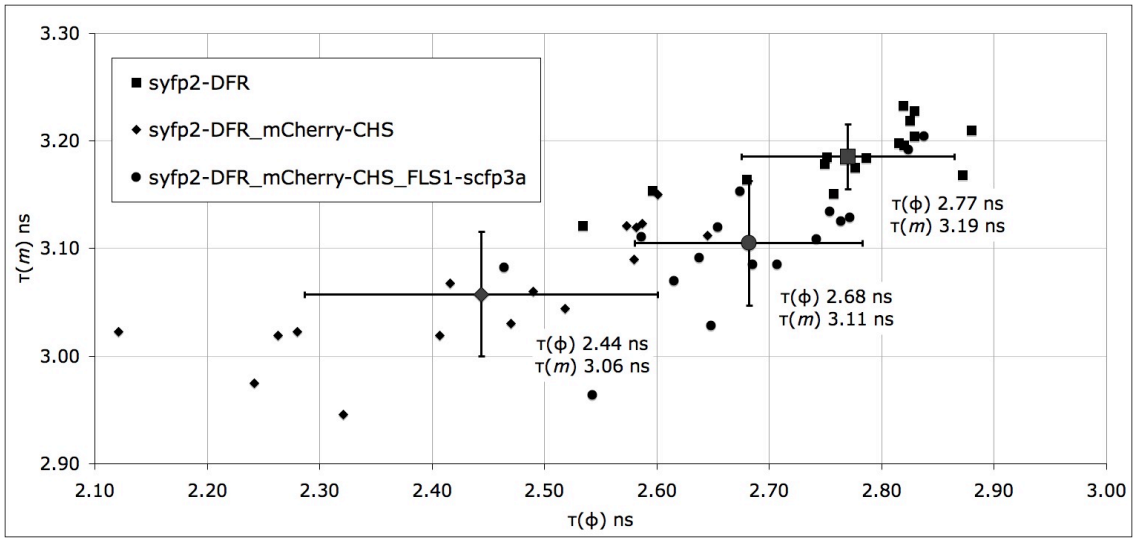

syfp2-DFR
syfp2-DFR_mCherry-CHS
syfp2-DFR_mCherry-CHS_FLS1-scfp3a
τ(ϕ) 2.77 ns
τ(m) 3.19 ns
τ(ϕ) 2.68 ns
τ(m) 3.11 ns
τ(ϕ) 2.44 ns
τ(m) 3.06 ns
τ(m) ns
τ(ϕ) ns
3.30
3.20
3.10
3.00
2.90
2.10
2.20
2.30
2.40
2.50
2.60
2.70
2.80
2.90
3.00

Figure 4-3: FLS1 interferes with DFR-CHS interaction. (A) Images for representative protoplasts showing intensity and lifetime data for syfp2-DFR. Top panels, syfp2-DFR; middle panels, syfp2-DFR+mCherry-CHS; bottom panels, syfp2-DFR+mCherry-CHS+FLS1-scfp3a. (B) Scatterplot showing distribution of phase lifetimes, $\tau(\varphi)$, plotted against modulation lifetimes, $\tau(m)$, for protoplasts expressing syfp2-DFR (smaller closed squares); syfp2-DFR+mCherry-CHS (smaller closed diamonds); syfp2-DFR+mCherry-CHS+FLS1-scfp3a (smaller closed circles). Mean lifetimes (larger square, diamond, and circle) and standard deviation for each sample set are also shown.

Table 4-2 Tabulation of competition data

Donor	Acceptor	Competitor	Exp	N_D	N_{DA}	N_{DAC}	$\Delta\tau(\varphi)_{DA}$	$\Delta\tau(\varphi)_{DAC}$	$E\,\tau(\varphi)_{DA}$	$E\,\tau(\varphi)_{DAC}$
syfp2-DFR	mCherry-CHS	FLS1-scfp3a	2	29	33	30	0.27ns	0.08ns	10.0%	2.8%
syfp2-DFR	mCherry-CHS	scfp3a	1	16	16	15	0.33ns	0.33ns	11.8%	11.8%
syfp2-DFR	mCherry-CHS	scfp3a-FLS1	1	16	16	17	0.33ns	0.12ns	11.8%	8.5%

In the case of multiple experiments, the $\Delta\tau$ and E values represent the weighted means for those replicates.

that showed only weak FRET with CHS (Fig. 2, Table 1), was co-expressed with the DFR/CHS pair. This suggests that FLS1 directly competes with DFR for binding to CHS.

Taken together, these findings provide support for a model in which the flavonoid biosynthetic enzymes assemble as a highly dynamic metabolic complex. FLS1 and DFR were both shown to interact with CHS in living protoplasts, which corroborates our previous *in vitro* data for associations between these enzymes (Burbulis and Winkel-Shirley, 1999; Owens et al., 2008a; Owens et al., 2008b). Because these protein interactions involve non-consecutive enzymes, our results provide further support for the existence of a globular superstructure with CHS functioning as a hub for the whole assembly. Furthermore, it appears that FLS1 and DFR bind to CHS in a mutually-exclusive manner. The structural basis for this interference, whether this is due to the two enzymes sharing a common binding site, overlapping binding sites, or some other mechanism, remains to be determined. Nevertheless, the functional implications of such a competition are clear. One of the proposed roles for metabolic complexes is to regulate

competition between branch pathways for common substrates. As DFR and FLS1 lie at a point of divarication between flavonols and the anthocyanins and proanthocyanidins, it is easy to envision one mode of regulating flux between these branches is by the association and dissociation of the relevant biosynthetic enzymes with a core complex.

It seems likely that multiple modes of regulation exist for controlling the types and amounts of endproducts that are produced within the flavonoid biosynthetic pathway at different times and locations within the plant. The expression of DFR and other enzymes involved in the synthesis of anthocyanins and proanthocyanidins is regulated differentially from CHS, CHI, FLS1, and other genes early in the pathway (Kubasek et al., 1998; Nesi et al., 2000; Pelletier et al., 1999; Pelletier and Shirley, 1996). There is recent structural evidence that DFR may be inhibited by some flavonols, the products of FLS1 (Trabelsi et al., 2008). Additionally, an association with CHS might be necessary to stabilize the DFR protein. Purified DFR is particularly unstable, and expression of the recombinant protein is problematic (Petit et al., 2007) while, in our experiments, expression levels - as judged by fluorescence - of both the scfp3a-DFR and the syfp2-DFR constructs were often higher when co-transfected with a CHS construct than when transfected alone in protoplast from CHS knockout plants (data not shown). Having many avenues of control, perhaps working in concert, may allow the cell to fine tune its response to developmental and environmental stimuli; clearly, further study is needed to unravel the intricacies of this regulation.

In summary, the data from these experiments provide us with the first evidence for the interaction of flavonoid enzymes in living cells. These experiments also suggest that FLS1 competes with DFR for binding to CHS, a result that supports a model that flux through pathway branch points may, at least in part, be controlled by the regulated association and dissociation of different biosynthetic enzymes with a core metabolic complex.

III. Materials and Methods

Plasmid constructs

Fluorescent proteins and multi-cloning sites (designated N1 and C1 for multi-cloning sites at the N- and C-terminus, respectively, of the fluorescent protein) were amplified from mammalian expression vectors (Kremers et al., 2006) using the primers 5'- CACCATGGTGAGCAAGG – 3' (forward C1), 5'-CACCGCTAGCGCTACC-3' (forward N1), and 5'-AAATGTGGTATGGCTGATTATGATC– 3' (reverse C1 and N1). The PCR products were then cloned into the Gateway entry vector, pENTR (Invitrogen) according to the manufacturer's protocol to generate syfp2-C1_pENTR, syfp2-N1_pENTR, scfp3a-C1_pENTR, and scfp3a-N1_pENTR vectors. cDNA sequences for CHS, FLS1, and DFR were amplified by PCR and cloned into pPCR-Script Amp SK(+) (Stratagene) using the primers 5'-CCCTTCAGATCTGTGATGGCTGGTG-3' (CHS forward C1), 5'-GGCGCGTGATCACTTGAGAGGAA-3' (CHS reverse C1), 5'-GCCCCCTCGAGCATGGTGATG-3' (CHS forward N1), 5'-AAGCTGGGTCCATGGGCCCAC– 3' (CHS/FLS1 reverse N1), 5'-CTTCTCCGGAGGGGTCGAAAGAG-3' (FLS1 forward C1), 5'-CACCCTTATCTAGAGGAAGTTTATTGAGC-3' (FLS1 reverse C1), 5'-CCCCCTCGAGCATGGAGGTC-3' (FLS1 forward N1), 5'-CACCAGATCTAGTCAGAAAGAGAC-3' (DFR forward C1), and 5'-CCCACCTCTAGAACACATCTGTTG-3' (DFR reverse C1). The following restriction sites were then used to clone the cDNA into the fp-pENTR vectors: *Bgl*II – *Bam*HI (CHS-C1), *Xho*I – *Nco*I (CHS-N1 and FLS1-N1), *Bsp*E1 – *Xba*I (FLS1-C1), *Xho*I – *Xba*I (DFR-C1). The resulting FP fusion constructs were then recombined using LR Clonase II enzyme mix (Invitrogen) into the plant expression vector, p2~gw7, which contains the enhanced cauliflower mosaic virus (CaMV) promoter and CaMV terminator. The p2~gw7 was created by removing the ECFP tag from the Gent Systems Biology vector p2CGW7 (Karimi et al., 2002)(see Supplementary Methods). The mCherry-CHS fusion construct was built by swapping the syfp2 sequences in the syfp2-CHS(C1) construct with the mCherry coding sequences (Shaner et al., 2004) from the mammalian expression vector mCherry-C1 (Goedhart et al., 2007) using the restriction sites *Nhe*I/*Spe*I (compatible overhangs) and *Bgl*II.

Preparation and transient transfection of *Arabidopsis* protoplasts

Arabidopsis mesophyll protoplasts were prepared from three to four-week-old Columbia wild-type or *tt4* (Salk 020583) plants grown under short-day (11 h light) conditions and transfected as described in Sheen, J. 2002 (http://genetics.mgh.harvard.edu/sheenweb/). Transfected protoplasts were incubated overnight for scfp3a/syfp2 pairs and for 36 hrs for syfp2/mCherry pairs prior to analysis.

Fluorescent lifetime imaging microscopy

Transfected protoplasts were mounted directly in eight-well Lab-Tek chambered coverglass (Nunc) for observation. Frequency-domain FLIM measurements were performed using the instrumental setup described in detail by van Munster and Gadella (Van Munster and Gadella,

2004). The instrument was calibrated using the average of three reference measurements of a freshly prepared 1 mg/ml solution of erythosine B (Sigma) with a known fluorescent lifetime of 86ps. The objective used was a Zeiss plan Neofluar 40x 1.3 NA oil-immersion. Samples with a scfp3a donor were excited with either a 442nm helium-cadmium laser (Melles-Griot) or a 440nm diode laser (PicoQuant) modulated at 75.1 MHz and a BP 440-500 nm emission filter. Samples with a syfp2 donor were excited using a 514 nm Argon laser (Melles-Griot) modulated at 75.1 MHz and a BP 530-560 emission filter. FLIM stacks of 12-36 phase steps permutated in recoding order to reduce artifacts due to photobleaching (van Munster and Gadella, 2004) with an exposure time from 100-900 milliseconds each. Software for the control, acquisition, processing, and analysis of the data was written in MATLAB 6.1 (Mathworks). Average lifetimes for individual cells and pseudo-colored lifetime maps and 1D histograms were generated by an ImageJ macro. Data was tabulated and graphs and tables were all prepared in Excel (Microsoft). FRET efficiency was calculated using the formula $E = 1-(\tau_{DA}/\tau_d)$.

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Chapter 5

Summary and Outlook

I.Context

The interior of the cell is a densely crowded and complex arena, full of a vast and diverse array of molecules and macromolecules. It is readily apparent that a fundament understanding of cellular physiology will depend not only upon a reductionist analysis of the chemistry, structure, and function of individual components and subsystems, but also a sagacious exegesis of the dynamic and emergent properties that characterize the higher-level system of living cells. Here, we have described work on two aspects of the supramolecular organization of the cell: the controlled assembly of the mitotic spindle during cell division and the regulation of cellular metabolism through the formation of multienzyme complexes.

II. Shaggy: Summary and Outlook

During division, the cell undergoes a profound morphological and molecular reorganization involving the creation of the mitotic spindle, a process that must be highly regulated in order to ensure that accurate segregation of hereditary material. In Chapter 2 we show results that implicate the kinase, Zeste-white3/Shaggy (Zw3/Sgg), the *Drosophila* homologue of the mammalian protein, Glycogen synthase kinase 3-β (GSK3-β), as having a role in regulating centrosome migration and by extension, overall spindle morphology. In the time since this research was done, a handful of papers have appeared in the literature investigating possible roles for GSK3-β during mitosis and cytokinesis, including Cheng et al. (2008), Izumi et al. (2008), and Saurin et al. (2008). (Cheng et al., 2008; Izumi et al., 2008; Saurin et al., 2008). Of particular relevance are two reports published within the last year that have helped to clarify how Zw3/Sgg might function in controlling centrosome positioning (Buttrick et al., 2008; Rusan et al., 2008).

The localization of the Adenomatous polyposis coli (APC) protein during mitosis has been shown to be regulated by Zw3 (McCartney et al., 2001). Researchers from Mark Peifer's group have tested the hypothesis that APC is required for neuronal polarity as well as axon growth and targeting in the developing central nervous system of *Drosophila* larvae (Rusan et al., 2008). While the investigators concluded that, contrary to the previously-accepted view, APC was not

necessary for these processes, they also observed that a significant number of dividing neuroblasts in larvae lacking functional APC exhibited a curved spindle phenotype identical to the aberrations we observed in the *zw3* mutants. Live cell time-lapse imaging revealed that these defects were generally corrected before the onset of anaphase, although the duration of mitosis was significantly prolonged.

In another study, James Wakefield's group studied the mitotic effects in early syncytial *Drosophila* embryos of mutations in the protein kinase, Akt/Protein Kinase B, which is an upstream regulator of Zw3/Sgg (Buttrick et al., 2008). Mutant embryos exhibited bent, ill-formed mitotic spindles and incomplete centrosome separation; some of these defects in centrosome migration were mitigated in embryos that had mutations in both *akt* and *zw3*, suggesting that Akt acts through Zw3 to modulate centrosome positioning. The localization patterns for the proteins APC2, Armadillo (Arm, the *Drosophila* homologue of β-catenin), and microtubule End-binding protein 1 (EB1), which are normally found concentrated at the cortical actin caps of the dividing embryo, were found to be disrupted.

Both these studies support a role for Zw3 in mitosis. The curved spindles described by Peifer's group convincingly corroborate our earlier findings. When they monitored cell division in living cells, these defects appeared to be transient, suggesting that there are backup mechanisms in place that allow the cell to compensate for errors in spindle formation caused by the lack of Zw3 and APC2 (Rusan et al., 2008). This would explain the prolonged duration of mitosis and the increased metaphase to anaphase ratio. In our study, we evaluated the robustness of the spindle assembly checkpoint by analyzing the rate of premature sister chromatid separation (PSCS) in *zw3* mutant larval neuroblasts. We found that these levels were elevated compared to wild type, but not nearly to the levels seen when there is a complete inactivation of the checkpoint (Basu et al., 1999; Scaerou et al., 1999; Williams and Goldberg, 1994). We originally weighed two possibilities: either Zw3 might function as part of the checkpoint, but was partially redundant with other players, or that Zw3 was not involved in the spindle assembly checkpoint and that the increased occurrence of PSCS was an indirect consequence of the defects induced by loss of Zw3 function. In light of the results shown by the Peifer laboratory (Rusan et al., 2008), it

appears that the latter is the case. The delay in cell cycle progression noted by their group and ours is indicative of a delay caused by the activation of the spindle assembly checkpoint and the triggering of an as-yet-unidentified corrective process.

The authors of the Akt study (Buttrick et al., 2008) propose that complete centrosome migration is dependent upon the attachment of microtubules to cortical actin, which in turn is mediated by a protein complex consisting of APC2, Arm, and EB1. This complex is stable as long as Zw3 remains inactivated by Akt phosphorylation. However, if Zw3 is in its active, unphosphorylated state, it can phosphorylate both APC2 and Arm, destabilizing the complex. In the absence of this anchoring complex, microtubules cannot form firm attachments to the actin of the cell cortex, inhibiting the ability of the motor protein, cortical dynein, to generate the force required to achieve complete centrosome separation. This model, although implicating Zw3 as a player in spindle positioning, may seem at first glance to run counter to our results, which suggests that the observed spindle defects are due to the loss of Zw3 function, rather than a consequence of its constitutive activity. However, it is important to note that these studies were done in two very different systems. Mitoses in the early *Drosophila* embryo differ from the canonical cell cycle, in that a series of nuclear divisions take place without cytokinesis, with a quick progression between S and M phases (Foe and Alberts, 1983). It is possible that proper centrosome positioning in somatic cells, like the larval neuroblasts examined in both our study and by Peifer's group, requires more fine-tuned regulation than what is needed in the rapid-fire divisions that occur in the syncytial *Drosophila* embryo. Force modulation might be precisely controlled by maintaining an equilibrium between anchored and unanchored microtubule ends. In the absence of Zw3 function, the APC2/Arm/EB1 complex may be locked in its stable conformation, preventing normal microtubule detachment from the actin cortex. Alternatively, Zw3 and APC2 may act together to mediate another independent process, which when perturbed in larval neuroblasts might temporarily disrupt microtubule tension and dynamics, and possibly microtubule-kinetochore attachments, which are manifested in the curved spindle phenotype until redundant mechanisms can be activated to correct these defects.

III. The flavonoid biosynthetic complex – recap

The congregation of metabolic enzymes into macromolecular complexes is a key feature of cellular physiology. Given the apparent pervasiveness of these assemblies, it seems likely that some of the mechanisms involved in their organization and regulation might be conserved across a range of biosynthetic pathways in diverse organisms. Our laboratory makes use of the flavonoid biosynthetic pathway in *Arabidopsis* as an experimental model for studying the architecture, dynamics, and functional roles of metabolic complexes. Over the past several years, we have accumulated substantive and compelling evidence indicating that a number of these enzymes directly interact, perhaps as part of a dynamic globular complex involving multiple points of contact between proteins (Chapter 1 and references therein).

In Chapter 3, we demonstrate that although there are six potential *flavonol synthase* (*FLS*) homologues in *Arabidopsis*, a number of which appear to be expressed, only the FLS1 isoform showed any enzymatic activity. Notably, however, in yeast-two hybrid assays, not only FLS1, but also FLS3 and FLS5 were able to interact physically with other members of the central flavonoid pathway. This opens up the possibility that these catalytically-inactive proteins might play some structural and/or regulatory role in the flavonoid metabolon.

The first evidence for the interaction of flavonoid enzymes in living cells is presented in Chapter 4. Using fluorescent lifetime imaging microscopy fluorescent resonance energy transfer analysis (FLIM-FRET), we were able to detect interactions between CHS and both FLS1 and DFR, which are key branch point enzymes in flavonoid biosynthesis. We also show results indicating that FLS1 interferes with DFR binding to CHS, data that we speculate points to a mechanism for controlling flux to different classes of endproducts.

IV. Future directions

i. *In planta* functional analysis

In the light of mounting evidence, we can be far less equivocal in our assertion that the core enzymes of the flavonoid biosynthetic pathway associate as part of a macromolecular

agglomerate. What has still not been completely resolved is the most biologically-relevant question: is the flavonoid metabolic complex functionally significant? One of the fundamental tenets of the metabolon theory is that these multienzyme assemblies play a crucial role in cell function through the regulation of a number of facets of metabolism: the active channeling of potentially unstable or toxic metabolites, control of flux through competing branch pathways through the selective association and dissociation of component enzymes, and the delivery of specific endproducts with a high degree of precision to specific subcellular sites (Mathews, 1993; Ovádi, 1991; Srere, 1985; Winkel, 2004). If these criteria are met, then there is the expectation that the flavonoid multienzyme complex exists in different configurations, depending upon the needs of the cell, and that these configurations would change based on developmental or environmental cues. Furthermore, if any of these processes is disrupted by interfering with the dynamics or assembly of the metabolic complex, then there should be observable ramifications for cellular metabolism.

Recent evidence from work done in our laboratory suggests that this may in fact be the case. Intriguing results have come from studies involving an engineered variant of the CHS protein where an alanine for arginine substitution was made at a residue that is both part of a putative nuclear localization signal (Saslowsky et al., 2005) and is an integral component of the predicted interface between CHS and CHI (C.D. Dana, J.I. Watkinson, and B.S.J. Winkel, manuscript in preparation). While *in vitro* assays utilizing recombinant protein showed that catalytic activity was retained, surface plasmon resonance experiments indicated that binding to CHI was completely abrogated (C.D. Dana, J.I. Watkinson, and B.S.J. Winkel, manuscript in preparation). Using the native CHS promoter, this modified enzyme was expressed in stably transformed *Arabidopsis* plants that did not produce the native version of the protein. Metabolic analysis by HPLC revealed that the ratios and levels of the flavonol endproducts, kaempferol and quercetin, were markedly different than in extracts from wild-type plants (M.V. Ramirez and B.S.J. Winkel, manuscript in preparation). The subcellular distribution of flavonols, as assayed by DPBA staining, was also altered. This suggests that perturbations in either the localization and/or the ability of CHS to complex with other enzymes in the pathway can have a profound effect on the capability of the cell to regulate flavonoid metabolism.

The relative abundance of the different flavonoids varies across tissue type and developmental stage (Peer et al., 2001; Pelletier et al., 1999; Winkel-Shirley, 2001). These levels can also change rather substantially in response to a range of external stimuli (Winkel-Shirley, 2002). The apparent competition between FLS1 and DFR for binding to CHS described in Chapter 4 is the first *in vivo* evidence for a that there might be a structural mechanism - the selective association of key branch point enzymes with the core metabolon - for the regulation of flux though competing branches of the flavonoid pathway. The next stage in this work will be to test whether this proposed model can be correlated with fluctuating levels of specific classes of flavonoids and if the association of different enzymes is responsive to environmental cues.

FRET microscopy has proven to be a powerful tool for monitoring protein-protein interactions in living cells and has the advantage that it can also be used to study dynamic processes, so that the association and disassociation of protein pairs can be tracked in real-time. Depending upon the type of sample and the mode of microscopy used, FRET can be analyzed at subcellular resolution and be used to determine if the configuration of the flavonoid metabolon varies between different cellular compartments (e.g., in the cytoplasm, along the ER, in the nucleus).

Our initial FRET studies have utilized primary plant cell cultures. This method is extremely useful and had been widely used to study a variety of cellular processes, including the interaction of transcription factors (Immink et al., 2002), vesicle trafficking (Galperin et al., 2005), receptors and signal transduction (Adjobo-Hermans et al., 2006), and metabolite sensing (Okumoto et al., 2005). However, the eventual goal is to replicate and extend these experiments in an *in planta* system by generating stably transformed plant lines. The FRET constructs generated for the protoplast studies were intentionally assembled within a Gateway-compatible entry vector to facilitate their transfer to vectors appropriate for generating transgenic plant lines. Ideally, expression of these fluorescent protein (FP) fusion constructs would be driven by the gene's endogenous promoter in plant lines that contain a null mutation in the corresponding native gene. This will allow us to test to see if our FP tagged enzymes retain their function and complement the mutation, while also avoiding any potential competition from the endogenous protein during

interaction studies. Once the *in planta* interaction patterns of the flavonoid enzymes is established under normal physiological conditions, tests can be performed to determine whether these associations change in response to external stimuli. This can be accomplished through the application of various phytohormones, indole acetic acid (auxin) or jasmonic acid (a wound responsive element) for example. Plants can also be directly subjected to environmental inputs such as mechanical wounding or re-orientation in relation to the gravitational field.

These specific types of experiments are of particular interest in the context of the proposed competition between FLS1 and DFR discussed in Chapter 4. Extensive work in recent years has established a relationship between flavonoids and the auxin mediated gravitropic response; it is the flavonol products synthesized by FLS1 that have been implicated in the regulation of auxin transport (Brown et al., 2001; Buer and Muday, 2004; Jacobs and Rubery, 1988; Peer et al., 2004). Conversely, the expression of a number of flavonoid genes, including *DFR* but not *FLS1*, are induced by factors that cause mechanical damage to the plant and it is DFR that catalyzes the production of leucoanocyanidins that are the precursors to the anthocyanins and proanthocyanidins, which are found to accumulate in response to plant stress (Devoto et al., 2005; Owens et al., 2008; Winkel-Shirley, 2002).

Quantitative FRET methods can be used to analyze and measure the relative frequencies of FLS1-CHS and DFR-CHS interactions under different conditions. The apparent FRET efficiency is a function of both the intrinsic FRET efficiency and the fraction of the donor undergoing FRET and there are a number of different techniques that can be utilized to discern the stoichiometric distribution of the interacting components (Hoppe et al., 2002; Kremers et al., 2008; Padilla-Parra et al., 2008). If we are able to observe changes in the fraction of donor and acceptor molecules in complex, this would lend strong support to a model where the production of differing classes of end products is controlled, at least in part, by the regulated association and dissociation of the relevant biosynthetic enzymes with a core complex.

ii. Single-molecule FRET to probe the architecture and dynamics of the flavonoid metabolon

We have repeatedly averred that the flavonoid biosynthetic pathway is an ideal model for the study of enzyme complex assembly and function. By implication, this means that some of the mechanisms that regulate these processes in this specific system might be applicable to a range of other metabolic networks. In order to begin to illuminate these fundamental principles, we must continue to apply the integrative approach advocated in Chapter 1. This will require that we delve into the *in vivo* behavior of the flavonoid complex (Chapter 5.IV.i), while at the same time making efforts to discern as precisely as possible the molecular level characteristics of the composition, binding sites, interaction modes, and overall architecture of the flavonoid metabolon.

Currently, a number of efforts are underway to map the structural interfaces between the various binding partners in the flavonoid complex. These include hydrogen/deuterium exchange mass spectrometry (Law et al., 2005; Zhou and Zhang, 2007)(P.A. Bowerman and B.S.J. Winkel, unpublished data), computational docking simulations (Ritchie, 2008)(C.D. Dana, J.I. Watkinson, and B.S.J. Winkel, manuscript in preparation), and surface plasmon resonance (P.A. Bowerman and B.S.J. Winkel, manuscript in preparation). Site-directed mutagenesis can be utilized to assess the validity of the predicted interfaces using analyses both *in vitro* using SPR and in living cells employing FRET. FRET experiments can be preformed initially in *Arabidopsis* protoplasts to evaluate the effects of these amino acid substitutions on protein-protein interactions, while subsequent *in planta* work (as described in Section IV.I above) could assay the impact of residue changes on localization and overall flavonoid metabolism.

The strategy outlined in the paragraph above should yield a great deal of data on the molecular architecture of the flavonoid metabolon, however there are still some limits to this approach. *In vitro* mapping of the interactions between enzymes, however rigorous, will only provide a static snapshot of the structure of the complex. While the dynamics of the association and disassociation of individual components with the complex can perhaps be mathematically simulated (Karplus and Kuriyan, 2005), this will be computationally demanding and any

predictions will need to be experimentally confirmed. FRET analysis in living cells allows us to examine the average interaction state(s) of a population of enzymes, yet it is impossible to descry the true kinetics of complex formation and disassembly at the level of the individual metabolon from ensemble measurements. For example, the consequences of this averaging might be reflected in the relatively low FRET efficiencies we detected in the studies described in Chapter 4, which we speculate are due to the transient nature of these interactions, rather than the inherent strength of the interactions. However, single-molecule FRET (smFRET) techniques have the capability to make quantitative measurements in real-time, tracking the association and disassociation of individual enzymes (Joo et al., 2008; Wennmalm and Simon, 2007). smFRET has been used to monitor the residence time of transcription factors associated with RNA polymerase (Kapanidis et al., 2005), analyze the dynamics of tRNA during translation (Marshall et al., 2008), measure conformational changes in the enzyme upon substrate catalysis or ligand binding (Antikainen et al., 2005; Majumdar et al., 2007), and determine mechanistic dynamics of a number of different motor proteins (Kimura-Sakiyama et al., 2008; Yildiz et al., 2004; Zimmermann et al., 2005). Although these types of experiments present a number of technical challenges, the resources required for this approach have become much more accessible (Roy et al., 2008; Walter et al., 2008). We hope to utilize smFRET studies to probe the dynamics of metabolon, perhaps across a number of conformational states and, when combined with H/D exchange and docking simulations, to illuminate details of the macromolecular architecture of the complex.

Currently, smFRET is most widely utilized as an *in vitro* methodology. There have been recent efforts to extend single molecule analysis into living cells, however there remain a number of inherent obstacles that make this impractical for our studies (Joo et al., 2008). In our experiments, we would utilized specific site-labeled recombinant proteins; we have been successful in purifying all the enzymes of the central flavonoid pathway in catalytically active forms, with the exception of DFR and F3'H, although there has been promising progress in developing systems for the expression of these two recalcitrant proteins. Because we have the advantage of possessing structural data for all the enzymes of the central flavonoid pathway, either in the form of authentic structures or homology-based models, we will be able to choose

labeling sites that take advantage of the exquisite sensitivity of FRET to changes in distance at the sub-nanometer scale. Unlike ensemble-based measurements, where distance estimates are at best, approximations, smFRET can provide quantitative details on the intermolecular distance between fluorophores (Deniz et al., 2008). The cyanine dyes (Cy3, Cy5) have been the most commonly used fluorophores for single molecule studies, although the Alexa and Atto dyes have also proven popular (Joo et al., 2008; Roy et al., 2008). Fluorescent proteins and quantum dots are additional options, but their applicability is hampered by size, photostability, and limited attachment strategies (Douglass and Vale, 2008; Hohng and Ha, 2005). There are a number of strategies for the bioconjugation of fluorescent probes; the most commonly used method involves attaching the dye to surface cysteine residues with a thiol reactive group (Kapanidis and Weiss, 2002). In cases where there are no exposed cysteines, or more than one present, on our protein of interest, the residue(s) can be added or removed by site-directed mutagenesis; alternatively, there are other labeling schemes that can be utilized (Kajihara et al., 2006; Kapanidis and Weiss, 2002; Roy et al., 2008). In all cases, the labeled enzyme can be tested for activity *in vitro* before proceeding with smFRET studies.

There are two primary modes of microscopy used in single molecule fluorescence imaging, laser scanning confocal or total internal reflection (TIR). Confocal SM detection relies upon tightly focused laser excitation to illuminate a detection volume in the range of one femtoliter (Deniz et al., 2008). Data is collected as single molecules freely diffuse through the excitation volume. The major drawback of this method is that it requires the use of an avalanche photodiode (APD), a very sensitive and fast point detector, and because the diffusion time though the detection volume usual takes only a few milliseconds, the observation time is limited, precluding the tracking of longer dynamic events (Mukhopadhyay and Deniz, 2007). Total internal reflection fluorescent microscopy (TIRFM) makes use of the evanescent excitation field that occurs when an incident beam of light undergoes total reflection at the boundary between a material of high refractivity and one of low refractivity. There are either prism-based or objective-based means to achieve this reflection, but in each case it results in an excitation field that only extends 50 to 200 nm into the sample, depending upon the incident angle of reflection (Axelrod, 2001). The majority of TIRFM based smFRET studies have made use of surface tethered molecules and this

technique has proven quite useful in investigating a number of biological processes (Joo et al., 2008). This technique permits a long timescale of observation, but has the caveat that there may be some surface induced artifacts (Friedel et al., 2006). A promising new system has been developed whereby the molecules of interest are encapsulated in an immobilized phospholipid nano-containers (Rhoades et al., 2003). Recent improvements have enabled the creation of porous vesicles, which allow for the exchange of metabolites and other small molecules (Cisse et al., 2007).

The smFRET technique would allow us to investigate multiple aspects of the flavonoid metabolon. In one set of experiments, we propose to evaluate the structural role that CHS might play as the hub of the multi-enzyme complex. Based on the protein-protein interfaces that are predicted by H/D exchange and docking simulations, we will generate a series of sites-specifically labeled enzymes (as described above). Utilizing prism-based TIRFM, encapsulated labeled enzyme pairs would be analyzed to determine residence time of binding, detect possible transient intermediate conformational states, and estimate inter-dye distances to see if they correlate with what we predict based on our models. We could also use this approach to complement SPR and *in vivo* FRET to evaluate the impact of amino-acid substitutions at putative interfaces, with the added advantage that at the single molecule level we may be able to detect subtle, incomplete perturbations in binding that would be unperceivable in ensemble-based measurements. The competition dynamics between FLS1 and DFR could also be explored in more detail, because we could control the stoichiometric ratios of these proteins more precisely than in *in vivo* experiments. A clearer picture of the role that F'3H plays in recruiting the complex, or certain configurations of the complex, to the endoplasmic reticulum might also emerge from this approach. In this case, utilizing the surface tethering approach, we could attempt to mimic the anchoring of F3'H in the endoplasmic reticulum and then study the occurrence of FRET between various enzyme pairs.

Intra-molecular smFRET - labeling a single protein with both a donor and acceptor fluorophore - can be a valuable tool in discerning whether an enzyme undergoes conformational changes upon protein-protein binding or interaction with a metabolite or other small molecule (Antikainen et

al., 2005; Arai et al., 2006; Majumdar et al., 2007). In this way, we could determine if any of the flavonoid enzymes undergo an alteration in conformation when associated with the metabolic complex. This is particularly of interest with regard to DFR, which may be structurally unstable in the absence of CHS (Chapter 4). There is also some recent evidence that DFR might be inhibited by a structural change induced by the binding of flavonols (the product of FLS1). While the minor conformational changes that might occur in these events may be beyond the sensitivity limits of smFRET, which can detect changes in inter-dye distances of approximately 0.3 nm (Roy et al., 2008), optimal dye placement might allow such processes to be observed.

The advent of a variety of three-color FRET methodologies, both ensemble-based (Galperin et al., 2004) and at the single-molecule level (Hohng and Ha, 2005; Lee et al., 2007), will markedly expand the capabilities of this technique, giving researchers the ability to track multiple configurations or multiple components of a enzyme complex in three dimensions. Currently, this technology is still in the proof-of-concept phase and for us to attempt to utilize these approaches to their fullest potential would necessitate the recruitment of collaborators with the instrumentation and expertise currently required to perform these experiments. However, if realized, the eventual implementation of these types of studies could provide valuable details on the architecture and compositional diversity of the flavonoid metabolon.

iii. Protein complementation screens to map the larger flavonoid network

A recent set of yeast two-hybrid (Y2H) screens done to identify potential interactors with CHS have revealed a number of novel associations with proteins that are not part of the flavonoid pathway. Preliminary results suggest that these putative interacting partners may have functions associated with gravitropism and auxin transport (J.I. Watkinson and B.S.J. Winkel, manuscript in preparation). As was noted earlier in Chapter 5.IV.i, there is an established connection between flavonoid metabolism and auxin efflux. There is an ongoing effort to map this expanded interaction network. There are large-scale projects underway in other groups to delineate portions of the *Arabidopsis* "interactomes," although the representation of flavonoid enzymes in these programs has been minor to date and any salient results from these endeavors is still a long way off. Our laboratory has made extensive use of the Y2H system and we are

pursuing TAP-tagging as a complementary *in vivo* approach to isolate members of the flavonoid interaction network. However, this technique may not be appropriate for the transient and weak interactions that may characterize our target system.

As a complement to the traditional Y2H scheme, we could utilize an *in vivo* library screen protein fragment complementation assay (PCA) that depends upon the reconstitution of the murine dihydrofolate reductase (mDHFR) (Subramaniam et al., 2001). This method has the advantage of relying upon an endogenous milieu (plant protoplasts) for expression and allows the interaction to take place in its native cellular compartment. The drawback of this technique is that detection of an interaction requires that the protoplasts be treated with fluorescein-conjugated methotrexate (fMTX) as a reporter, which binds to the reconstituted mDHR. After overnight incubation, the protoplasts then need to be extensively washed to remove the unbound substrate, which could become quite arduous during a large scale screen. An alternative PCA based library screen is one based on bimolecular fluorescence complementation (BiFC) (Bracha-Drori et al., 2004; Walter et al., 2004). This approach would share the advantages of the mDHFR assay, but because the reconstituted protein tag is inherently fluorescent, the protoplasts would not require any additional chemical treatment or manipulation following transfection. Furthermore, a protocol could be developed to allow protoplast transfection and detection to be carried out in a 96-well plate format, facilitating rapid screening of a library. Whichever of these screening strategies is implemented, potential interactors could then be evaluated further using some of the approaches outlined in Chapter 5.IV.i and Chapter 5.IV.ii.

V. Parting Remarks

Research programs in medicine, agriculture, and bioengineering are increasingly focused on physiology and metabolism at the molecular level. As more and more studies emerge - such as those showing that protein-protein interactions play a role in certain neurodegenerative disorders (Ovádi et al., 2004), have a profound impact on the efficacy of cancer therapeutics (McDonnell and Norris, 2002), and are critical to the success or failure of numerous efforts in metabolic engineering (Conrado et al., 2008; Libourel and Shachar-Hill, 2008) - it becomes apparent that progress in any of these endeavors will require an understanding not just of the functions of

individual components, but of how they interact as part of larger cellular networks. Ultimately, the most comprehensive, holistic approach to untangling the operation of the multitudes of physiological networks that exist within a single cell will lie in a rigorous application of systems biology modeling and simulations. Yet, as we have noted earlier, there needs to be a strong experimental foundation upon which to base such models.

Here, we have outlined a number of experimental approaches that we are either utilizing currently or propose to undertake. These include molecular level "reductionist" techniques, such as mapping the structural architecture of the flavonoid complex through a combination of single-molecule FRET, HDX, and computational docking simulations. The specific kinetics of these interactions can then be calculated by *in vitro* methods such as surface plasmon resonance. This work will both inform, and be complemented by, studies focused on how the flavonoid multi-enzyme complex functions within living cells, using non-invasive FRET and other fluorescent techniques to track interactions between component enzymes and discern their physiological significance. Meanwhile, library-based screens will expand our knowledge of the global interaction network in which flavonoid metabolism is situated. In collaboration with five other research groups from Virginia Tech and Wake Forest University, the Winkel lab has also embarked upon an NSF funded effort that will extend our analysis of flavonoid and phenylpropanoid metabolism with a systems biology approach. The integrative, pluralist research agenda that we are pursuing in regards to the flavonoid metabolic complex could very well serve as a model for attempts to understand how other metabolic systems function.

VI. **References**

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Appendix

The work of Donald Poulson: A prelude in the study of developmental genetics in *Drosophila melanogaster*

Kevin C. Crosby and Richard M. Burian[1],

[1]Department of Philosophy, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

The decade spanning the early 1980s to the early 1990s was marked by a revolution in developmental biology that saw the wholesale integration of genetics into the study of development (Anderson and Ingham, 2003). The foundations of this revolution lie in work done from the late 1960s through the 1970s, when a number of researchers working with *Drosophila*, began to integrate the techniques of classical and molecular genetics with those of embryology (Ashburner, 1993; Wolpert, 1994). It is sometimes forgotten that these accomplishments were foreshadowed decades earlier by the efforts of a handful of scientists, who attempted to answer the same questions, and in some cases, using some of the same techniques, as their later counterparts. One such individual was Donald Poulson. Poulson began his career as a student of the Morgan group at Caltech in the 1930s, steered by Alfred Sturtevant to the study of the embryological development of *Drosophila*. Between 1937 and 1945, Poulson produced a series of studies that attempted to dissect part of the genetic basis for development through an analysis of the effects of chromosomal deficiencies on embryogenesis. Although he has been alternatively described as "the founder of *Drosophila* developmental biology" (Ashburner, 1993) and as "a pioneer and an outlier" (Keller, 1996), Poulson's work did not have a major impact on his contemporaries. While his descriptive work on *Drosophila* embryogenesis remained the standard reference in the field for decades (Poulson, 1937a; Poulson, 1950), his attempts to understand the genetic basis for early development remained largely unnoticed.

Poulson began his studies on the genetics of development at the California Institute of Technology, as an undergraduate during the 1930s. This was at a time when T.H. Morgan's "transplanted" fly room was at its height, so he was in an ideal place for someone wishing to explore developmental questions from a genetic standpoint. Yet, originally Poulson had very little interest in studying biology. In high school his interests lay most strongly with chemistry and physics, and when he enrolled at Caltech in 1929 as an undergraduate, it was with the intention of becoming a chemist. In his second year he took a general biology course in which T.H. Morgan delivered a number of lectures, followed by a genetics course taught by Dobzhansky, which sparked a keen interest in biology and genetics. Several decades later, he would still describe Dobzhansky's course as "...one of the most exciting experiences in my life" (Poulson, oral history). Dobzhansky also influenced Poulson's career during an informal conversation on invertebrate metamorphosis that inspired his young student to begin thinking

about the developmental processes of insects. One of Poulson's first major research projects as an undergraduate involved the study of the rates of respiration and oxygen consumption of *Drosophila melanogaster* and *D. pseudoobscura* during metamorphosis (Bonner et al., 1981).

After completing his undergraduate studies, Poulson stayed at Caltech and began graduate work under the direction of Sturtevant. Sturtevant encouraged Poulson to undertake a detailed study of embryonic development in *Drosophila melanogaster*, which, aside from some preliminary work done by A.F. Huettner in the early 1920s (Huettner, 1923; Huettner, 1924), remained largely unelucidated. Rather than a purely descriptive exercise Poulson embarked on this work with the intention of laying a foundation for subsequent studies of the genetics of development, emphasizing that it is "…essential to have complete information concerning development before it is possible to speak intelligibly about genes and development." (Poulson, 1937a)

Poulson was intrigued by the genetics of development during his years as a graduate student. He believed that the real key to a true understanding of development would be found in the underlying genetic controls. But what were these controls and how did they work? Many years later, Poulson would comment that the "most significant question" of his oral examinations was poised by the noted embryologist, Albert Tyler, – "If the genes are the same in all the cells, how does development occur?" (Bonner et al., 1981) This very question in fact, was one of the major sticking points in the divide between traditional embryologists and geneticists. To many of those engaged in the study of development, the science of genetics seemed to have no relevance to their work, and by some was seen as a potential threat to the integrity of their discipline (Allen, 1986; Burian, 2004 (in press); Gilbert, 1978). Conversely, while many geneticists acknowledged the importance of understanding the mechanisms of development and felt that the study of genetics offered the key to a more complete knowledge of these processes, the technical constraints required to do this work with an organism suited for genetics, such as *Drosophila*, in contrast to the embryos of frogs or marine invertebrates, organisms more traditionally used by embryologists, were daunting. The small size, fragility , and opaqueness of the *Drosophila* embryo made the study of its development very slow and impractical (Wright, 1970).

Poulson, however, felt strongly that the key to unlocking the mysteries of development lay in a genetic dissection of the process, stating in a later monograph that:

> Any full analysis of development and differentiation must rest ultimately on the nature of the hereditary materials, the genes, their reproduction and their activities in the physiological economy of the cell (Poulson, 1945).

Beginning in the 1930s and continuing through the next decade, Poulson undertook a systematic study of the effects of various X-chromosome deletions on embryological development (Poulson, 1937b; Poulson, 1940; Poulson, 1945). Poulson explicitly acknowledged that the ideal set of experiments would involve the removal of individual genes, one at a time, followed by knocking out specific combinations of genes (Poulson, 1937b). Given the technology of the time, this approach would have been, at best, impractical. Fortunately, the previous twenty plus years of genetic work on *Drosophila* had yielded an extensive catalogue of small-scale chromosomal deficiencies, which provided a promising substitute for his experiments.

Poulson chose to focus his initial studies on deficiencies of the X-chromosome. Because their effects were revealed so readily in males, there was already a large catalogue of X-chromosome deficiencies available. For this reason as well, the crosses required to obtain zygotes that exhibited the lethal effects of the deficiency could be done with relative ease. Not surprisingly, severe defects, apparent in the earliest cleavage stages were found to result from the complete absence of the X chromosome. Likewise, striking defects were evident early in embryogenesis when the zygote was deficient for half of the X chromosome. Initially, Poulson was unable to distinguish between the two half-X deficiencies (Poulson, 1937b), but as his cytological techniques became more sophisticated, he was able to discern distinct morphologies for each (Poulson, 1945). Poulson was, in fact, able to refine his technique to the point of resolving single gene effects on development in his studies of the *notch* deficiencies, a result thought to be technologically impossible at the time.

It was not unexpected that large deletions produced major disruptions of development, but Poulson's analyses of smaller deficiencies at the *notch* loci produced much more revealing, and surprisingly detailed, results. Embryos with the *notch* deficiency exhibited an over-abundance of neuroblasts, produced at the expense of the mid-gut, epidermis, and other ectodermal tissues. While some differentiation of cell types occurs in the tissues of the embryonic nervous system, it

is highly abnormal. Yet despite these defects, some tissues of *notch* deficient embryos, such as the hind-gut and portions of the malpighian tubules, develop relatively normally (Poulson, 1945). Poulson tested a series of *notch* deficiencies, with deletions ranging in extent from several bands to those which were not cytologically visible. Because each of these yielded an identical phenotype, Poulson reached the important conclusion that it was likely that his studies had revealed a single gene, whose normal function it was to control the development of the future mesoderm and endoderm cells of the ventral blastoderm at the time of germ-layer separation. As the mesoderm develops, he speculated, it might in turn induce the formation of the early nervous system.

Poulson also examined a number of deficiencies in the *white* locus. These resulted in a phenotype where the ectoderm and resulting organs developed normally, but the mid-gut and other tissues of mesodermal or endodermal origin showed marked abnormalities (Poulson, 1945). Defects caused by the *notch* deficiencies first begin to appear around the mid-point of embryonic development about 12 hours after fertilization (Poulson, 1937b). Those aberrations caused by deletions in the *white* locus appeared later in development, between 12 and 16 hours following fertilization. Another series of small deletions near the tip of the X chromosome were found to produce effects only very late in embryogenesis. Based on these results, Poulson concluded that the *notch* gene acted early on in the development of the embryo, while those genes, or clusters of genes, at the *white* locus and near the tip of the X chromosome acted later in embryogenesis (Poulson, 1945). Poulson's model presaged much later work done in developmental genetics by highlighting not only the importance of understanding the functions of genes active during development, but the timing of their activation as well.

As interesting and exciting as Poulson's experiments and conclusions may seem in hindsight, few within the *Drosophila* community seemed eager to follow up on his work, which was, at the time, quite painstaking and slow. Many felt that he was laboring in "an impossible field," ((Keller, 1996) and that more productive and fundable work was to be found elsewhere (Ashburner, 1993; Keller, 1996). His work only gathered a handful of passing citations in the subsequent literature and it was a reflection of the gulf between embryology and genetics at the time that none of these references came from traditional embryologists.

Compounding the apparent reticence among the *Drosophila* community to follow Poulson's lead, genetics was going through a period of rapid change. *Drosophila* was no longer the cutting edge instrument it had once been. The leading work was now being done in simpler organisms, such as fungi, *E. coli*, and bacteriophage, where molecular level analysis and single gene dissection was possible (Morange, 1998). Although Poulson's work on the *notch* locus showed that "old fashioned" techniques *could* get to single genes, few wanted to use these methods when other organisms were more adaptable to the goal of getting down to the level of the gene. For example, George Beadle and Boris Ephrussi's transplantation work in *Drosophila* had traced the effects of a couple of genes that had diffusible products (*cinnabar* and *vermilion*), but had not discriminated control of enzyme formation from hormonal action at a distance. Beadle's switch from *Drosophila* to *Nerospora* and Ephrussi's imitative switch to yeast show that the attempts to work at the level of the single gene was also pushing toward working with "simpler" organisms (Gayon, 1994; Gayon and Burian, 2004 (in press)).

It would, of course, be inaccurate to claim that the study of *Drosophila* development and embryology stagnated during the next three decades. A number of investigators continued to work in this area (Ashburner, 1993), but it would not be an exaggeration to say that progress was slow in comparison to other areas of investigation. A rather cogent example of this involves a set of experiments Poulson proposed to test the ability of mutant embryos to be rescued by cytoplasmic transplant from normal, unfertilized embryos. It wasn't until 30 years later that these experiments were actually performed by Alan Garen and Walter Gehring at Yale when they showed that the developmental defects of embryos altered by the maternal effect mutation, *deep orange,* were repaired by the injection of cytoplasm of wild-type donors (Garen and Gehring, 1972). Although, the authors were colleagues of Poulson at Yale, there is no indication that their work was directly influenced by Poulson's research. Particularly revealing is an interview of Alan Garen conducted by E.F. Keller in 1992 when she asked him why, when the experiments had been technically feasible since the 1930s, no one had attempted them earlier. Garen responded that, in his opinion, the *Drosophila* community lacked sufficient interest in the subject (Keller, 1995; Keller, 1996). Of course, Poulson could have done these experiments himself yet failed to do so. He continued to work on characterizing the embryonic defects

caused by the *notch* deficiency, but this work remained largely unpublished, except for some material that was cited in the review of one of his students (Wright, 1970). The reasons for this are difficult to discern from the material available, but what seems clear is that he had a certain reluctance to publish or push ahead his own agenda. His colleagues regarded him as capable, patient, and thorough, although perhaps not remarkable (Keller, 1996). He may have lacked a degree of natural hubris that would have been necessary for him to drive forward his own research program in the absence of much interest or support from the wider scientific community.

Poulson had few students, but a handful, particularly Theodore Wright, did continue his work on the genetics of embryogenesis in *Drosophila*. Even into the 1970s, work in this area was frustratingly tedious. In a review published in 1970, Wright, echoing what must have been a common sentiment, commented that "from a genetic point of view [*Drosophila*] may be the system of choice, but it is clear that in many other ways it leaves a lot to be desired….As a subject for experimental embryology, the *Drosophila* embryo is rather frustrating to say the least". However, the field was progressing, and Wright was able to sound an optimistic note when he argued that although this research was beset with technical problems, most of these could probably be solved "if enough talented people think it worth the effort" (Wright, 1970) Yet, few embryologists were willing to work on any traditional embryological problem with an organism as difficult for those purposes as *Drosophila*. Consequently, there was little hope of getting help from embryologists with these projects. Indeed, during the late 1960s and 1970s, the stage began to be set for a revival of interest in *Drosophila* developmental biology. One factor contributing to a renewed interest in work on the fruit fly was the fact that many of the pioneers of molecular genetics, such as Seymour Benzer and Sydney Brenner, were looking to tackle more complex questions involved in the study of behavioral or developmental biology that required the use of more complex systems then the phage or *E. coli*. Additionally, during the late 1960s new biochemical and molecular methods began to be applied in *Drosophila*, opening the door for many of the techniques that would propel *Drosophila* to the forefront of developmental biology in the subsequent decades (Anderson and Ingham, 2003; Ashburner, 1993).

One of the most influential experiments of this period, however, did not rely on any of these new technologies. In the late '70s, Christiane Nüsslein-Volhard and Eric Wieschaus began work on a mutation screen designed to genetically dissect the developmental process responsible for the segmentation and polarity of the *Drosophila* embryo (Nüsslein-Volhard and Wieschaus, 1980). Nüsslein-Volhard was a biochemist who had become interested in *Drosophila* development while working as a post-doctorate in Walter Gehring's laboratory in Switzerland. Wieschaus had been Poulson's last student at Yale, taken in as a favor to Harvey Bender, who was worried about Wieschaus draft status during the Vietnam war. Ironically, Wieschaus after three years of washing out fly bottles in Bender's lab as an undergraduate, had absolutely no interest in working with *Drosophila*. Yet the year he spent working with Poulson proved to be seminal. As Wieschaus would later relate:

> Until that point, I had thought all developmental genetics of flies involved eye colors and bristles and other aspects of adult morphology. It had never occurred to me that flies had embryos, or that Drosophila embryogenesis was characterized by the same kinds of spectacular cell movements seen in the classically studied embryos of vertebrates. I learned all that from Poulson (Wieschaus, 1995).

During his second year at Yale, Wieschaus switched labs (with Poulson's approval) in order to work with Walter Gehring. Wieschaus finished his Ph.D in Basel, Switzerland, after Gehring moved his lab there in 1972 and it was during this time that the collaboration between he and Nüsslein-Volhard was established (States and Kafatos, 1994). The mutagenic screen that was the fruit of this collaboration is generally regarded as one of the most significant influences on the direction of *Drosophila* research in the realm of development (Ashburner, 1993; Keller, 1996) – a sentiment confirmed by the awarding of the Nobel prize in 1995 (along with Edward Lewis for his work).

It seems unlikely that Poulson or his students would have had the technical resources to conduct a saturation screen on the scale done by Nüsslein-Volhard's and Wieschaus. However, putting aside the intellectual brilliance of the research and the ambition of the scientists, it can be argued that more then anything else it was also the scientific environment into which that idea and its results were presented that made all the difference. Poulson's work, although innovative, was

done in an era when there was little interest in such efforts. When Nüsslein-Volhard and Wieschaus began their experiments some thirty years later, the tools they possessed were not radically different then what were available to Poulson, but what they did have was a receptive scientific community, both in general and among drosophilists in particular. Perhaps more importantly, these experiments took place in a historical context with highly developed interest in both genetics and development. Gehring, who sponsored and encouraged the breakthrough work done by Wieschaus and Nüsslein-Volhard, had studied with the Swiss geneticist Ernst Hadorn, who in turn was a student of Alfred Kühn and was also in the lineage from Theodor Boveri. Kühn was a direct student of August Weismann and had an active program in physiological-developmental genetics and did the work on the meal moth *Ephestia* with Ernst Caspari and others that helped set the stage for the work of Beadle and Ephrussi. Therefore Wieschaus and Nüsslein-Volhard work was in a straight lineage from Weismann and Boveri in the Kühn-Hadorn-Gehring tradition (Wehner et al., 1990). At last Poulson's research program had met a context in which its potential for understanding the genetics of early embryonic development of *Drosophila* was not only appreciated, but actively fostered.

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