The regulation of genes involved in trichome development

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THE REGULATION OF GENES INVOLVED IN TRICHOME DEVELOPMENT

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

Matthew Lloyd Brown
B.S., Louisiana State University, 1996
May, 2006
This work is dedicated to my mother and father, Brenda and Jerry Brown, for the support they have given me in all my endeavors.
ACKNOWLEDGEMENTS

There are so many people that contributed to my professional and personal development over the last seven years that it would be difficult to mention them all. My parents have been, and continue to be, a constant source of encouragement and support. My friend Jared Patterson provided an important role model for my pursuit of this degree. My fiancé, Emily McMains, has provided an enormous amount of emotional support to me for the past three years. My labmates, Ginger Brininstool, Michelle Speckhart, and Remmy Kasili, have all provided me with counsel and assistance in the laboratory for my entire graduate career. I feel especially lucky that I chose to study at Louisiana State University. I feel that all of the professors and graduate students with whom I interacted were always eager to make available their knowledge and resources to me. This spirit of cooperation makes LSU a special place. Lastly, I would like to thank my major professor, John Larkin. Without his direction my graduate career would have been much less fulfilling.
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ABSTRACT

_Arabidopsis thaliana_ is an organism that can be used as a model for most of the processes that occur in flowering plants. The leaf hairs, or trichomes, of _Arabidopsis thaliana_ are macroscopic single cells that have been used as a model system for cell fate determination, cell expansion, cell cycle regulation, cell wall deposition, as well as other processes. Initiation of the trichome cell fate is controlled by a complex of genes including _GLABRA1_ (GL1), _TRANSPARENT TESTA GLABRA_ (TTG), and _GLABRA3_ (GL3). This work examines the role of GL3 in trichome initiation and uses plants expressing varying levels of GL3 to determine if genes involved in trichome development are regulated by GL3. Though several genes are given a cursory examination, the regulation of two genes, an α–carbonic anhydrase and a novel cell-cycle regulator called _SIAMESE_, are given a thorough examination. The α–carbonic anhydrase At2g28210 was previously not known to be involved in trichome development. Its involvement in trichome development was discovered with the aid of an enhancer trap line with robust reporter gene expression in developing trichomes. Pharmacological studies indicate that this α–carbonic anhydrase may play a role in trichome expansion. _SIAMESE_ (_SIM_) was first identified in a mutant screen in the Larkin lab. This dissertation demonstrates that this gene encodes a novel type of cell-cycle regulator with several homologs in Arabidopsis and other plant species. _SIM_ and one of its homologs in Arabidopsis were shown to be expressed in a trichome-dependent manner. These investigations shed new light into the molecular process of trichome development.
CHAPTER 1. INTRODUCTION

A central question of biology is that of development. Even the most complex organism begins life as a single cell, but in most multicellular organisms, this cell’s descendents differentiate into a myriad of cell types required to produce a mature organism. How these different fates are realized when all of the daughter cells of that original zygote have the same genes is the result of two general processes: cell fate selection and activation of a discrete subset of genes once this cells fate is determined. These processes have been investigated in many different systems from heterocyst formation in cyanobacteria (Meeks and Elhai, 2002) to neural development in the mouse brain (Hirabayashi et al., 2004). These investigations have revealed many different strategies of differentiation ranging from those which are entirely lineage-dependent to those which depend solely upon positional cues provided by neighboring cells. Trichome development in Arabidopsis thaliana has been studied for over fifteen years (Haughn and Somerville, 1988; Herman and Marks, 1989). This system is an excellent model for developmental questions because trichome development is confined to the two-dimensional plane of the developing epidermis and trichomes are not essential to the survival of the plant. In this work, I investigate both the machinery that governs trichome differentiation and the genetic consequences of adopting the trichome cell fate. To address these issues, I have examined downstream events regulated by the transcription factors controlling trichome initiation.
1.1 Arabidopsis as a Model Organism

*Arabidopsis thaliana* is one of the most studied organisms in the world. This plant has not received this attention because of its agricultural importance, but rather because of its use as a model organism. A model organism is one that is used as the focus of intense study by investigators with the assumption that discoveries in the model will help to elucidate similar properties in related organisms. *A. thaliana* is a small winter annual crucifer, a member of the mustard family *Brassicaceae*. It is related to several crop plants such as broccoli, cabbage, and radish.

*A. thaliana* was first described by Johannes Thal in 1577 in a book describing the plant life of the Harz Mountains (Koncz et al., 1992). It was not until 1907, however, that Friedrich Laibach published the first paper describing experimental research using Arabidopsis thaliana. In 1935, Koncz and colleagues published a paper suggesting Arabidopsis as a model organism for the study of plants analogous to the use of Drosophila for the study of animals (Koncz et al., 1992). However, widespread adoption of *Arabidopsis thaliana* as a model organism would not be realized for several decades. In the early 1970’s, two particularly influential reviews by G.P. Rédei proclaimed the benefits of using *Arabidopsis thaliana* as a plant model system (Rédei, 1970; Rédei, 1975). In addition to the lobbying by Rédei and subsequent investigators, the production of a linkage map of *A. thaliana* by Koornneef et al (1982) helped to establish it as a widely used research tool. Application of molecular biology methods during the 1980’s and 1990’s (Chang and Meyerowitz, 1986) and the subsequent
sequencing of the genome (The Arabidopsis Genome Initiative, 2000) also served as powerful catalysts to attract interest in Arabidopsis. Changes in the scientific culture of the 1980s which paralleled these technological advances also increased interest in the plant (Fink, 1998) and set the stage for a boom in Arabidopsis research in the proceeding decade (Figure 1-1).

*Arabidopsis thaliana* has many qualities that make it an organism well-suited for scientific research. Unlike crop plants, *A. thaliana* is very small and it requires only a minimal amount of care. Furthermore, *A. thaliana* can produce a prodigious amount of seed (over 50,000 per plant, Redei, 1975); it can be grown year-round under laboratory conditions, and it has a relatively short life cycle of 6-8 weeks. It has a small, relatively compact genome (Pruitt and Meyerowitz, 1986; Sparrow and Miksche, 1961) consisting of 5 chromosomes (Steinitz-Sears, 1963). In 1999-2000, the Arabidopsis genome sequence was completed (Lin et al., 1999; Mayer et al., 1999; Odell et al., 1985; Salanoubat et al., 2000; Tabata et al., 2000; Theologis et al., 2000), and the total number of base pairs was found to be 125 Mbp, only about 25 times greater that of *E. coli* (Blattner et al., 1997), and twenty times smaller than maize (Palmer et al., 2003). As of July 2004 there were 31,270 genes annotated in Arabidopsis (http://www.arabidopsis.org/info/agilinks.jsp) illustrating the compact nature of the genome. *Arabidopsis thaliana* is also easy to transform with foreign DNA by utilizing *Agrobacterium tumefaciens*-mediated floral dip transformation.
The increasing research interest in Arabidopsis thaliana in the last 15 years.

Figure 1-1: Number of citations found on PubMed for Arabidopsis and Drosophila every 5 years for the past 15 years. This is a graph of the number of publications resulting from a PubMed search containing the word Arabidopsis or Drosophila in the title within the calendar year listed.
(Clough and Bent, 1998; Desfeux et al., 2000; Feldmann and Marks, 1987), thus eliminating the need for more labor-intensive tissue culture transformation to generate transgenic organisms.

The greatest resource to an Arabidopsis researcher today is a website called The Arabidopsis Information Resource, or TAIR (www.arabidopsis.org). TAIR contains the complete annotated genome sequence indexed by several different criteria, including name, accession number, or keyword. An investigator can also use an Arabidopsis-specific version of the Basic Local Alignment Search Tool (BLAST) to explore the genome. TAIR also includes collections of tools available for a nominal fee to any researcher, such as library clones and seed stocks. This website also acts as a repository of Arabidopsis knowledge and a focal point so that investigators can find others working on the plant. For all of the reasons stated above, Arabidopsis thaliana was the obvious choice as the organism upon which to base this body of work.

1.2 Trichomes as a Model System

The epidermal surfaces of the leaves and stem of Arabidopsis thaliana are covered by hair-like projections called trichomes. In Arabidopsis thaliana, trichomes are large single cells that project perpendicularly from the epidermis; these structures are so large that they can be easily seen with the naked eye and a dissecting microscope allows for easy description. Trichomes located on different tissues have different shapes. Leaf trichomes have two to four branches while trichomes found on the stem are typically unbranched (Marks et al., 1991). Trichomes are found on the adaxial surface of early leaves, and on both surfaces
of leaf pairs that arise later in the development of the rosette. Importantly, 
trichomes are not essential for the survival of the plant. This allows for easy 
genetic manipulation of processes involving trichome development (Haughn and 
Somerville, 1988). Because trichomes are easy to observe and can be 
manipulated with minimal effect on the physiology of the plant, these cells make 
an excellent model system for studying a variety of processes including cell fate 
determination, cytoskeletal function, and cell cycle regulation.

1.3 Generation of the Trichome Spacing Pattern

The mechanism responsible for generating the spacing pattern on the 
surface of first true leaves has been intensely investigated during the past 
decade (Larkin et al., 2003; Marks, 1994; Marks, 1997). Trichomes arising on the 
leaf surface are found adjacent to one another less than one percent of the time 
(Larkin et al., 1994). Trichome initiation occurs in a non-random, non-cell lineage 
dependent manner (Larkin et al., 1996). Currently, a lateral-inhibition model is 
used to explain cell fate determination in Arabidopsis trichomes (Larkin et al., 
1997). A spacing pattern governed by lateral inhibition would consist of an 
initiation factor and inhibitory factor acting within a field of equipotent precursor 
cells. The initiation factor would be produced by all cells in an auto-regulatory 
manner and would be cell-autonomous. The activity or synthesis of the inhibitory 
factor would be under the control of the initiation factor and this inhibitory factor 
would be able to diffuse to other cells to counteract the action of the initiation. 
This would set up a “dead-locked” situation by which all cells are producing the 
initiation factor, but are being inhibited from adopting the specified fate by their
neighbors until the stalemate is broken by one cell producing more of the initiation factor than its neighbors though stochastic changes in gene expression. This would cause some cells among the equipotent precursors to adopt a particular identity while maintaining a minimum distance between cells that adopted this fate (see Figure 1-2). This basic mechanism has also been shown to explain the distribution of Drosophila neural bristles (Portin, 2002) and the spacing of cyanobacteria heterocysts (Wilcox et al., 1973).

Several genetic components that could initiate or inhibit Arabidopsis trichome production have been discovered. There is a great deal of redundancy in both types of components. The initiation decision requires products from at least five different genes: GLABRA1 (GL1), AtMYB23 (MYB23), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), and TRANSPARENT TESTA GLABRA (TTG). GL1 encodes a R2R3 MYB transcription factor (Oppenheimer et al., 1991). Mutants of GL1 have no trichomes at all on early leaves, but do have trichomes on the edges of later rosette leaves. This lack of trichomes (making the plants bald, or glabrous) is the only reported phenotype of gl1 (Koomneef et al., 1982). MYB23 also encodes a R2R3 MYB transcription factor (Kirik et al., 2001), and the protein is functionally equivalent to GL1 with respect to trichome initiation (Kirik et al., 2005). MYB23 mutants display reduced trichome branching, while gl1 myb23 double mutants lack all trichomes, including those few trichomes found on the edges of later leaves of gl1 mutant plants (Kirik et al., 2005). GL3 is a basic helix-loop-helix (bHLH) transcription factor (Payne et al., 2000). GL3 loss-
Figure 1-2: Model of the lateral inhibition mechanism of trichome development. Early in leaf development all cells produce about the same amount of initiation complex, but the cell is prevented from adopting the trichome fate by inhibitor elements produced by its neighbors. Slight increases in the amount of initiation complex or decreases in inhibitor activity could easily disrupt this equilibrium owing to the auto-regulatory nature of the initiation complex and its subsequent control over the inhibitor. This would quickly give rise to a single cell which adopts the trichome fate while strongly inhibiting its neighbors from adopting the same fate. Black arrowheads denote activation and red blunt arrows denote inhibition.
of-function mutants have a reduced number of trichomes, and the trichomes that do form have fewer branches than do wild-type trichomes (Bowman, 1994). *EGL3* is also a bHLH transcription factor that is 75% similar to *GL3* at the amino acid level (Zhang et al., 2003). *EGL3* mutants have slightly reduced trichome initiation, and they exhibit a subtle branching defect as well as reduced anthocyanin pigmentation, reduced seed coat mucilage and altered root hair positioning. Double mutants of *gl3 egl3* are completely glabrous (Zhang et al., 2003). *TTG* encodes a WD40 repeat protein (Walker et al., 1999). *TTG* loss-of-function mutants are not only glabrous, but they also lack anthocyanin pigment and seed coat mucilage (Koornneef, 1981), and they have defects in root-hair patterning (Galway et al., 1994). GL1, MYB23, GL3/EGL3, and TTG are thought to act together to promote transcription of downstream genes required for trichome initiation (Ramsay and Glover, 2005).

The inhibitory module of trichome initiation appears to be comprised of a highly redundant group of proteins that contain a R3 MYB-DNA activation domain, but lack a transcription activation domain. So far, four genes have been found that play a role in trichome inhibition: *TRIPTYCHON (TRY)*, *CAPRICE (CPC)*, *ENHANCER OF TRY AND CPC (ETC)*, and *ENHANCER OF TRY AND CPC2 (ETC2)*. *TRY* loss-of-function mutants have larger trichomes with more branches than wild-type and these trichomes often are found in clusters of adjacent trichomes (Hulskamp et al., 1994). *CPC* was first identified because it has a reduction in the amount of root hairs that it produces (Wada et al., 1997), but a more careful examination of its phenotype revealed that it produces more
trichomes than wild type (Schellmann et al., 2002). The trichomes of *try cpc* double mutants exist primarily in clusters and these plants have no root hairs, indicating the redundant nature of these two proteins (Schellmann et al., 2002). In both *try* and *try cpc* trichome clusters, the extra trichomes at a single initiation site seem to arise in the location normally occupied by one of the accessory cells found at the base of the trichome. *ETC1* has no apparent single mutant phenotype, but enhances either the *cpc* phenotype (with respect only to root hairs) or the *try cpc* double mutant phenotype (Kirik et al., 2004a). The *try cpc etc1* triple mutant phenotype is dramatic: giant clusters consisting of hundreds of trichomes cover the entire leaf surface leaving only the midrib and most basal portion of the leaf bare (Kirik et al., 2004a). The only *ETC2* mutant phenotype is a small increase in trichome production which is enhanced when combined with a *cpc* mutant (Kirik et al., 2004b). Combining the *etc2* mutation with *try* and *cpc* does not produce the great trichome clusters seen in *try;cpc;etc1* triple mutants; rather the phenotype of the *try;cpc;etc2* mutant resembles the *try;cpc* double but with trichome clusters also appearing on the petiole (Kirik et al., 2004b). Thus, these four genes seem to function as partially redundant inhibitors of trichome initiation.

The key to understanding the process by which a cell adopts the trichome fate lies not only in finding the components of the initiation and inhibitory elements, but also in uncovering how these elements interact. Based upon data found in Arabidopsis and other species, a model of the interaction of these elements is shown in Figure 1-3. A functional trichome initiation element is
thought to be created by a complex of two bHLH proteins (GL3 or EGL3) with a single GL1 and TTG protein attached to this dimer. MYB-bHLH-WD40 protein complexes are involved in controlling developmental and biochemical processes in several species (Ramsay and Glover, 2005). In Petunia, maize and snapdragon a network of proteins that includes a WD repeat protein, an R2R3 MYB transcription factor, and two bHLH transcription factors is involved in regulating anthocyanin pigmentation (Mol et al., 1998). The fact that these proteins have been found in some cases to be interchangeable between these three species emphasizes the universality of this motif (Mol et al., 1998, Ramsay and Glover, 2005). Using the preceding information and data presented below, I have made a model of the process of the molecular basis of trichome cell fate selection, which is shown in Figure 1-3.

The function of TTG in this process is rather mysterious. It has been suggested that TTG-like WD repeat proteins mediate protein-protein interactions (Mol et al., 1998), or possibly function in a signal transduction pathway (Walker et al., 1999). The ttg loss-of-function phenotype is phenocopied by a gl3 egl3 tt8 triple mutant (TT8 is TRANSPARENT TESTA 8; a gene putatively encoding a bHLH protein similar to GL3 and EGL3) (Zhang et al., 2003) and ttg mutants can be rescued by the maize R gene under the control of the cauliflower mosaic virus 35S promoter (Lloyd et al., 1992; Zhang et al., 2003), which is a promoter that directs constitutive, ectopic expression of genes under its control (Benfey and Chua, 1990). Given that GL1 over-expression has no effect on the ttg phenotype (Larkin et al., 1994) while GL3 or EGL3 over-expression can suppress the ttg
phenotype (Lloyd et al., 1992; Zhang et al., 2003), it is likely that TTG somehow affects the bHLH proteins in such a way as to lower the amount of these proteins needed to generate the initiation signal. This idea is supported by yeast two-hybrid studies that show that GL3, but not GL1 interacts with TTG (Payne et al., 2000). The sub-cellular localization of TTG has not been determined directly. Preliminary evidence, such as lack of an identifiable nuclear localization signal, an unpublished result indicating cytoplasmic localization, and the localization of the TTG homolog AN11 to the cytoplasm in Petunia, all imply that TTG may be restricted to the cytoplasm and thus its exact role in regulating bHLH/MYB transcription factor complexes remains to be determined (Mol et al., 1998; Walker et al., 1999).

The exact assortment of MYB and bHLH proteins that make up the trichome initiation complex is unknown, but it seems as though both bHLH proteins are interchangeable with one another. Expression of EGL3 under the control of the 35S promoter appears to be functionally redundant with expression of GL3 under control of the same promoter (Zhang et al., 2003). A similar situation exists among the MYB proteins, though expression of MYB23 using the 35S promoter only rescues the trichome initiation phenotype; the resulting trichomes from these plants still have fewer branches (Kirik et al., 2005). These collected facts imply a molecular mechanism of trichome initiation in which any combination of the bHLH proteins GL3 or EGL3 form a dimer in the cytoplasm, which is facilitated by TTG. Simultaneously, a MYB protein, either GL1 or MYB23, associates with the bHLH dimer, and if this MYB/bHLH/bHLH complex
Figure 1-3: Hypothetical model of epidermal cell fate determination by interactions between proteins involved in trichome patterning. A combination of a MYB protein (either GL1 or MYB23) with a bHLH dimer (any combination of GL3 or EGL3) produces the trichome initiation complex. This complex activates genes involved in trichome development as well as both initiation genes and an inhibitory signal. This inhibitory signal would be exported to neighboring cells, where it would interact preferentially with the bHLH dimer, preventing expression of genes involved in trichome development. TTG would act in the cytoplasm most probably aiding in the association of the bHLH dimer.
enters the nucleus it will initiate transcription of the genes responsible for trichome development (Figure 1-3).

As mentioned before, the inhibitory signal appears to be comprised of a single type of protein; an R3 MYB lacking an activation domain. TRY has been shown to inhibit GL3/GL1 association in a dosage-dependent manner in the yeast two-hybrid system (Esch et al., 2003). TRY, CPC, and ETC2 (Esch et al., 2003; Kirik et al., 2004b) have all been shown to associate with GL3 in this system as well. These data suggest that these inhibitory MYB proteins inhibit trichome initiation by competing with GL1 for binding to the bHLH dimer preventing transcription from occurring when this complex binds its target DNA. The differences between these proteins lie in their expression patterns and the range at which they act. ETC1, ETC2 and CPC seem to act redundantly in providing long range inhibition (Kirik et al., 2004a), while TRY provides more localized inhibition of trichome formation (Schellmann et al., 2002). The expression of ETC2 inhibits trichome formation on the leaf edges and petiole and the expression pattern of this gene indicates ETC2 may play a role in stomata patterning (Kirik et al., 2004b). This data allows one to create a model of trichome cell fate determination that is complex and highly redundant (Figure 1-3).

Other factors have been shown to have an effect on trichome patterning in Arabidopsis. Normally, epidermal cells have the potential to adopt the trichome fate during a specific window during development (Larkin et al., 1996; Lloyd et al., 1994). The locus REDUCED TRICHOME NUMBER (RTN) plays a role in
regulating this window. Plants with the \textit{RTN} allele from the Landsberg \textit{erecta} background have a shorter period of trichome development compared to plants with the \textit{RTN} allele from the Columbia (Col) ecotype (Larkin et al., 1996). The gene \textit{COTYLEDON TRICHOME 1} (\textit{COT1}) plays an inhibitory role in trichome development. Plants homozygous for the \textit{cot1} mutant allele have no apparent phenotype alone, but in conjunction with \textit{GL1} expression controlled by the 35S promoter, these mutants produce trichomes on the normally glabrous cotyledons as well as other ectopic locations (Szymanski et al., 1998b). Unfortunately these genes have not yet been cloned and characterized, preventing a more thorough understanding of their interactions with other known regulators of trichome patterning. Mutations in the gene \textit{FIDDLEHEAD} (\textit{FDH}) also retard trichome development. This gene encodes a protein with similarity to condensing enzymes involved in lipid biosynthesis and plants without functional copies of \textit{FDH} undergo ectopic fusion of their aerial organs (Yephremov et al., 1999). No other investigations into the mechanism behind this effect have been made, and one can only postulate that this abrogation of trichome initiation is somehow related to the spread of the trichome inhibitory proteins.

\textbf{1.4 Process of Trichome Morphogenesis}

Once a cell is committed to the trichome fate it undergoes a dramatic morphological change. The first obvious change in shape is an increase in diameter with respect to surrounding epidermal cells. Then the incipient trichome begins to protrude perpendicularly from the epidermis of the leaf, forming what will become the stalk of the trichome. After the young trichome has begun to
grow out from the leaf, branches begin to develop (Szymanski et al., 1998a). The canonical branching pattern of wild-type trichomes consists of two distinct branching events. The first branch forms at an approximately 119° angle from the original stalk and this branching event is aligned with the leaf in a proximodistal manner. A second projection subsequently branches from the main stalk at about an 83° angle (Folkers et al., 1997). The trichome continues to grow both in circumference and height and the end of the branches, which were initially blunt, become pointed. After trichome growth is over, the outer surface of the cell wall becomes covered with numerous bumps called papillae (Szymanski et al., 1998a). Compared to the process of trichome cell fate determination, trichome development is much more complex. This complexity makes a complete molecular picture of trichome development more difficult to discern.

Two additional genes have been found that seem to lie downstream of the trichome initiation signal, but upstream of many processes in trichome development. Plants lacking GLABRA2 (GL2) expression appear to have defects in trichome cell expansion, branching and cell wall thickening (Koornneef et al., 1982; Rerie et al., 1994). GL2 encodes a protein with similarity to homeodomain and leucine zipper proteins (Rerie et al., 1994). A pGL2::GUS reporter construct shows GL2 is expressed in trichomes throughout their development (Szymanski et al., 1998a). These phenotypes suggest that GL2 plays an upstream regulatory role in the process of trichome development. TRANSPARENT TESTA GLABRA 2 (TTG2) shares a similar mutant phenotype with GL2. TTG2 encodes a WRKY type transcription factor and is expressed strongly in trichomes throughout their
development (Johnson et al., 2002). *gl2 ttg2* double mutants have an additive phenotype in regard to trichome development, but not in respect to trichome initiation, indicating that the processes these two proteins regulate may overlap (Johnson et al., 2002).

As would be expected from a structure that has such a radically different shape from its parent cell, cytoskeletal reorganization plays a key role in trichome development. Drugs affecting either filamentous actin or microtubules affect trichome development which demonstrates that these structures play a role in trichome formation. Agents that depolymerize actin do not affect the ability of trichomes to form branches, but do prohibit the branches formed from expanding as they do in wild-type trichome branches. The result is a trichome that undergoes irregular radial swelling and severe reductions in branching; much like the “distorted” class of mutants (Szymanski et al., 1999). Application of microtubule destabilizing drugs caused trichomes to become bloated and, most significantly, lose their capability to form branches (Mathur et al., 1999). Correspondingly, when the *sti* (STICHEL) and *zwi* (ZWICHEL) mutants, which have branch defects, were transiently treated with microtubule stabilizing drugs during trichome development new branch points were created (Mathur and Chua, 2000).

Consistent with the pharmacological evidence, genes defined by mutations in cell shape typically encode either cytoskeletal components or proteins that interact with the cytoskeleton. Several genes have mutants with phenotypes that resemble the effect of actin destabilizing drugs (Hulskamp et al.,
Many of these mutations lie in genes that encode subunits of actin-related protein 2/3 (ARP 2/3), which serves to nucleate actin filaments, or WASP family verprolin homologous protein (WAVE) complexes that regulate the ARP2/3 complex (Szymanski, 2005). Several mutants with branch defects have been traced to genes that are linked to the microtubular cytoskeleton. *LEFTY1* and 2 encode α-tubulin proteins. *lefty1 lefty2* double mutants have trichomes with reduced branch number (Abe et al., 2004). *KATANIN1 (KTN a.k.a. FRAGILE FIBER 2 and FURCA2)* encodes a microtubule severing protein; plants without KTN1 function have trichomes with only 2 branches (Burk et al., 2001). *ZWICHEN*, whose mutants have only two branches, was the first branch mutant to be cloned. *ZWI* encodes a calmodulin-binding kinesin-like motor protein (Oppenheimer et al., 1997). ZWI interacts with two other proteins involved with trichome branching: ANGUSTAFOLIA and KCBP-interacting Ca2+ binding protein. All three of these proteins are thought to be involved in microtubule-associated vesicular trafficking in trichomes (Folkers et al., 2002; Oppenheimer et al., 1997; Reddy et al., 2004). Based upon this knowledge it seems that microtubules are responsible for branch initiation, possibly playing a role in transporting materials to the site of branch initiation, while proper regulation of filamentous actin is required for proper branch extension. Both systems seem to be required for maintenance of proper stalk shape and ablation of both microtubules and actin prevent trichome outgrowth (Mathur et al., 1999).

While cytoskeletal reorganization gives the trichome its shape, cell expansion processes involved in trichome development must make a cell larger
than its precursor. The process of plant cell expansion is driven chiefly by cell wall formation and extension (Cosgrove, 1997). The cell wall is composed primarily of bundles of cellulose that condense into crystalline structures called microfibrils (Richmond, 2000). Microfibrils are synthesized by a structure called the rosette terminal complex which contains cellulose synthase catalytic subunits (Kimura et al., 1999). The rosette terminal complex spans the plasma membrane and apparently is associated with microtubules in the cytoplasm, which are involved in guiding them as they lay down the cell wall (Wasteneys, 2004). When the cell wall is initially formed, it can be loosened to accommodate further cell expansion via the action of expansins, but the wall loses this ability after it matures (Cosgrove, 1997). Though there are 6 families of “cellulose synthase-like” genes in Arabidopsis (Richmond, 2000), a search of the literature did not reveal a cellulose synthase reported to be expressed in a trichome specific manner. However, the gene $KORRIGAN2$, which encodes a membrane-anchored endo-1,4-β-D-glucanase, is expressed in a trichome specific manner on the leaf epidermis and is thought to be involved in cell wall assembly during growth (Molhoj et al., 2001).

As indicated by this complexity in coordinating the cytoskeletal and cell growth processes required to produce a trichome, it is daunting to think of all of the processes that must be modified in addition to the cytoskeleton to produce this structure. To accommodate such massive cellular restructuring other physiological processes must be affected as well. However, it will be difficult for traditional genetic screens to detect these proteins if they are functionally
redundant with other proteins or if they are essential for survival of the plant. New techniques will need to be adopted. For instance, cell-specific protein profiling was used to identify a group of proteins involved in sulfur metabolism in trichomes (Wienkoop et al., 2004). Also, as described in this dissertation, an enhancer trap has led to the discovery of a carbonic anhydrase that is specifically expressed in trichomes.

1.5 Modulation of the Cell Cycle During Trichome Development

As a cell develops into a mature trichome, it undergoes many structural and physiological changes. One of the most interesting of these changes has to do with the regulation of the cell cycle. Early in their development trichomes replicate their chromosomes several times without undergoing mitosis (Hulskamp et al., 1994). This alternate cell cycle is called endoreplication, or endoreduplication (Edgar and Orr-Weaver, 2001). Endoreplication is common in plants (Joubes and Chevalier, 2000), and Gailbraith et al. (1991) reported that cells from every tissue in Arabiodpsis possesses endoreplicated cells with the exception of the inflorescence. Endoreplication also has been found to occur in many other systems including: mammalian megakaryocytes (Zimmet and Ravid, 2000), placental trophoblasts (Zybina and Zybina, 1996), vascular smooth muscle cells (Berk, 2001), cancer tumors (Schwerer et al., 2003), spiders (Rasch and Connelly, 2005), and endoreplication has been extensively studied in Drosophila (Edgar and Orr-Weaver, 2001).

Though the function of endoreplication has not been unequivocally established, there is a positive correlation between the amount of endoreplication
and cell size in many systems (Sugimoto-Shirasu and Roberts, 2003). This
correlation may be a result of a long observed karyoplasmic ratio; an observation
that states that the cell physiologically maintains a constant ratio of DNA to
cytoplasm (Wilson, 1925, Sugimoto-Shirasu and Roberts, 2003). Another
hypothesis is that endoreplication occurs in preparation for rapid growth or high
metabolic activity that a cell will undergo (Edgar and Orr-Weaver, 2001). Trass et
al. (1998) theorized that this correlation between cell size and polyploidy may be
the result of asynchronous arrests of the cell cycle and cell expansion within cells
in a tissue. Whatever causes this correlation between cell size and
endoreplication level, it is readily apparent on the leaf epidermis of Arabidopsis.
The DNA content among epidermal pavement cells on Arabidopsis leaves
ranges from 4C to 16C (or 4 to 16 copies of the genome) and cells with higher
ploidy levels are larger than those with less copies of the genome (Melaragno et
al., 1993). This correlation also persists in trichomes, which are much larger than
epidermal pavement cells and have an even higher ploidy level averaging 20-
32C (Hulskamp et al., 1994; Melaragno et al., 1993).

A number of mutants have been identified that affect endoreplication in
trichomes. It should be noted that these mutants seem to have a positive
correlation between endoreplication amount and branch formation i.e. try mutants
have extra branched trichomes and have increased DNA content, while gl3
mutants show the opposite phenotype in both respects (Hulskamp et al., 1994).
Mutations in KAKTUS (KAK) and SPINDLY (SPY), RASTAFARI (RFI), and
POLYCHOME (PYM) also have increased branching and increased
endoreplication (Perazza et al., 1999). KAK encodes a HECT-class E3 ubiquitin ligase that regulates endoreplication in several tissues throughout the plant; probably through regulation of ubiquitin-mediated proteolysis of cell cycle proteins (Downes et al., 2003; El Refy et al., 2003). SPY encodes a putative O-linked N-acetyl-glucosamine transferase that is involved in the gibberellin (GA) signaling pathway (Jacobsen et al., 1996; Swain et al., 2002). The protein products of RFI and PYM have not yet been identified. It is worth noting that trichomes of Arabidopsis tetraploids also have more branches than those found on diploid plants (Perazza et al., 1999).

Changes in the expression of various cell cycle regulators can lead to cell division in the normally unicellular trichome. Expression of either CYCLIN B 1;2 (CYCB1;2) or CYCLIN D 3;1 (CYCD3;1) under the control of the GL2 promoter created multicellular trichomes (Schnittger et al., 2002a; Schnittger et al., 2002b). Interestingly, the trichomes still retain the same basic morphology. This has led investigators to propose that branching in Arabidopsis trichomes is a left-over function of a cell division pattern (Schnittger and Hulskamp, 2002). During my time of study, our laboratory has discovered a mutant in a gene called SIAMESE (SIM) that is involved in repressing mitotic divisions in trichomes (Walker et al., 2000). In this work I show evidence that SIM encodes a protein similar to a cyclin-dependent kinase inhibitory protein (CKI) that is localized to the trichome nucleus.
1.6 Function of Trichomes

A common question asked of investigators that study trichomes is “What do trichomes do?” Trichomes have been ascribed many potential useful attributes (Larkin et al., 2003) and a popular theory is that trichomes play a role in defense against insect herbivory. In Arabidopsis genes responsible for methylsalicylate biosynthesis are expressed in the support cells of trichomes (Chen et al., 2003). Methylsalicylate production is increased in response to wounding and insect herbivory and has been proposed to be involved in defense (Chen et al., 2003). The glandular trichomes of tobacco are the structures that secrete nicotine, which is a potent insecticide as well as having a narcotic effect on humans (Laue et al., 2000). Non-glandular trichomes on Dutchman’s pipe, Aristolochia elegans, have been shown to either retard the rate in which the caterpillar Battus philenor consumes the plant, or to make this caterpillar more exposed to predators (Fordyce and Agrawal, 2001). It has been shown that beetles prefer portions of a leaf of Salix borealis in which the trichomes have been mechanically removed to portions with trichomes still remaining (Zvereva et al., 1998), but another group showed that Datura wrightii did not benefit from having trichomes as compared to being glabrous (Elle and Hare, 2000). Ecological studies with Arabidopsis indicate that there is selective pressure for increased trichome density in the presence of insect herbivores (Mauricio and Rausher, 1997). There are other possible functions of trichomes as well. Trichomes have been implicated in temperature regulation (Klich, 2000), regulation of water loss and gas exchange (Schreuder et al., 2001), and as a
place for plants to store toxic metals (Salt et al., 1995). Like their role in the wild, the role of trichomes in the laboratory is varied as well. A model for cell fate determination, cell cycle regulation, a bellwether of cytoskeletal or physiological change; trichomes could be said to serve any number of functions, and for that reason they are a cell worthy of more through investigation.
CHAPTER 2. MATERIALS AND METHODS

2.1 Recombinant DNA Construction Techniques

2.1.1 Restriction Enzyme Digests

Restriction enzyme digests were performed by adding an appropriate amount of DNA to a 40µl total reaction containing 1µl of the restriction enzyme 4 µl of the appropriate 10X buffer and 4µl 10X BSA if necessary. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). After the reagents were combined the restriction digest was incubated at the manufacturer’s recommend temperature for 2 hours. When cutting a vector molecule, 1µl of calf alkaline intestinal phosphatase (CIP) (New England Biolabs, Ipswich, MA, catalog #M0290S) was added to the reaction mixture to dephosphorylate the vector molecule. After digestion the reaction mixture was incubated at 70°C for 15 minutes to deactivate the enzymes.

2.1.2 Purifying Vector and Insert DNA.

Gel extraction was performed by excising a slice of an agarose gel containing the DNA of interest using a razor blade and the purifying this DNA using a QIAprep Spin Miniprep column (QIAGEN, Inc., Valencia, CA, Catalog #27106) according to the instructions provided by the manufacturer. Ethanol precipitation was accomplished by adding 2 volumes of 95% ethanol to a volume of DNA-containing solution along with 0.1 volumes of 3 M sodium acetate. This solution was placed in the -20°C freezer for 30 minutes; then spun for 30 minutes in a microcentrifuge at maximum speed. After this spin, the supernatant was decanted and discarded and 100µl 70% ethanol was added to the pellet to wash
it. Once the 70% ethanol was added the pellet was not disturbed and the tube was re-spun for five minutes at maximum speed. After this spin the supernatant was again decanted and discarded and the tube containing the DNA pellet was inverted on the benchtop and allowed to dry at room temperature.

2.1.3 Ligation of DNA Fragments

All recombinant DNA constructs were made with the indicated DNA fragments using T4 DNA ligase (New England Biolabs catalog number M0202S). The insert fragments were mixed with the vector fragments in an approximate 3:1 molar ratio for sticky-end restriction fragments and a 10:1 insert to vector ratio for blunt-end fragments. These concentrations were estimated by running the vector and insert DNA fragments together on a 0.8% TAE gel containing ethidium bromide and comparing the intensity of the bands generated DNA fragments to one another. 1µl of ligase along with 2µl of T4 ligase buffer were added to the DNA fragments and the mixture was incubated at 14°C for 12-16 hours. After this time 10µl of the ligation reaction was used directly in the transformation of chemically competent cells or the entire reaction was ethanol precipitated and resuspended in 2 µl of ddH₂O if used to transform cells via electroportation.

2.1.4 Transformation of *Escherichia coli* and *Agrobacterium tumefaciens*

The *E. coli* strain DH5α and the *Agrobacterium tumefaciens* strain LBA4404 were used as hosts for all constructs. Transformation of *E.coli* was carried out primarily using the heat shock transformation technique, while transformation of *A. tumefaciens* required electroporation to take up foreign DNA (Sambrook et al., 1989). Transformation of *E. coli* by heat shock was
accomplished by incubating the chemically competent *E. coli* along with the DNA of interest on ice for 30 minutes, then transferring the tube to a 42°C water bath for 30 seconds. The cells were then added to a sterile test tube along with 1 mL of LB broth. After this the cells were allowed to recover in a shaking 37°C incubator for 40 minutes. The cells were then plated on LB 1.5% agar plates containing the antibiotic corresponding to the vector used and placed in a 37°C incubator overnight, after which colonies could be seen.

Electroporation was done using electroporation cuvettes with a 200 mm gap distance (USA Scientific catalog # 9104-5050) and an electroporator (Bio-Rad model # 1652102). Both the cuvette and the electrocompetent cells were always kept on ice until used. The electroporator was set to 2.50 volts and activated. Immediately after the electrical pulse 1 mL of LB broth was added to the cuvette and this broth/cell mix was transferred to a 15 cm test tube using a sterilized Pasteur pipette. The cells were then allowed to recover for 60 minutes by shaking at 37°C. The cells were then plated on LB 1.5% agar plates containing the antibiotic corresponding to the vector used and placed in either a 37°C (for *E. coli*) or 28°C (for *A. tumefaciens*) incubator overnight or until colonies appeared.

2.1.5 Molecular Analysis of Bacterial Transformants

Once colonies appeared on the antibiotic plates after transformation, they were inoculated into 3 mL of LB broth containing the appropriate antibiotic using a sterile toothpick stuck into a bacterial colony. The LB broth inoculated with a candidate colony was placed in a 37°C shaking incubator shaker overnight. DNA
was extracted from the culture by alkaline lysis method (Birnboim and Doly, 1979). 1 mL of culture was added to a 1.5 mL Eppendorf tube and spun at 10,000 rpm for one minute. The supernatant was discarded and the cellular pellet was resuspended 100 µl GTE buffer (50mM glucose, 25 mM Tris pH 8.0 10mM EDTA). After resuspension, 200 µl NaOH/SDS (0.2 N NaOH 1% SDS) solution was added and this mixture was mixed by vortexing. Then 150 µl sodium acetate/acetic acid solution (100 mL 5 M potassium acetate and 172 ml 5 M acetic acid) was added, the solution was mixed, and left at room temperature for 5 minutes. Then the solution was spun at maximum speed in a microcentrifuge for 5 minutes, after which 350 µl of supernatant was transferred to a fresh tube. To this new tube 700 µl 95% ethanol was added, the solution mixed by inversion and then spun for 5 minutes at maximum speed. The supernatant of this solution was decanted and 500 µl of 70% ethanol was added to wash the pellet. After spinning the 70% ethanol containing tube for 5 minutes, the ethanol was decanted, the tube inverted and the pellet was allowed to dry on the bench top for 1 hour. Afterwards the pellet was resuspended in 50µl TE buffer pH 7.7. 3 µl of this DNA solution was digested in a 10 µl total volume restriction enzyme reaction containing 1 µl RNase A (10 mg/ml). One half of this reaction was run on a 0.8% TBE gel to check for the presence of the appropriate DNA construct. Once the correct construct was found, a glycerol stock was made using 1 ml of the remaining cell culture combined with 500 µl 50% glycerol. This stock was cataloged and placed into the -80°C freezer for permanent storage.
2.2 Plant Growth Methods

2.2.1 Plant Growth Conditions

Seeds were sown on a combination of potting soil and vermiculite in 2” x 2” x 2 1/8” pots. The pot was filled approximately 2/3rds full with extra coarse vermiculite (Sun Gro Horticulture, Pine Bluff, AR) and covered with potting soil. After the seeds were sown, the pots were place in trays into which water and Hoagland’s solution (Epstein, 1972) were added. Then the plants were placed into a growth chamber where they were exposed to constant illumination of 40-W Sylvania Cool White fluorescent bulbs (~100 μE m\(^2\) s\(^{-1}\)) at 21°C. Until germination, the plants were kept under a humidity dome. Plants were watered and fertilized periodically throughout their life cycle.

2.2.2 Agrobacterium-Mediated Transformation of Arabidopsis

Plants were transformed by the floral dip method (Clough and Bent, 1998). The binary vector containing the DNA of interest was transferred to Agrobacterium by electroporation as described above. The presence of the target DNA in a specific Agrobacterium clone that would be chosen for later use was confirmed using PCR. This clone was then inoculated into 500µl of LB broth supplemented with 50µg/ml kanamycin and placed into a 28°C shaking incubator for 24 hours. After incubation, the culture was centrifuged at 6,000g for 15 minutes to pellet the cells. The cell pellet was then resuspended in 500 ml 5% sucrose solution with 0.2% Silwet L-77 (Setre Chemical, Memphis TN) added immediately before the cells were exposed to the plant. Before transformation, the already developed siliques of plants that were to be transformed were
removed to enrich the proportion of transformant seeds by eliminating untransformed seeds. Once the plants were prepared, their floral organs were soaked in the Agrobacterium-containing sucrose solution for one minute. After soaking, plants were returned to the growth chamber and allowed to grow until the seeds were ready to harvest. Seeds from these plants were sown on ½ strength MS, 0.9% agar plates supplemented with 50 µg/ml kanamycin or 20 µg/ml hygromycin depending upon the selectable marker in the vector. For vectors conferring hygromycin resistance, the plates contained no MS salts, only 0.9% agar and 20 µg/ml hygromycin. Plants that survived the antibiotic treatment were transferred to soil and allowed to go to seed.

2.3 Polymerase Chain Reaction (PCR) Techniques

2.3.1 Standard PCR Reactions

Unless otherwise indicated PCR reactions conducted in this work consisted of the following: 1X Buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3 @ 25°C), 2 units Taq DNA polymerase (New England Biolabs, catalog #M0267S), 200 µM dNTPs (New England Biolabs catalog #N0447S), 500 nM of each primer (Integrated DNA Technologies, Coralville, IA) in 25 µl sterile ddH2O. Primer sequences are listed in Appendix A. Reactions were assembled on ice and template was added to each tube once the reactions were aliquoted. Reactions were then placed into the thermal cycler (MJ Research, Watertown, MA, Model # PTC-100) and 35 cycles of the following program were performed: 30 seconds at 94°C, 30 seconds at the annealing temperature appropriate for the
primers used, and 30 seconds at 72°C. 5µl of this reaction was run on a 0.8%
TAE gel to assess the success of the reaction.

2.3.2 Quantitative PCR Techniques

Quantitative PCR reactions in this work were performed using either the
Quantitect SYBR green kit (QIAGEN catalog #204143), if using the SYBR green
chemistry (Schneeberger et al., 1995) or the Quantitect Probe PCR kit (QIAGEN,
catalog #204343), if using the TaqMan® chemistry (Gibson et al., 1996). The
reaction mix for SYBR green reactions consisted of 1x SYBR green master mix,
500 nM of each primer, and 1µl of template in a total reaction volume of no less
than 30 µl. TaqMan® reactions contained 1x probe master mix, 50 nM of each
primer, 1.67µM FAM/BHQ1 probe (Biosearch technologies, Novato, CA), and 1µl
of template in a total reaction volume of no less than 30 µl. Primers for any given
reaction are named based upon the gene whose quantity they were intended to
analyze, and the sequence of these primers are listed in Appendix A. Reactions
were aliquoted into wells of a 96-well PCR plate made from optically neutral
plastic (USA Scientific, Ocala, FL, catalog #1402-9708), template added and the
plate was sealed with TempPlate RT Optical transparent film (USA Scientific
catalog #2978-2100). All samples were run in triplicate. Both SYBR green and
TaqMan® reactions were performed using an ABI 7000 Sequence Detection
System (Applied Biosystems, Foster City, CA). Reactions using the SYBR green
chemistry were run using the following thermal cycler program: 10 minutes at
95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 57°C, and 30
seconds at 72°C. Reactions using the TaqMan® chemistry were run using the
following thermal cycler program: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C. Both SYBR green and TaqMan® thermal cycler programs instructed the ABI 7000 to emulate the cycling conditions of the ABI 9600 machine. After the reactions were complete they were examined on a 2% TAE gel to make sure that each reaction produced only a single band with no extraneous reaction products. This step was found to be crucial to obtaining reproducible results.

In this work, both relative and absolute quantitative PCR were used to determine differences in the amount of expression of a given gene or the T-DNA content of a transgene in a transgenic plant (Bustin, 2002; Ginzinger, 2002). Relative quantitation requires the analysis of both the gene of interest and a separate gene which is presumed not to change under the given experimental conditions. In all cases where relative quantitation was used, TUBULIN9 was used as the standardizing gene, in both genomic DNA and cDNA preparations.

Once the reaction was completed, data were analyzed by first taking the derivative of the raw fluorescence values, then defining the baseline fluorescence values either by trial and error or by an automatic algorithm provided within the ABI software. After this the threshold value for these reactions was set manually so that the threshold would cross all the reactions within their logarithmic phase. This generated a Ct, or cross threshold, value by which different reactions could be compared. The difference in the amount of a particular template in two samples was calculated by the ddCt method. If the Ct value of the test gene is referred to as T and the Ct of the standardizing gene is referred to as S and the
two samples are referred to as A and B, then the ddCt is calculated by the formula: \( \text{ddCt} = (T_A - T_B) - (S_A - S_B) \). This value corresponds to the actual amount of starting material in a logarithmic fashion \( 2^{\text{ddCt}} \); i.e. a ddCt of 2 corresponds to a 4 fold difference between samples.

Absolute quantitation in this work was used exclusively with the TaqMan® chemistry to quantify mRNA levels via cDNA content in some experiments. Absolute quantitation requires the construction of a standard curve using ten-fold serial dilutions of DNA containing the amplicon of interest, whose concentration has been determined spectrophotometrically. Five different ten-fold dilutions were used to make the curve as well as a no template control which was used to check for contamination. Using the base pair content and concentration of this DNA the number of copies of this DNA per µl can be calculated. These serial dilutions are used as templates in quantitative PCR and the Ct values derived from the qPCR reaction combined with the calculated DNA concentration can be used to form a standard curve. A calculated least squares fit through this data produces a slope for use in the equation \( y = m \ln x + b \), where “\( m \)” is the slope and “\( b \)” is the y-intercept. Therefore, by using linear regression a discreet number of copies of starting DNA can be reported using a given Ct value for an experimental sample. However, owing to the vagaries of PCR, these experimental samples must be run concurrently with the standard curve. Before cDNA synthesis, the RNA is quantified spectrophotometrically and this calculation is used to normalize the samples to one another after the initial data has been obtained. A sample standard curve is shown in Figure 2-1.
Figure 2-1 Sample standard curve for absolute quantitation. The Ct values were plotted on the Y axis and the numbers of copies of the At5g02420 amplicon were plotted on the X-axis, which is shown in logarithmic scale.
2.4 DNA, RNA, and cDNA Techniques

2.4.1 RNA Purification

RNA was harvested from plants by first freezing the plants in liquid nitrogen and then pulverizing them using an extruded plastic dingus (XPD). RNA was purified using the Plant RNeasy kit (QIAGEN, catalog #74903) following instructions provided by the manufacturer. DNA was removed from the sample by using the DNase kit for the Plant RNeasy kit (QIAGEN, catalog#79254) following instructions provided by the manufacturer. Once RNA was purified, it was resuspended in RNase-free water and stored at -80°C.

2.4.2 Reverse Transcription

Reverse transcription was performed using the Omniscript reverse transcription kit (QIAGEN, catalog #205111) following instructions provided by the manufacturer using “anchored” poly-T as a primer (NVTTTTTTTTTTTTTTTTT). The specific amounts of RNA used in the reaction varied from experiment to experiment. Once cDNA synthesis was complete the cDNA reaction was diluted either 1:2 or 1:5 and stored at -20°C. Before storage a PCR reaction using a primer pair that flanked an intron was performed to check for genomic DNA contamination.

2.4.3 Genomic DNA Extraction

Genomic DNA was extracted using the Sigma GenElute kit (Sigma Chemical Co. St. Louis, MO, catalog # GE-G2-N) following instructions provided by the manufacturer.
2.4.4 Nucleic Acid Quantification

DNA and RNA were quantified using a Beckman DU-65 spectrophotometer. Twenty µl of a given DNA or RNA sample was added to 500 µl of water and the A260 and the A280 of this sample were recorded. The amount of nucleic acid was calculated using the equivalence of one absorbance unit equals 50 µg DNA or 40 µg RNA.

2.5 Microscopy Techniques

2.5.1 Light Microscopy and Leaf Measurement

Larger specimens were observed using a Nikon (model # SMZ-U) dissecting microscope. These samples were usually left unprepared. Images were captured by a microscope mounted digital camera (model #3.2.0, Diagnostic Instruments, Sterling Heights, MI) controlled using the SPOT software. DIC microscopy was performed to visualize developing trichomes on young leaf tissue. The young leaves were exposed by removing one cotyledon mechanically with forceps. The plants were then transferred to a microscope slide and if necessary the leaves were spread apart using microforceps with the aid of a dissecting scope. The tissue was immersed in a solution of eight grams chloral hydrate, one ml glycerol, and two ml water, which decolorized the developing leaf tissue, and a coverslip was placed over the tissue. A Nikon brightfield stereomicroscope (model # Microphot FX-A) was used to visualize the samples. Images were captured using the SPOT digital imaging system (model # 2.3.1, Diagnostic Instruments, Sterling Heights, MI). Leaves were then measured in a manner described by Larkin et al (1996).
2.5.2 GUS Staining

Plants containing a GUS reporter construct were treated in a method described in Larkin et al (1993) prior to visualization on either a compound stereomicroscope or a dissecting microscope.

2.5.3 GFP and Fluorescent Imaging

Fluorescent microscopy was performed on a Leica TCS SP2 laser scanning confocal microscope. Live samples were mounted in either water or 50% glycerol before imaging.

2.5.4 Scanning Electron Microscopy (SEM)

Samples were prepared for SEM by fixation in FAA (37% formaldehyde, 100% ethanol, glacial acetic acid), and water in a ratio of 2.5:9:1:7.5), dehydrated through an ethanol series to 100% ethanol, critically point dried, sputter coated with gold, and viewed at 15kV using a Cambridge (LEO) 260 Stereoscan scanning electron microscope. Images were captured digitally.

2.6 Specific Methods for Each Chapter

2.6.1 Chapter 3 Methods

2.6.1.1 Construction of GL3 Genomic Rescue Fragment

The MYC6 P1 library clone was ordered from the Arabidopsis Biological Resource Center (www.arabidopsis.org/abrc/). *E. coli* containing MYC6 were grown in LB broth supplemented with 50 µg/ml kanamycin and harvested using a QIAGEN Maxi prep kit (QIAGEN Inc. Catalog number 12263) following the instructions provided by the manufacturer. Approximately 2 µg of this DNA was cut with 200 units of EcoRI (New England Biolabs catalog # R0101S) and the
entire reaction was run on a 0.8% TAE gel. A gel slice containing a 6kb fragment was excised using a clean sharp razor and the DNA contained therein was purified using the QIAEX II kit. Two µl of the vector pBluescript SK+ (Strategene) was also cut with 200 units of EcoRI and dephosphorylated using 50 units of CIP along with the enzyme reaction. This DNA was ligated following the procedure described above and transformed DH5α with it. The presence of the insert was confirmed in ampicillin-resistant colonies by digesting the resulting clones with HindIII (New England Biolabs catalog # R0104S). A glycerol stock was made from one of the clones that had the correct restriction enzyme digest pattern. This clone was called pMB01. pMB01 was amplified in LB broth containing 100 µg/ml ampicillin and plasmid DNA was harvested using a QIAGEN maxi prep. The 6kb EcoRI fragment was then subcloned into the binary vector pBIN19 (Bevan, 1984) by digesting pMB01 with EcoRI and purifying the 6 kb fragment as described above. pBIN19 was also cut with EcoRI in a reaction including CIP and this cut DNA was ethanol precipitated. The fragment containing GL3 was ligated to pBIN19 and the DNA from kanamycin-resistant colonies was isolated. This DNA was digested using HindIII to identify which clones contained the correct DNA. A glycerol stock of a clone containing the correct DNA was made and named pMB02. This clone was introduced into Agrobacterium and used to transform gl3-1 mutants plants.

2.6.1.2 Construction of pGL3::GL3::GR

A plasmid containing the rat glucocorticoid receptor (GR), pCaMV-GR, was obtained as a gift from Dr. David Oppenheimer (who received it from Dr.
Mark Schena). This plasmid was used as a template in a 3 primer PCR reaction (Karreman, 1998) to make an in-frame translational fusion between a region of the GR containing the steroid binding domain and the last codon of the last exon of GL3. This PCR reaction contained 100 pg of pMB01, 400 pg pCaMV-GR as templates, and the primers GR-A, MCY6.1A-L, and mega2, as well as the standard ingredients described above. GR-A hybridizes 22 bp from the stop codon of the rat GR coding region, MCY6.1A-L hybridizes 473 bp from the stop codon of GL3, and mega2 is a primer that hybridizes with the 3’end of the 6th exon of GL3 and the 5’ end of the steroid binding domain of the glucocorticoid receptor. This primer serves as a complement to both GR-A and MYC6.1A-L during PCR and during later cycles, PCR fragments that have incorporated the GL3GR primer actually serve as huge primers. This causes three bands to appear on the gel: one band corresponding to the fragment generated by MYC6.1A-L and mega2, another from GR-A and GL3GR and a third corresponding to the fusion of both fragments. Because of PCR kinetics, the amount of this third fragment is scarce, so a 1/100 dilution of this reaction was used as a template using just the GR-A and MYC6.1A-L primers. The product of this reaction was a single band of the appropriate size for a fusion of the 3’ end of the GL3 genomic fragment and the steroid binding region of GR. This PCR product was cloned into the vector pcDNA3.1/V5-His-TOPO using a TOPO cloning kit (Invitrogen, Carlsbad, CA, catalog # K4500-01) to form the plasmid GL3GR-TOPO. To facilitate recombination of this fragment with the entire genomic GL3 fragment, the 6kb EcoRI fragment containing GL3 from pMB01
was subcloned into Litmus29© (New England Biolabs) to form the plasmid GL3-LIT. There is a XmnI site 27 bp from the 3’ terminus of the GL3 coding region and there is no XmnI site in the 866 bp region of GR used to make GL3GR-TOPO. GL3GR-TOPO was cut with XmnI and EcoRV which would create a 890 bp blunt-ended DNA fragment containing the steroid binding region of GR and 8 terminal codons of GL3. This 890 bp fragment was inserted into the single XmnI fragment in GL3-LIT creating pMB03. pMB03 was sequenced and the following mutations were found: G>A 827 bp from the 3’ end of the GR coding region, C>G 569 bp from the 3’ end of the GR coding region, T>C 514 bp from the 3’ end of the GR coding region, and C>T 484 bp from the 3’ end of the GR coding region. All of these mutations were in silent positions of codons. The pGL3::GL3::GR region was removed by digesting the pMB03 plasmid with SacI and StuI (New England Biolabs). This fragment was inserted into the SacI and SmaI sites in the binary vector pBIN19 creating a plasmid called pMB014.

### 2.6.1.3 Construction of 35S::GL3::GR

Attachment of the CaMV 35S promoter to the coding region of GL3::GR was complex. First a region containing the 5’ end of GL3 was amplified using the primers GL3-5’ EcoRI and MYC6 D using the reduced error polymerase ThermalAce (Invitrogen, catalog #E0200). This product was inserted into the vector pBLUNT II TOPO© using a TOPO cloning kit (Invitrogen, catalog #K2830-20) creating pGL3-5’ which was sequenced and found to contain no discrepancies with the amplified region. pGL3-5’ was then cut with EcoRI and EcoRV and religated into the vector. This changed the orientation of the insert
and removed an extra EcoRI and EcoRV site and this plasmid is called pGL3-5’-X. pMB03 was cut with EcoRV, which creates a 4570 bp fragment that contains the 3’ end of GL3 that is not contained within pGL3-5’-X as well as the GR region. This 4570 bp fragment was cloned into the single EcoRV site of pGL3-5’-X to create a promoterless GL3::GR; this plasmid was named pMB004. The 35S CaMV promoter was excised from pBI121 (Clontech, GenBank accession # AF485783) and placed into pBluescript (Strategene, GenBank accession # X52325) creating pMB008. pMB008 was cut with EagI, the single stranded DNA overhang created by the EagI digestion was made double stranded with T4 DNA polymerase and cut with BamHI. pMB004 was cut with XbaI and polished with T4 DNA polymerase and then cut with BamHI; then this was inserted into pMB008. This created a 35S::GL3::GR in pBluescript and was named pMB015. The 35S::GL3::GR construct was then transferred to the binary vector pCAMBIA1301 ((Hajdukiewicz et al., 1994) by cutting pMB015 with ScaI and SalI, cutting pCAMBIA1301 with the same enzymes and inserting 35S::GL3::GR into pCAMBIA1301 creating the plasmid pMB016.

2.6.1.4 Creation of the gl3 egl3 Double Mutant Containing pGL3::GL3::GR

The line SALK_019114, which contains a T-DNA insert in exon six of ENHANCER OF GLABRA3 (EGL3), was ordered from the ABRC (Alonso et al., 2003). This line was crossed to a gl3-1 pGL3::GL3::GR line, and a line homozygous for gl3-1 SALK_019114 and pGL3::GL3::GR was selected from the F2. Lines homozygous for gl3, egl3 and pGL3::GL3::GR were used in all dex induction experiments except where noted.
2.6.1.5 Examination of T-DNA Levels Using Quantitative PCR

To examine the number of copies of T-DNA in each line transformed with pMB02, the amount of GL3 present in the genome of each line was compared to the amount of GL3 present in *gl3-1*, the parent plant, using relative quantitation. The primers GL3 qPCR and TUB9 were used to assess the amounts of these genes within the genome of each line.

2.6.1.6 Analysis of Gene Expression Between *gl3 egl3*, col, GL3OE, and pMB02 Transformants and in the Dex-Inducible Experiment

Relative quantitation was used to determine the difference in GL3 expression in the different pMB02 transformant lines. RNA was harvested from the aerial organs of plants of the various genotypes and cDNA was made from this RNA. For the dex-inducible experiment, RNA was harvested from the aerial organs, including first true leaves, cotyledons, and approximately half of the hypocotyl of plants submerged in the various solutions required for this experiment. Relative quantitative PCR using SYBR green was used to assess the level of a particular message within this cDNA pool and the fold change between either transformed lines or various genotypes was reported using the ddCt method (see above). The primers used in the qRT-PCR reaction were named according to the gene whose level they were intended to measure and the sequences of these primers are listed in Appendix A.

2.6.2 Chapter 4 Methods

2.6.2.1 Comparison of Expression of Genes Flanking the E938 Insert

cDNA was synthesized from RNA harvested from complete shoots of at
least four plants with three true leaves. Relative qRT-PCR was performed using SYBR green using TUB9 as a standardizing gene.

### 2.6.2.2 Analysis of At2g28210 Message Levels in Different Plant Tissues and in the Dex-Inducible System

Absolute qRT-PCR was used to measure At2g28210 levels in GL3:GR plants exposed to either dex, dex and chx, or control situations and different organs of the plant. All plants were of the col ecotype. Tissue labeled “roots” consisted of all the plant tissue below the hypocotyl. “Stem” tissue consisted of inter-nodal stem tissue which was prepared by chopping the stem into very small pieces (less than one cm in length). “Silique” tissue consisted of siliques with the pedicel removed. To acquire enough RNA to perform qRT-PCR for the aforementioned tissues, tissue from dozens of plants had to be collected. “Rosette” tissue was collected from all the aerial organs of at least four different plants which each had three true leaves. Tissue marked “flowers” consisted of the terminus of a developing stem and included developing floral organs as well as the meristematic tissue at this terminus of the plant. Tissue from at least four plants was combined for each replicate. All tissue samples were collected in triplicate at the same time and the analysis of At2g28210 expression was performed on the same plate using the same standard curve for all tissues. For the induction experiment, only the first true leaves of plants dipped in the various solutions were harvested for RNA extraction. These leaves were between 200 and 400 µm long and leaves from at least thirty different plants had to be collected to get enough RNA to perform the analysis.
2.6.2.3 Effect of Ethoxyzolamide on Trichome Growth

GL3::GR gl3 egl3 plants were used to determine the effect of a carbonic anhydrase inhibitor on trichome development because these plants allowed the initiation of trichome development and exposure to EZ to be coupled. EZ powder was suspended in DMSO to make a 100 mM stock solution. It should be noted that only fresh solutions of EZ were used, because after suspension in DMSO and storage at 4°C for more than two weeks, the EZ seemed to lose its potency. The EZ was diluted to a concentration of either 1 mM or 100 µM in a solution of 1% sucrose, 10µM dex, and MS salts. GL3::GR plants were grown on solid media containing 0.8% agar, 1% sucrose, and MS salts until the plants had formed leaves which were competent to form trichomes; i.e. at least 100µm in length. At this time the whole plants were transferred to the appropriate EZ-containing solution or a control solution. Leaf length was measured using an ocular micrometer and DIC microscopy as described previously. At least 20 leaves were measured for each point on the graph detailing the length of leaves exposed to various solutions.

2.6.3 Chapter 5 Specific Methods

2.6.3.1 Construction of a SIM Rescue Plasmid

A 3504 base pair region of the genome that included the coding region of SIM along with 2870 base pairs upstream DNA and 250 base pairs downstream DNA was amplified using the primers SIM whole L 3500 and SIM whole R. This PCR fragment was TOPO cloned, creating the plasmid named pMB065, and DNA sequencing found that the coding region of SIM in this construct did not
differ from that listed for the genomic sequence found on TAIR. A fragment containing the amplicon created by SIM whole L 3500 and SIM whole R was excised from pMB065 using BamHI and XhoI sites which flanked this region of the plasmid. This fragment was inserted into BamHI and SalI sites of pBIN19, creating pMB068, which was subsequently used to transform sim-1 plants. Three kanamycin-resistant lines were obtained and all of these lines rescued the sim phenotype.

2.6.3.2 Absolute Quantitation of SIM and its Arabidopsis Homologues in Various Tissues

Absolute qRT-PCR was used to measure SIM, At1g08180, At3g10525, and At5g02420 transcript levels in different organs of wild-type plants of col ecotype. Tissue labeled “roots” consisted of all the plant tissue below the hypocotyl. “Stem” tissue consisted of inter-nodal stem tissue which was prepared by chopping the stem into very small pieces (less than one cm in length). “Silique” tissue consisted of siliques with the branch connecting the silique to the stem removed. To acquire enough RNA to perform qRT-PCR for the aforementioned tissues, tissue from dozens of plants had to be collected. “Rosette” tissue was collected from complete shoots of at least four different plants which each had three true leaves. Tissue marked “flowers” consisted of the terminus of a developing stem and included developing floral organs as well as the meristematic tissue at this terminus of the plant. Tissue from at least four plants was combined for each replicate of these tissues. All tissue samples were collected in triplicate at the same time. The qRT-PCR analysis of these samples were all performed on the same plate with the same standard curve.
2.6.3.3 Absolute Quantitation of SIM in *gl3 egl3*, *sim-1*, wild-type, SIM$^\text{OE}$, and GL3$^\text{OE}$ Plants

To measure the amount of SIM in *gl3 egl3*, *sim-1*, wild-type, SIM$^\text{OE}$, and GL3$^\text{OE}$ plants, RNA was extracted from the aerial organs of plants with at least three true leaves. For each genotype, the tissue of at least four plants was combined before RNA extraction. Absolute quantitation of SIM levels in the subsequent cDNA reaction was measured using TaqMan® qRT-PCR.
3.1 Introduction

Though many genes have been found to be involved in the process of trichome development, very little is known about the regulation of these genes. The group of genes that form the complex to initiate trichome cell fate, hereafter referred to as the trichome initiation complex, is best characterized. The trichome initiation complex is comprised of GL1, a MYB transcription factor; TTG, a WD40 protein; and GL3, a bHLH transcription factor (Payne et al., 2000). It is thought that GL1 and GL3 are the only proteins which directly regulate transcription. TTG lacks a nuclear localization signal, and WD40 proteins are thought to mediate protein-protein interactions and not to function as direct transcriptional regulators (Ramsay and Glover, 2005). The trichome initiation complex should regulate all genes involved in trichome development either directly, through direct contact with the regulatory sequences of trichome-specific genes, or indirectly, through production of transcription factors which up-regulate the expression of trichome-specific genes. We wished to identify genes that are regulated by the trichome initiation complex, and determine whether they are regulated in a direct or indirect manner, with the ultimate goal of discovering new genes previously not known to be involved in trichome growth.

This chapter describes the development of a system to determine whether a gene is regulated by the trichome initiation complex. This system is based upon alterations in the expression and behavior of GL3. GL3 was mapped and cloned
in the Larkin lab coincidentally by Payne et al (2000), and several results from this effort proved useful in developing this system. First, a gl3 elg3 double mutant was used to produce completely glabrous plants. Then, in the course of cloning GL3, several plants with hypermorphic trichome phenotypes were obtained, and these were used in comparisons of gene expression in different plants based upon trichome amount. Finally, GL3 was modified to create an inducible system in which trichome formation is dependent on the addition of a steroid hormone with the intended goal of identifying direct targets of GL3.

Many genes are known to be involved in trichome development (see Chapter One). Several of the genes with trichome mutant phenotypes, such as KAK and KTN1 (Burk et al., 2001; El Refy et al., 2003), are ubiquitously expressed in the epidermis rather than in a trichome-specific manner. It is therefore unlikely that these genes are controlled by the trichome initiation complex; the fact that defects in the function of these genes cause a trichome phenotype probably owes to the fact that alterations in trichome morphogenesis is more easily observed as compared to other cell types on the leaf epidermis. Based upon the lateral inhibition model of trichome cell fate selection, genes involved in both initiating and inhibiting trichomes should be regulated in some fashion by the trichome initiation complex. The known reporter construct phenotypes of the genes involved in cell fate determination make it likely that they are controlled by the trichome initiation complex (Larkin et al., 1993; Schellmann et al., 2002; Bernhardt et al., 2003; Zhang et al., 2003; Kirik et al., 2004; Kirik et al., 2005). Based upon their mutant phenotypes and reporter
construct data, the transcription factors GL2 and TTG2 are good candidates for regulation by the trichome initiation complex (Szymanski et al., 1998; Johnson et al., 2002). These transcription factors and the genes involved in trichome cell fate determination provide several candidates for direct regulation by the trichome initiation complex.

In this chapter, we also attempt to determine which genes that appear to be regulated by the trichome initiation complex are direct targets of GL3. To accomplish this, the GL3 protein was fused to the rat glucocorticoid receptor (GR) steroid binding domain. Conveniently, the steroid binding domain of GR is physically and functionally separable from the DNA binding domain (Rusconi and Yamamoto, 1987). This allows attachment of the steroid binding domain to another protein via recombinant DNA techniques to create a steroid-inducible fusion protein. The primary benefit of using this system is that under these conditions, the GL3::GR protein will already be expressed and loitering in the cytoplasm. This allows for the addition of a protein synthesis inhibitor, cycloheximide (chx), to be added to the dexamethasone-containing media to prevent expression of genes under control of transcription factors downstream of the GL3 initiation complex. This strategy was shown to work for LEAFY, a gene involved in flower production (Wagner et al., 1999). This should provide a situation where direct targets of the trichome initiation complex can be discriminated from those which are under the control of transcription factors which are themselves produced by the trichome initiation complex.
3.2 Results

3.2.1 Complementation of GLABRA3

The gl3-1 mutant allele was uncovered in an EMS mutagenic screen reported by Koornneef et al. (1982). The trichomes of the gl3-1 mutant have fewer branches, occur in fewer numbers, and occasionally occur in clusters (Figure 3-2 A, G, and M). GL3 was mapped using molecular markers to a region on chromosome V between the markers AtS0191 and DFR by Jason Walker in the Larkin lab. A P1 artificial chromosome clone within this region is named MYC6. A bHLH protein from maize named R was previously shown to spur trichome production in the normally glabrous ttg mutant by Lloyd et al. (1992). For this reason the bHLH on MYC6 was a good candidate to be GL3. The bHLH gene on MYC6 is flanked by two EcoRI restriction sites, 1798 base pairs upstream of its ATG and 776 base pairs downstream of its stop codon. The EcoRI fragment containing the bHLH gene was inserted into the binary vector pBIN19 to create pMB02 (Figure 3-1). Introduction of pMB02 into gl3-1 mutant plants rescues the gl3 phenotype, restoring wild-type trichome production (Figure 3-2 C, I and O). This gene was sequenced in a gl3-1 mutant and a mutation was found in codon 378 that converts a glutamine residue to a stop codon. This mutation would truncate the C-terminal portion of this gene which contains the bHLH DNA binding region. These findings are consistent with work described by Payne et al. (2000) in their published account of the cloning of GL3.

Some gl3-1 plants harboring the pMB02 construct have phenotypes that do not resemble wild type (Figure 3-2 E and F). These lines have trichomes with
Figure 3-1 Schematics of the pMB02, pMB014, and the pMB016 constructs.
The black arrows indicate exons and these arrows are pointing from the 5' end of GL3 to the 3' end of the gene. Promoters are identified by crosshatched markings, while the glucocorticoid receptor is denoted by vertical lines. Other entities are identified on the figure. A. pMB02 was made by excising a 6 kb EcoRI fragment from the MYC6 library clone and inserting this EcoRI fragment into a binary vector. This creates a fragment that can rescue the gl3-1 mutant. B. pMB014 was constructed by inserting a PCR product of a 3-primer amplification that made a 3' fusion of GL3 and the steroid binding site of GR into a XmnI site that is unique in pMB02. This creates a GL3::GR fusion under the control of the genomic GL3 promoter. C. The pMB016 construction was complex (see Materials and Methods chapter); essentially it removes the GL3 promoter and replaces it with the CaMV 35S promoter.
Figure 3-2 Phenotypes of plants transformed with pGL3::GL3. *gl3*-1 plants transformed with the pMB02 construct had several different phenotypes. Whole leaf phenotypes are shown in the left column, a close-up showing clusters of trichomes is seen in the center column and close-ups of individual trichomes are shown in the left column. Line 5.0 (C, I and O) is an example of plants that exhibited a simple rescue of the *gl3* phenotype. The other lines represent other phenotypes seen within the transformant lines. Increases in trichome number, branching, and clustering were frequently seen. The most extreme case, line 11.5,(F, L and R), had misshapen trichomes that resembled those found in the *dis* class mutants. Size bars A-F 1 mm, G-L 500µm, M-R 100µm.
increased branching, frequent trichome clusters, and sometimes the trichomes have a distorted shape. These phenotypes occur at varying severity between lines; some lines have just a few clusters and trichomes with extra branches, while others have large clusters of extra-branched trichomes. Three of these lines are described in this work. Line 5.2 shows a very slight change in trichome phenotype: it has more frequent occurrences of 4 branched trichomes and a few clusters of trichomes. Line V10 shows a more severe phenotype: there are far more trichomes than found on a wild type leaf, most of the trichomes have more than 4 branches and there are many trichome clusters, especially near the edges of the leaf. Line 11.5 displays the most severe phenotype: not only are there an overall increase in trichome number, many trichomes appear in clusters and most trichomes have either extra branches or no branches at all; they just assume a globular phenotype reminiscent of the trichomes found on the “distorted” class of mutants.

Agrobacterium-mediated transformation frequently produces multiple T-DNA insertion events (Grevelding et al., 1993). An examination of the number of copies of the GL3 gene construct in these lines was performed using quantitative PCR. The numbers of copies of the GL3 genes was compared to the number of copies of TUBULIN9 in the genome, which allowed for an approximation of the number of copies of GL3 present in each transformant line. This technique shows a positive correlation between trichome phenotype severity in the transformed lines and pMB02 T-DNA content (Figure 3-3). Subsequently, RNA content was
Figure 3-3 Comparison of the number of copies of GL3 DNA within different transformant lines. Quantitative PCR was used to examine the amount of T-DNA inserts in transformed lines. Copies of GL3 DNA were compared between a transformant line and an untransformed *gl3-1* plant. Genome copies were normalized using TUBULIN 9 as a reference gene. The Y-axis represents the fold change in GL3 content, and therefore the numbers of copies of GL3 DNA, between *gl3-1* and the transformant line. A is a graphical representation of the numerical data contained in B.

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<th>Line</th>
<th>Fold increase in GL3</th>
<th>standard deviation</th>
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<tr>
<td>T 5</td>
<td>1.9</td>
<td>1.4</td>
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<tr>
<td>T 5.2</td>
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<td>V 10</td>
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<td>T 11.5</td>
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analyzed using qRT-PCR. The amount of GL3 message was compared in each transformant line to the amount of message in the parent plant of these transformants, gl3-1. The two most severe lines, 11.5 and V10, showed a large increase in GL3 expression compared to their gl3-1 parent, correlating with their increase in GL3 copy number, but the lines with weaker phenotypes, such as lines 5 and 5.2 showed very little change in GL3 expression (Figure 3-4). The expression in WT col plants was similar to that seen in line 5, which was the transformant line that most closely resembled the wild-type phenotype. These data shows that increasing the copies of GL3 in the genome has a corresponding effect on GL3 expression and this increase in expression can dramatically alter the trichome phenotype.

3.2.2 Plants with Both 35S::GL1 and Extra Copies of GL3 Produce Ectopic Trichomes

A plant that showed a strong supernumerary trichome phenotype following transformation with the GL3 genomic fragment (plant V10 from Figure 3-2 hereafter referred to as GL3OE, standing for GL3 over-expressor) was crossed with a plant containing the 35S::GL1 construct in order to further characterize the behavior of GL3. The 35S promoter directs expression in all tissues to varying degrees (Benfey and Chua, 1990). Though 35S::GL1 plants have fewer trichomes than wild-type on their true leaves they produce a few trichomes on the cotyledons, as well as other tissues which are normally glabrous (Szymanski and Marks, 1998). When GL3OE is crossed to 35S::GL1, the heterozygous lines produce trichomes in ectopic locations that otherwise lack trichomes in both wild-
Figure 3-4 Change in GL3 expression between GL3 transformants and gl3-1 mutant plants. GL3 message level was examined in the 4 GL3 transformants described above and wild-type plants of the col ecotype by comparing the amount of GL3 message in these plants to that found in gl3-1 mutant plants using relative qRT-PCR. TUB9 was used as a reference gene to normalize the amount of input RNA in the analysis. The Y-axis describes the fold change in GL3 expression between a particular line and the gl3-1 mutant. This data shows very little change in GL3 expression between gl3-1 and WT, T5, or 5.2 plants. A large change in expression is seen between gl3-1 and lines V10 or 11.5, which are two lines that show the strongest phenotypes. A is a graphical representation of the numerical data contained in B.
type and the GL3^{OE} line (Figure 3-5). While 35S::GL1 plants have less than one trichome per cotyledon (Larkin et al., 1994), plants containing both 35S::GL1 and GL3^{OE} have several trichomes on the adaxial surface of the cotyledons (Figure 3-5 E). These plants also had many more trichomes on the abaxial surface of the first leaves than plants containing only 35S::GL1 (Figure 3-5 F). Upon examination it was found that these tissues are glabrous in plants carrying only the GL3^{OE} construct (Figure 3-5 D). The adaxial surface of first leaves in these plants was indistinguishable from GL3^{OE} alone by visible inspection. These results indicate that the GL3 promoter may direct expression of GL3 in the abaxial surface of the first leaf and the cotyledon, while the expression of GL1 is normally restricted in these tissues.

3.2.3 Identification of Genes Exhibiting Trichome-Specific Expression

The expression of genes involved in trichome production was examined in gl3 egl3, WT (col ecotype), and GL3^{OE} plants using quantitative RT-PCR. These three genes represent a dosage series of functional GL3. All plants were approximately two weeks old and had three true leaves. Plants of this age have trichomes of many different developmental stages, from incipient to fully mature, so a comparison of mRNA levels between glabrous and pubescent plants should show a difference in expression in a given gene regardless of the point in development at which the gene is active. If a gene is expressed in a trichome specific fashion one would expect to see a greater amount of this gene’s message in WT plants as compared to glabrous plants. Similarly, an even
Figure 3-5 Plants harboring 35S::GL1 and multiple copies of pMB02 have ectopic trichomes. Trichomes are not found on cotyledons or abaxial surface of first leaves of wild-type plants. Plants containing 35S::GL1 have trichomes on the cotyledons, but they are very rare and no trichomes were observed on the abaxial surface of the first leaves of these plants. No trichomes have been observed on either of these tissues in GL3\textsuperscript{OE} plants. When GL3\textsuperscript{OE} and 35S::GL1 plants were crossed many trichomes were seen on both the cotyledons and the abaxial surface of first leaves. A. col plant with the 35S::GL1 construct showing the cotyledons and emerging first leaves. B. The abaxial surface of a 35S::GL1 plant. C. The cotyledon of GL3\textsuperscript{OE}. D. The abaxial surface of the first leaf of GL3\textsuperscript{OE}. Note the lack of trichomes in both cases. E. A young 35S::GL1/ GL3\textsuperscript{OE} plant showing vigorous trichome production on the cotyledons. F. The abaxial surface of a 35S::GL1/ GL3\textsuperscript{OE} first leaf showing trichome formation. G. SEM of a 35S::GL1 cotyledon. H. An underdeveloped trichome found on the leaf shown in G.
greater amount of this gene’s expression should be seen in GL3\textsuperscript{OE} plants. \textit{TUBULIN9 (TUB9)} was used as an internal standard for relative quantitation. Since trichomes grow very rapidly, it would be reasonable to assume that the expression of many genes involved cell structure and maintenance, which are normally chosen as standards for relative quantitation would also increase during the course of trichome development. \textit{TUB9} should be unaffected by the presence or absence of trichomes because it is expressed in the vasculature of the leaf rather than the epidermis (Cheng et al., 2001).

Figure 3-6 shows that two genes, \textit{GL2} and \textit{MYB23} are expressed much more strongly in pubescent plants than in those without trichomes. \textit{TTG} expression seems to be unaffected by trichome production, implying that it is not controlled by \textit{GL3}. \textit{TRY}, \textit{CPC}, and \textit{ETC1}, which are three of the genes involved in trichome inhibition, showed a slight increase in expression between \textit{gl3;egl3} and col plants, but they showed a much larger increase in expression between glabrous plants and GL3\textsuperscript{OE}. Of these inhibitory genes, \textit{CPC} seems to show the largest increase between glabrous and pubescent plants. \textit{TTG2}, whose phenotype is similar to the \textit{GL2}, shows a moderate increase in expression as the number of trichomes increases. \textit{GL1} shows a slight increase in expression while \textit{GL3} and \textit{EGL3} show no increase in expression between wild-type and glabrous plants. The 16-fold increase in expression between GL3\textsuperscript{OE} and glabrous plants is to be expected because these plants are over-expressing \textit{GL3}. The \textit{STI}, \textit{ZWI}, \textit{KTN1}, and \textit{AN} genes all show no increase in expression between glabrous and
Figure 3-6: Fold change in expression of genes involved in trichome initiation and development. Quantitative RT-PCR results showing relative change in expression of various genes between glabrous gl3;egl3 plants and either col or GL3OE plants. TUB9 was used as a standardizing gene for relative quantitation. A. Bar graph of data. The Y-axis represents the fold change in the number of copies of a particular transcript between either gl3;egl3 and col or gl3;egl3 and GL3OE as noted by the color of the bar on the graph. B. Numerical values represented on bar graph.
pubescent plants. This result is expected, because these genes have been shown to be expressed throughout the epidermis rather than in a trichome-specific manner (Oppenheimer et al., 1997; Burk et al., 2001; Folkers et al., 2002; Ilgenfritz et al., 2003). These data indicate that GL1, GL2, TTG2, TRY, CPC, ETC and MYB23 are expressed in a GL3-dependent fashion, while the other genes examined in this study are probably expressed in a less specific fashion in the plant.

3.2.4 Construction of a Dex-Inducible GL3 for Use in Identifying Downstream Targets

To further delineate the relationship between various genes and the trichome initiation complex a plant was prepared that was glabrous until the introduction of a chemical signal, at which time it begins developing trichomes. This plant was made by adding a construct consisting of a translational fusion of the steroid binding region of the rat glucocorticoid receptor (GR) to the carboxy-terminus of GL3. The glucocorticoid receptor (GR) is a steroid hormone receptor that activates target genes in a ligand-dependent manner (Yamamoto, 1985). In the absence of glucocorticoids, this receptor is tethered to the 90kD heat shock protein (HSP90) (Sanchez et al., 1987), which resides in the cytoplasm. Upon addition of a glucocorticoid (the synthetic glucocorticoid dexamethasone (dex) is used in this and other studies) the receptor is released and is free to migrate to the nucleus where its DNA binding domain can attach to glucocorticoid responsive elements (GREs) (Yamamoto, 1985). The steroid-binding and transcription activation domains of the rat GR are found in separate domains
Figure 3-7: Proposed GR mediated GL3 action. In the absence of the steroid ligand dexamethasone, GL3::GR should remain in the cytoplasm associated with HSP 90 and be unable to direct transcription. When dex is added, GL3::GR would dissociate from HSP 90 and would be able to direct transcription of its target genes. These genes could subsequently activate their target genes. Exposure to both dex and the protein synthesis inhibitor cycloheximide would allow for transcription of genes regulated by GL3, but prevent these proteins from being made. This ensures that transcripts from genes regulated by these indirect targets are not made while expression of only direct targets of GL3 will be seen to increase in this case.
which are functional when physically separated (Rusconi and Yamamoto, 1987).
This trait has been exploited previously to construct a fusion of the steroid-
binding element of GR to the maize R protein, which only showed R function in
the presence of dex (Lloyd et al., 1992). The fact that this chimeric protein is
synthesized before the induction complex acts allows for the use of this method
to discriminate between direct and indirect targets of a transcription factor. Direct
promoter targets of the trichome initiation complex will show an increase in
expression in plants exposed to both dex and the protein synthesis inhibitor
cycloheximide, while promoters regulated by transcription factors whose genes
are targets of the trichome initiation complex will show no increase in expression
under these conditions. A schematic of this procedure is shown in Figure 3-7.

Because pMB02 appears to function properly in complementing GL3
function, it proved to be a reliable starting point for the addition of a steroid
regulatory region. A region of DNA encoding the C-terminal 288 amino acids of
the rat glucocorticoid receptor which contains the steroid binding domain
(Rusconi and Yamamoto, 1987) was attached to the genomic GL3 fragment in
such a manner as to create a C-terminal translational fusion (see Chapter 2:
Materials and Methods for details of the construction of this plasmid). This
construct is called pMB014 (Figure 3-1), and it was introduced into gl3-1 plants.
gl3-1 mutant plants containing the pMB014 construct have a wild-type trichome
phenotype when grown on media supplemented with dex (Figure 3-8). In the
absence of dex many of the plants do not show the gl3 mutant phenotype;
instead they exhibit a far more severe deficiency in trichome production (table 3-
Figure 3-8 Behavior of the pGL3::GL3::GR construct in *gl3-1*. All plants were grown upon solid media containing MS salts, 1% sucrose and 10µM dex where indicated. All size bars represent 2mm. A. *gl3-1* plants containing pGL3::GL3::GR grown on media without dex. B. *gl3-1* plants containing pGL3::GL3::GR grown on media with dex. C. *gl3-1* plants grown on media with dex. Note that *gl3-1* plants containing the construct have fewer trichomes when grown on media lacking dex than do *gl3-1* plants lacking the construct.
Table 3-1 Trichomes found on the first leaves of pGL3:GL3:GR plants grown in the presence or absence of dex. Plants were grown on media either containing or lacking the inducible agent, dex. Trichomes from first leaves were counted after the plants had begun to produce its third true leaf. The gl3-1 plants containing the pGL3:GL3:GR construct grown on media lacking dex were expected to have similar amounts of trichomes as gl3-1 plants lacking this construct. However, gl3-1 plants containing the pGL3:GL3:GR construct produced fewer trichomes than those without the construct.

<table>
<thead>
<tr>
<th>Genotype and growth condition</th>
<th>Average trichome number per leaf</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL3:GL3:GR in gl3-1 dex</td>
<td>16.7</td>
<td>1.2</td>
</tr>
<tr>
<td>pGL3:GL3:GR in gl3-1 NO dex</td>
<td>0.83</td>
<td>1.5</td>
</tr>
<tr>
<td>gl3-1 dex</td>
<td>5.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>
gl3-1 plants with the pMB014 construct were crossed with a plant from the line SALK_019114 which contains a T-DNA insertion in the sixth exon of EGL3. gl3 egl3 pMB014 homozygotes were glabrous when grown on media without dex (Figure 3-9 A), but developed trichomes normally when grown on media containing dex (Figure 3-9 D). These plants provide a system that allows trichome initiation, and the subsequent production of genes associated with this event, to be triggered upon addition of dex while plants unexposed to dex should lack any trichome-specific expression.

The expression of genes involved in trichome production was examined using this dex-inducible trichome initiation system. This allows discrimination between genes directly and indirectly activated by the trichome initiation complex. Chx is used to stop protein synthesis, which provides a way to discern between direct targets of the transcription factors controlling trichome initiation and genes up-regulated by transcription factors induced by the trichome initiation complex. The plants used in these induction experiments had very young first leaves, which presumably would have the largest potential population of cells competent to become trichomes. In some experiments these developing leaves were exclusively harvested, in others all the aerial organs were harvested. Wagner et al (1999) used this system, which serves as a template for design of our experiment. Ten µM dex and 10 µM chx were used to initiate trichome production and halt protein synthesis, respectively. In the experiment of Wagner
Figure 3-9 Behavior of the pGL3::GL3::GR construct in the gl3 egl3 background. Exposure to dex initiated trichome production in these plants. A. A plant grown on media lacking dex. B. The second leaf of a plant transiently dipped into a 10 μM dex solution and then allowed to resume growth on media lacking dex. The plant was dipped when it was approximately the age of the plant in E. C. The first leaf of a plant that was dipped in 10 μM dex and then transferred to media containing dex. Note the presence of trichomes along the basal portion of the leaf while the distal portion remains glabrous. D. Leaf of a plant grown on dex. E. Approximate size of plants in B and C before they were transferred to dex.
et al (1999), plants were dipped into their various treatments twice during a four-hour period and then RNA from the dipped tissues was harvested. When developing plants were treated in this manner and then grown on dex-free media, trichomes developed on the plants that were exposed to dex and chx (data not shown). Also, when gl3 egl3 pMB014 plants were transferred from normal media to media containing dex during this window of leaf development trichome production proceeded normally on the portion of the leaf developmentally competent to form trichomes (Figure 3-9 C). When similar plants at this same point in development were transiently exposed to dex by dipping them in a solution of 10 µM dex and then returning them to dex free media the plants produced a few trichomes on the second leaf. These trichomes had fewer branches than plants continually exposed to dex, and they appeared only on the tip of the first leaf (Figure 3-9 B). These experiments indicate that the time frame of the effect of chx exposure is much shorter than that of dex exposure, and transient exposure to dex may not be sufficient to induce proper trichome formation. Therefore, instead of dipping the plants in the various chemicals, the plants were soaked for four hours, and then RNA was harvested from their leaves or total shoots. Plants with developing leaves were soaked in solutions containing either dex and chx or dex alone for 24 hours. Plants exposed to dex alone initiated trichome production, while plants exposed to chx and dex were glabrous (Figure 3-10). It is important to note, however, that the leaves of the plants exposed to the protein synthesis inhibitor were much smaller (please note
Figure 3-10 Cycloheximide exposure inhibits trichome formation. *gl3;egl3* pGL3::GL3::GR plants were soaked for 24 hours in a solution containing either dex and cycloheximide (A) or dex alone (B). The white arrow in B indicates a developing trichome. No trichome production was seen on plants exposed to chx and dex. Though not shown well in these micrographs, plants exposed to chx had much smaller leaves than plants not exposed to chx suggesting that these plants either did not grow or grew very slowly in the presence of dex.
the size of the calibration marks in each photo), suggesting that leaf development was generally retarded.

The expression of \textit{GL2} and \textit{MYB23} was measured in plants exposed to dex, dex and chx together, chx only, or water only using qRT-PCR. These genes were chosen because they displayed the greatest change in expression between glabrous and pubescent plants in the experiment described above. At least three experiments in pGL3::GL3::GR \textit{gl3 egl3} plants appeared to show that both of these genes were expressed more in plants exposed to dex as compared to controls, regardless of the presence of chx. The increase in expression of \textit{GL2} and \textit{MYB23} seen in plants exposed to both dex and chx indicates that these genes are direct targets of the trichome initiation complex. The results of a representative experiment are shown in Figure (3-11). These results proved very difficult to replicate, however, and the expression of \textit{GL2} could not be monitored in several experiments owing to technical difficulties. Other genes that showed a significant increase between glabrous and pubescent plants, such as \textit{CPC} and \textit{TTG2}, were also examined, but no consistent trends in expression of these genes were observed. Also, in several experiments, the expression of genes exposed to chx alone was much higher than the expression of these genes in plants exposed to neither dex nor chx, and even dex alone. Because of these inconsistencies these results have not been reported.

3.2.5 Expression of GL3::GR Using the 35S Promoter

In order to express the GL3::GR construct in a constitutive, ectopic manner, GL3::GR was placed under the control of the cauliflower mosaic virus
Figure 3-11 Preliminary experiments indicate that MYB23 and GL2 levels increase significantly after exposure to dex regardless of the presence of chx. Plants bearing young leaves competent to produce trichomes were soaked in dex, dex and chx, or control solutions for four hours. Afterward the expression of MYB23 and GL2 and EGL3 was examined using qRT-PCR. Both GL2 and MYB23 showed at least a seven-fold greater amount of expression in plants exposed to dex as compared to controls. A similar increase was observed in the presence of chx, indicating that these genes are direct targets of the TIS. EGL3 showed no change in expression in plants exposed to dex as compared to those that were exposed to only control solutions. Please note that the values reported on this graph were determined using relative quantitation, so the levels of the bars representing chx alone or water control solutions are set arbitrarily at one for comparison.
35S promoter (Odell et al., 1985) creating the plasmid pMB016. Using this method should produce greater expression of genes responsive to the trichome initiation complex, which could overcome the technical difficulties associated with the pMB014 construct. pMB016 was introduced into gl3;egl3 double mutants and col plants harboring the 35S::GL1 plasmid. The pMB016 plasmid was introduced into plants containing the 35S::GL1 construct because over-expression of both GL1 and GL3 was shown to cause supernumary, ectopic trichome formation by Payne et al (2000). None of the lines seemed to respond to dex when seeds with 35S::GL3::GR were sown on dex-containing media in a manner similar to the pMB014 lines. The four lines of gl3 egl3 mutant plants containing pMB016 were glabrous on media lacking dex (Figure 3-12 A). When these seeds were sown on dex many phenotypes were seen: several of the plants were mostly glabrous with some sparse trichome production on the base of the leaf and the petiole (Figure 3-12 B); some had no evidence of trichome production, while others exhibited a variety of developmental abnormalities (Figure 3-12 C and D). When the two lines of 35S::GL1 plants containing pMB016 were sown onto dex-containing media most of the plants did not live, and those that do show no alteration in trichome phenotype from plants containing only 35S::GL1. However, one line of 35S::GL1 35S::GL3::GR plants seems to initiate trichome production in a dex-dependent manner. On dex-free media, this line of plants has more trichomes than plants with only the 35S::GL1 construct and these trichomes appear somewhat larger and tend to form clusters (Figure 3-12 E). However, trichomes are only found on the adaxial surface of the leaf, just as wild-type. When
35S::GL1/35S::GL3::GR plants are grown on media lacking dex and then transferred, either transiently or permanently to media containing dex, trichome production is initiated on a fantastic scale (Figure 3-12 G and H). Trichome density is greatly increased on the adaxial leaf surface, though some cells on the epidermis appear to have escaped a trichome fate. The abaxial leaf surface also begins to develop trichomes in great number upon exposure to dex (Figure 3-12 G). The effect of induction on other organs was not assessed because plants that responded to dex at a young age all perished before they reached maturity.

3.3 Discussion

3.3.1 Determination of the Targets of the Trichome Initiation Complex

The purpose of this work was to determine which genes involved in trichome development are controlled by the trichome initiation complex. This work focused primarily on genes involved in trichome initiation because they should be up-regulated by the trichome initiation complex based upon our current model of trichome cell fate determination (Larkin et al., 2003), although other genes that have been shown to be involved in trichome development were examined as well. A plant that regulated trichome initiation in a steroid-inducible manner was also used to try to discriminate between genes regulated directly by the trichome initiation complex, and those regulated by protein factors produced by the trichome initiation complex. This steroid-regulated trichome initiation system was made by modifying GLABRA3, an integral part of the trichome initiation complex which was coincidentally mapped and cloned before this work began. During the cloning of GL3, several plants containing supernumerary
Figure 3-12 Behavior of the pMB016 construct. The pMB016 construct was introduced into both gl3;egl3 (A-D) and 35S::GL1 (E-H) lines. All size bars represent 1 mm except for H, which represents 200 µm. A. Plants with pMB016 in gl3;egl3 grown on media without dex are glabrous. B. When these plants are sown on media with dex some plants develop trichomes, but only on the petiole and base of the leaf. C. and D. Other plants whose seeds were sown of media with dex exhibit developmental abnormalities. A similar phenotype to B is seen when pMB016 in gl3;egl3 are transferred to dex-containing media. When pMB016 is combined with the 35S::GL1 construct more trichomes are produced on the adaxial surface of the leaf relative to the number found on 35S::GL1 plants lacking pMB016 construct (E) before exposure to dex. The abaxial surface of the leaf (F) remains glabrous. When these plants are germinated on dex-containing media, no plants containing the construct produce visible first leaves. When plants are germinated on dex-free media and then transferred to dex-containing media during leaf development a dramatic increase in trichome production can be seen. G. The abaxial surface of a leaf that was exposed to dex during leaf development. Numerous trichomes are developing on this normally glabrous surface of the leaf. H. A close-up view of the adaxial surface of the leaf showing accessory cells assuming the trichome fate (white arrow). Note that some cells still retain the epidermal fate.
copies of GL3 displayed a hypermorphic trichome phenotype. One of these plants, called GL3\textsuperscript{OE}, was used to further confirm whether specific genes were regulated by the trichome initiation complex. A comparison of the expression of a gene between glabrous and GL3\textsuperscript{OE} plants provides a confirmation of the comparison of this gene’s expression between glabrous and wild type plants. If a gene is expressed in a trichome-specific manner, then it will be expressed more in wild-type plants than glabrous plants and more still in GL3\textsuperscript{OE} plants as compared to wild-type or glabrous plants.

The expression of genes involved in trichome development was compared between the shoots of gl3 egl3 and wild-type or gl3 egl3 and GL3\textsuperscript{OE} using qRT-PCR. Quantitative PCR was used rather than just RT-PCR or semi-quantitative RT-PCR because it can detect more subtle changes in expression better than either of the other two techniques. TUBULIN\textsuperscript{9} was used to normalize mRNA levels between the various samples because it has been shown to be primarily expressed in the vasculature (Cheng et al., 2001) and should be unaffected by the presence or absence of trichomes.

Based upon expression patterns of their reporter constructs (Rerie et al., 1994; Kirik et al., 2001), GL2 and MYB23 were expected to have the greatest change in expression between glabrous and pubescent plants and this was confirmed by qRT-PCR (Figure 3-6). Both GL2 and MYB23 reporter constructs show expression only in trichomes in the rosette and show no expression in glabrous plants (Szymanski et al., 1998; Kirik et al., 2005). This expression pattern should create a much larger change in expression than seen in genes
that are expressed not only in trichomes, but other cell types as well. As seen in Figure 3-6, these genes indeed showed the greatest difference in expression between glabrous and trichome-bearing plants. These data would imply that GL2 and MYB23 are regulated either directly or indirectly by the trichome initiation complex.

GL1, TTG2, TRY, CPC, and ETC have reporter construct phenotypes that indicate that these genes are expressed not only in trichomes, but in other areas of the leaf as well (Larkin et al., 1993; Johnson et al., 2002; Schellmann et al., 2002; Kirik et al., 2004). The GUS reporter constructs of all these genes appear to show diffuse expression on the basal portion of the leaf, where epidermal cells are developing, with strengthening expression in trichomes. This strengthened expression persists for only a short while for GL1, but well past the maturity of the trichome for TTG2, TRY, ETC, and CPC. The expression of these genes is independent of the presence of trichomes, and it serves to attenuate the change in expression of these genes between glabrous and pubescent plants, which most likely explains the qRT-PCR results for this experiment. Measurement of the expression of these genes using qRT-PCR shows a reproducible increase in expression of GL1, TTG2, TRY, CPC, and ETC between glabrous and pubescent plants, but this increase is meager in comparison to GL2 and MYB23. This more subtle change in expression is probably the result of the low-level expression of GL1, TTG2, TRY, CPC, and ETC in glabrous leaves and probably indicates that these genes are at least partially under the control of the trichome initiation complex.
GL3 and EGL3 were reported by Zhang et al (2003) to have a GUS reporter expression pattern similar to GL1, but the data presented in this chapter show no change in expression for these genes between glabrous and pubescent plants (the increased level of GL3 in GL3\textsuperscript{OE} plants owes to the fact that these plants contain excess copies of GL3). It is difficult to explain this inconsistency. On the one hand, qRT-PCR results of a slight difference in expression could easily be underreported, but on the other hand there could be a subtle difference between the expression pattern of GL1 and these bHLH genes that could explain this variation. The fact that GL3 has been shown to function in other pathways in the leaf, such as anthocyanin production (Payne, 2000) make it seem likely that GL3 is expressed in cells other than just trichomes and the data gathered in this chapter indicate that the expression of GL3 and EGL3 is not trichome specific.

TTG, STI, ZWI, KTN1, and AN also showed no change in expression between glabrous and pubescent plants. This result was expected for all of these genes (with the exception of TTG) because previously published reporter construct data indicated that these genes are expressed throughout the epidermis rather than in any trichome-specific manner (Oppenheimer et al., 1997; Burk et al., 2001; Folkers et al., 2002; Ilgenfritz et al., 2003). The analysis used in this study should not detect a difference in expression between plants with and without trichomes for genes possessing this expression pattern. No reporter construct data are available for TTG, but this gene is involved in several different processes throughout the plant, so it remains possible that no difference in expression would be seen between glabrous and pubescent plants in this
work. It would be reasonable to assume based on these results that TTG, STI, ZWI, KTN1, and AN are all regulated by factors other than the trichome initiation complex.

3.3.2 Use of a Steroid-Inducible Trichome Initiation System to Determine Direct Targets of GL3

GL2 and MYB23 expression was analyzed in pGL3::GL3::GR gl3 egl3 plants, which are glabrous until exposed to dex, at which point they begin trichome production. Expression of these genes was examined in plants exposed to dex and plants exposed to dex with the protein synthesis inhibitor cycloheximide. When the expression of these genes was examined in plants exposed to these various conditions both of these genes were expressed more in plants exposed to dex as compared to controls. However, these results were difficult to replicate. While independent experiments did show a dex-dependent increase in MYB23 and GL2 expression, other experiments showed no difference in expression of these genes between plants exposed to dex and controls. There are many possible explanations for this inconsistency. The most probable is that there are so few cells on the developing leaf that are capable of adopting the trichome fate that the change in expression of these genes was borderline for detection using qRT-PCR. The protocol was altered to expose the plants to dex or a combination of dex and cycloheximide for an entire day to try to enrich this possible population, but these results showed a great amount of inconsistency as well. Another problem is that plants exposed only to chx often showed an increase in expression of these genes greater than that seen in plants exposed to dex alone (data not shown). In these situations the expression of these genes
was greater in plants exposed to both dex and chx than to just chx alone, but this chx-dependent increase in expression was unexpected. The use of chx in conjunction with a glucocorticoid receptor controlled transcription factor has been used several times (Wagner et al., 1999; Samach et al., 2000; Sakai et al., 2001), but only one reference could be found to this phenomenon (Ohgishi et al., 2001). In the case of Ohgishi et al, however, an explanation based upon the biology of the transcription factor in question was posited while no such explanation can be provided for the results presented in this chapter.

The main difference between the experiment described in this chapter and others described in the literature is the pGL3::GL3::GR construct uses its native promoter rather than the viral 35S promoter. Based upon the phenotype of p35S::GL3::GR 35S::GL1 plants, use of the 35S promoter would produce a much greater increase in expression of genes whose expression is trichome-dependent. By expressing GL3::GR under the control of its native promoter, only a small subset of the total amount of epidermal cells are able to assume the trichome fate while if the 35S promoter is used to drive the GL3::GR gene in many more cells, including those on the abaxial side of the leaf, and they would begin to assume the trichome fate. The only reason that these plants were not used in this study was that a functioning plant line was found only very recently. Repeating this experiment using a p35S::GL3::GR 35S::GL1 line holds great promise for success.
3.3.3 A Case for Auto-Regulation?

The lateral inhibition model for trichome cell fate determination predicts that the genes that promote trichome development should be auto-regulated. The GUS reporter constructs for GL1, GL3 and EGL3 show a very similar expression pattern to one another: expression throughout the developing leaf primordia with increased expression in developing trichomes. Based upon these data, it would seem that both GL1 and GL3/EGL3 should be auto-regulated. However, the data presented in the assessment of the expression of genes involved in trichome initiation (Figure 3-6) make a case for only one component of the trichome initiation complex to increase in expression in response to trichome production. While GL3, EGL3 and TTG show no change in expression between plants that lack or possess trichomes, GL1 is expressed more in pubescent plants as opposed to glabrous ones. While this contradicts the GUS reporter phenotypes seen for GL3 and EGL3, there is another piece of evidence which supports that idea that GL3 and EGL3 are not regulated by the trichome initiation complex. Plants expressing GL1 from the 35S promoter have a different phenotype than do 35S::GL3 plants. 35S::GL1 plants have fewer trichomes than wild-type plants (Larkin et al., 1994), while 35S::GL3 and GL3OE plants have more trichomes than wild-type plants (Payne et al., 2000). This reduction in trichome number found in 35S::GL1 plants was proposed by to be caused by a “squelching” effect in which an excess of GL1 physically titrates out another factor from being able to participate in further interactions (Larkin et al., 1994). If GL1, but not GL3, were auto-regulated then a surplus of GL1 would cause a squelching effect resulting in
a reduced trichome phenotype while an excess GL3 would cause a concomitant increase in GL1 levels, leading to increased trichome production. This “squelching” phenomenon is presumably caused by an excess of GL1 binding GL3/EGL3 proteins before they could dimerize with themselves. There is no obvious resolution for the discrepancy between the reporter construct data and the gene expression data. The increased GL1 expression reported in Figure 3-6 was small in comparison to other genes, but it was consistently observed and was sufficiently great as to not be considered in the range of “noise” for this system.

3.3.4 The Effect of Increasing Amounts of pGL3:GL3.

The effect of a genomic fragment containing the GL3 coding region upon gl3-1 mutants was examined in this work. Payne et al (2000) showed that a genomic fragment containing the bHLH gene located on the MYC6 clone along with ~1 kb of upstream and downstream sequence was sufficient to rescue the gl3 phenotype. This fragment, named pD2LX, differs from the construct described in this paper, named pMB02, in that pD2LX has ~800 bp less upstream and ~200 bp more downstream DNA than pMB02, and that pD2LX was PCR generated, while pMB02 was subcloned from MYC6 a genomic P1 clone. pMB02 also rescued the gl3 mutant phenotype, but several of the transformed plants had other trichome-related phenotypes. These phenotypes included increased trichome number, increased branching, an increase in the number of trichomes that occur in clusters and alteration of trichome morphology. These additional phenotypes were not reported to be associated with pD2LX.
transformation. Examination of T-DNA levels using quantitative PCR revealed a positive correlation between phenotype severity and pMB02 amount. Plants that showed a simple rescue phenotype (line 5) had 2 copies of the pMB02 construct (presumably one on each chromosome) while in the most severe phenotype (line 11.5) 66 copies were present. GL3 message level increased along with GL3 DNA copy number in the two strongest transformant lines, but there appeared to be little difference in GL3 expression between line 5.0 (which showed a simple rescue phenotype and contained 2 extra copies of GL3 DNA), line 5.2 (which showed a moderate increase in branching and clustering and had about 10 extra copies of GL3 DNA), col and gl3-1 plants.

The phenotypes of plants with an excess number of copies of pMB02, while superficially intuitive, are at odds with other observations concerning increasing levels of GL1 and GL3. A 35S::GL3 construct, called pD22, was made by Payne et al (2000), but the resulting phenotype does not closely resemble any of the lines in this study with supernumerary copies of GL3 under the control of its own promoter. Lines containing pD22 have a much greater number of trichomes on the leaf than do wild-type plants, but these trichomes appear to be evenly spaced, with no branching defects. The basis for this discrepancy probably lies within the differences in expression amounts and locations between the 35S and GL3 promoters. Genes attached to the CaMV 35S promoter are usually considered to be overexpressed (Benfey and Chua, 1990; Oppenheimer et al., 1991; Payne et al., 2000), but the most accurate description of 35S expression is that the genes are expressed constitutively throughout all tissues.
(Benfey and Chua, 1990). In contrast, the GL3 promoter drove expression of a GUS reporter construct on the basal portion of the developing leaf (the region of the leaf competent for trichome development) and in developing trichomes according to a report by Zhang et al. (2003). In older portions of the leaf, GUS activity was absent in areas between developing trichomes. Trichome development takes place in a discreet window during leaf development (Larkin et al., 1996). The phenotype of 35S::GL3 plants could be explained by altering the timing of GL3 expression from this brief period during leaf development to constitutive production throughout leaf expansion. GL3OE probably produces more GL3 per cell, but with the same temporal restrictions as found in the native GL3 promoter.

3.3.5 The Effect of GL3 Amount on Trichome Morphology

Altering the amount of GL3 available to form the trichome initiation complex appears to alter trichome morphology. Plants with extra copies of GL3, such as lines 5.2, V10 and 11.5 from Figure 3-2, produce trichomes that are larger and have more branches than wild-type trichomes. This phenotype is reminiscent of try mutants, which have defects in trichome spacing, but also are larger with more branches. In the case of line 11.5, many of the trichomes lose the ability for polarized expansion and become amorphous spheres. This phenotype is similar to the GL3 gain-of-function allele shapeshifter (gl3-sst) (Esch et al., 2003), which has a missense mutation in the region of GL3 that binds to GL1 and TRY. When pGL3::GL3:GR gl3;egl3 plants are transiently exposed to dex and then allowed to resume growth on media lacking dex (as in
Figure 3-9 B) the few trichomes that form are either unbranched or they have only one branch point. These trichomes are similar to those found on gl3 mutants. The fact that EGL3’s function is redundant with that of GL3 indicates that the gl3-1 phenotype is not a complete loss-of-function, but rather a reduction-of-function. These observations lead us believe there is a mechanism controlling branching which is controlled in some way by GL3 amount. It was postulated by Kirik et al (2005) that MYB23 may function in an alternate pathway with GL3/EGL3 to promote branching once a cell has adopted the trichome fate. A scenario could be imagined in which the amount of available GL3 is the limiting component in this branch promoting complex. If MYB23 binding is not interrupted in the gl3-sst mutation, then this model would explain the gl3-sst phenotype as well.

3.3.6 GL1/GL3 Association May Occur in the Cytoplasm

Yeast two-hybrid data in two publications show that GL1 and GL3 interact (Payne et al., 2000; Esch et al., 2003), but there are no data indicating whether this interaction takes place in the cytoplasm, or is restricted to the nucleus. The behavior of the GL3::GR protein in two situations provides indirect evidence that GL3 and GL1 can interact in the cytoplasm. The phenotype of pGL3::GL3::GR in gl3-1 when not grown on dex is unusual: these plants have almost no trichomes on the first leaves, which is significantly less than the number found on gl3-1 alone. Also, the phenotype of the 35S::GL3::GR 35S::GL1 plant when it is not exposed to dex is unexpected. In this situation the plant has more trichomes than 35S::GL1 alone. Both of these phenomena can be explained if GL3 binds to GL1
in the cytoplasm. In the case of pGL3:GL3:GR in gl3-1, GL3::GR competes with EGL3 for GL1 molecules in which to bind, effectively reducing the concentration of GL1. In the case of 35S:GL3:GR 35S::GL1 the opposite effect occurs, though probably owing to a similar phenomena. If the 35S::GL1 phenotype is caused by an imbalance in the stoichiometry of GL1 to GL3 molecules, then GL3::GR in the cytoplasm could bind these excess molecules of GL1 and the amount of functional initiation complex would increase.

3.3.7 GL1 Dictates Which Tissues in the Early Shoot Produce Trichomes

35S::GL1 plants have trichomes, though few in number, in ectopic locations in the developing shoot such as cotyledons and abaxial surfaces of first leaves. When GL3 is expressed under this same promoter, no ectopic trichomes were reported. Plants containing extra copies of pGL3::GL3 also lacked ectopic trichome production. As would be expected, 35S::GL1 35S::GL3 plant has plentiful trichomes on the cotyledon and the abaxial leaf surface. However, when 35S::GL1 plants were crossed with GL3OE, numerous trichomes could be seen on both the cotyledons and abaxial leaf surface in the F1 progeny (Figure 3-5 E and F). The combination of the fact that the GL3OE phenotype is caused by multiple copies of GL3 under the control of its own promoter and that trichomes can be found in ectopic locations on 35S::GL1 plants signifies that GL3 is normally expressed in these locations. This would implicate GL1 as the decisive factor in tissue-level control of trichome formation.
3.3.8 Plants Containing Both 35S::GL1 and 35S::GL3::GR Produce Prodigious Numbers of Trichomes in Response to Dex Exposure

When 35S::GL1 plants were transformed with the 35S::GL3::GR construct pMB016, a line was obtained which produced great numbers of trichomes in the locations in which trichomes normally form as well as in ectopic locations such as the abaxial surface of the leaf. This is the same phenotype as was seen with 35S::R::GR 35S::GL1 plants reported by Lloyd et al (1994). Since the differences between \( R \) and \( GL3 \) are unknown in respect to their ability to regulate the transcription of various genes, the 35S::R::GR 35S::GL1 plant was not used in this study. Unfortunately, a working 35S::GL3::GR 35S::GL1 line was not obtained until very recently, so it was not able to be used in determining if candidate genes were direct targets of the trichome initiation complex. It is unusual how difficult it was to obtain a plant carrying a functioning 35S::GL3::GR construct. Several wild-type plants that had apparently been transformed with 35S::GL3::GR were glabrous and not responsive to dex, though these were not mentioned in this work. Of the six lines discussed in this work only one actually responded to dex. Now that this line has been obtained, however, it is being crossed to glabrous \( gl3 \ egl3 \) plants and should provide an excellent method to test if a gene is controlled directly by the trichome initiation complex.
CHAPTER 4. IDENTIFICATION OF A CARBONIC ANHYDRASE IN TRICHOME DEVELOPMENT

4.1 Introduction

To date, most genes involved in trichome development have been identified by classical genetic approaches. However, since trichome development requires some essential processes such as cytoskeletal reorganization (Szymanski, 2005) and cell cycle modulation (Inze, 2005); mutations affecting these might be lethal or very deleterious. On the other hand, if two genes have duplicate function, loss of either function may have only a subtle phenotype or a phenotype that causes no observable change in trichome morphology. For this reason, reverse genetic approaches, such as enhancer trap screens, or direct assessment of either the proteins or RNA transcripts that exist in the trichome, such as that done by Wienkoop et al (2004) may be necessary to identify such genes.

For example, Gutiérrez-Alcala et al (2000) used in situ hybridization and other techniques to show that the four genes involved in both cysteine and glutathione biosynthesis were strongly upregulated in trichomes as compared to surrounding epidermal cells. The work leading to the discovery of increased expression of genes involved in glutathione biosynthesis in trichomes reported by Gutiérrez-Alcala et al was an extension of work originally begun to study sulfur metabolism in plants (Barroso et al., 1995; Barroso et al., 1999). Since glutathione and especially cysteine are required for the growth of all cells, it is highly unlikely that the involvement of this pathway in trichome development would have been discovered using traditional genetic screens. Instead, new
methods will need to be utilized to uncover the processes activated once a cell adopts the trichome fate.

The work presented in this chapter describes the identification of a carbonic anhydrase that is expressed preferentially in trichomes. This discovery was made through the use of a publicly-available enhancer trap line (http://enhancertraps.bio.upenn.edu/). Enhancers are cis-regulatory elements that function to regulate transcription in a manner independent of either orientation or position relative to the gene of interest (Jeang and Khoury, 1988). Enhancer traps were first used in Arabidopsis in works published in 1995 by Klimyuk et al and Sundaresan et al. The enhancer trap used in this work consists of GAL4/VP16 under the control of a minimal 35S promoter located very near the right border of the T-DNA, coupled with an endoplasmic reticulum-localized GFP gene under the control of the UAS promoter. GAL4 is a transcription factor from yeast (Giniger et al., 1985) and VP16 is a viral transcription factor from the herpes virus that possesses a strong transcription activation domain (Triezenberg et al., 1988) which is fused to the GAL4 DNA recognition sequence in this system. When GAL4/VP16 is expressed, it attaches to the UAS promoter, which is the target of GAL4 (Giniger et al., 1985), and drives expression of the reporter gene GFP. T-DNA insertion near an enhancer element will allow GAL4/VP16 to be expressed in the temporal and spatial manner dictated by this element. This will subsequently drive expression of the UAS-controlled GFP, producing a visible phenotype (http://www.plantsci.cam.ac.uk/Haseloff/geneControl/GAL4Frame.html). This
system has been used successfully in other organisms such as Drosophila
(Brand and Perrimon, 1993) and rice (Johnson et al., 2005). This work uses the
results of a large screen using this enhancer trap to identify a gene potentially
involved in trichome production.

4.2 Results

4.2.1 Identification of an Enhancer Trap Line with a Developing Trichome
Phenotype

Wild-type plants of the Columbia ecotype were transformed with an
eenhancer trap construct by Scott Poethig’s laboratory at the University of
Pennsylvania (Haseloff, 1999). The GFP expression patterns of these
transformants were assessed, the locations of the T-DNA responsible for the
phenotype were determined, and this information was indexed and made
available to the public via the internet (http://enhancertraps.bio.upenn.edu/).
Several enhancer trap lines that were listed as having expression in trichomes
were obtained and the GFP phenotypes of these lines were examined. Line E938
showed trichome-specific GFP expression in developing leaves (Figure 4-1 A). A
more thorough examination of the GFP expression pattern of E938 revealed
expression in the developing petiole (Figure 4-1 C), within the endodermis of the
root (Figure 4-1 B), sporadic patches of epidermal cells (Figure 4-1 D), in
stomatal guard cells and epidermal cells immediately adjacent to these guard cell
pairs (Figure 4-1 D) as well as during trichome development. In E938 plants GFP
expression in trichomes appears to be greatest at initiation of trichome formation
and the GFP expression fades as the trichome ages. GFP expression appears
Figure 4-1 GFP phenotype of E938. The phenotype of E938 was examined using confocal microscopy. A-C show the GFP image overlayed with the transmitted light image, while D shows the GFP image only. A. Developing trichomes on young first leaves show strong GFP expression. B. GFP expression was seen within the root endodermis. Staining was particularly strong near lateral root branch points. C. An older leaf showing a strong expression in the cells along the midrib and the petiole and midrib. Also note the lack of expression in the more mature trichomes near and on the edge of the leaf. D. Magnification of the area bounded by the white box in C. Some of the cells expressing GFP in this area are stomatal guard cell pairs and the epidermal cells immediately flanking these GFP-expressing guard cells (white arrows).
almost absent in mature trichomes (Figure 4-1 C). Based upon this expression pattern, E938 was selected for further characterization.

4.2.2 Determination of Which Gene Shares the E938 Expression Pattern

The location of the left border of the E938 T-DNA was reported on the enhancer trap website as lying on chromosome II approximately 1000 base pairs upstream of the start codon of the gene At2g28200. The location of this left border location was confirmed by PCR and the kanamycin-resistant phenotype of these plants was found to segregate in an approximate 3:1 ratio (52 kanamycin-resistant : 15 kanamycin-sensitive), indicating that there is only one kanamycin-resistance gene in E938 plants. Several efforts were made to locate the right border of the enhancer trap T-DNA, including TAIL PCR, inverse PCR, and adaptor PCR but these methods did not reveal the T-DNA/genomic-DNA junction of the right border (data not shown).

To determine if the expression of these genes was influenced by the presence or absence of trichomes, the expression of genes flanking the E938 insertion site was examined by qRT-PCR in glabrous gl3 egl3 plants and wild-type plants (col ecotype) (Figure 4-2). At2g28190 and At2g28220 did not show a change in expression between these conditions, while At2g28200, the gene closest to the known T-DNA border, showed only a modest (two-fold) increase in expression in pubescent plants. At2g28210 shows a striking (43-fold) increase in expression in pubescent plants as compared to glabrous ones, indicating that this gene may share the trichome-specific expression seen in the E938 enhancer trap line. This result was confirmed by subsequent repetitions of this experiment.
Figure 4-2 Comparison of expression of genes flanking the E938 insertion site in glabrous and pubescent plants. Top. The location, orientation and identity of the genes near the E938 T-DNA insertion site. Bottom. Relative quantitation comparing the expression these genes between gl3 egl3 and col plants. This comparison was done by relative quantitation, so the black bars (labeled col) represent the fold change in the expression of the listed gene between gl3 egl3 and col. The grey bar (labeled gl3 egl3) is shown at a value of one for reference.
Figure 4-3 At2g28210 expression as determined by *in situ* hybridization. At2g28210 expression was examined using *in situ* hybridization. Figure A shows the hybridization pattern using the labeled At2g28210 antisense strand, while figure B shows the negative control which uses labeled At2g28210 sense strand. Black arrows indicate trichomes. Developing vasculature, labeled with an empty arrow, and shoot apical meristem tissue, labeled with a red arrow, also show abundant labeling.
4.2.3 Expression of At2g28210 in the Plant

To further characterize the expression of At2g28210, two methods were used: in situ hybridization and tissue level quantitation of expression using qRT-PCR. In situ hybridization to the shoot apex and developing leaves shows that the expression of At2g28210 is strong in trichomes (Figure 4-3, black arrows). However, other tissues, such as the shoot apical meristem and the vasculature are strongly labeled as well. General tissue-level distribution of the At2g28210 transcript by qRT-PCR (shown in Figure 4-4) showed that expression was robust in the rosette leaves and stem, which are trichome bearing organs and very low in siliques and flowers, which are glabrous. However, this technique demonstrates that At2g28210 is expressed at the greatest concentration in the root. These results indicate that At2g28210 may be involved in other processes besides trichome development. Both of these techniques show an expression pattern for the At2g28210 transcript to be similar to that shown by the E938 GFP pattern in some respects, but also indicate that this gene may be expressed in tissues other than those designated by the E938 line as well.

4.2.4 Response of At2g28210 Expression to Trichome Induction

The strong difference in expression of At2g28210 between glabrous and pubescent plants suggested that the trichome-inducible system described in Chapter Three could be used to determine if this gene is a direct target of the trichome initiation signal. At2g28210 expression was measured in plants soaked for four hours in a solution of dex, dex and chx, chx alone, or none of these agents. As you will recall, trichome initiation will be dependent upon the addition
Figure 4-4 Expression of At2g28210 in various organs of the plant. Expression of At2g28210 was measured by qRT-PCR absolute quantitation. Trichome-bearing organs had the highest amount of At2g28210 expression among tissues in the shoot, but the root had the greatest concentration of At2g28210 message among the tissues examined.
Figure 4-5 Analysis of the expression of At2g28210 using the dex-inducible system. The amount of At2g28210 transcript per ng RNA was measured by qRT-PCR. Much higher levels of At2g28210 transcript are seen in plants exposed to dex than to plants not exposed to dex. However, a substantial increase in At2g28210 transcript was also seen in plants exposed only to chx, the protein synthesis inhibitor.
plants that were not exposed to either dex or chx, while much higher expression is seen in plants exposed to dex. This indicates that At2g28210 expression responds to the trichome initiation complex. However, it is unclear whether or not this gene is a direct target of the trichome initiation complex because of the high level of expression in the chx only control, making it difficult to interpret the strong expression seen in the presence of dex and chx. Nevertheless, these data do indicate that At2g28210 begins to be expressed very early in the development of the trichome.

4.2.5 Identification of the At2g28210 Gene

Analyses of PCR-amplified transcripts of At2g28210 indicate that the gene consists of seven exons with a coding region of 831 base pairs (Figure 4-6). The start of the At2g28210 transcript is 92 base pairs upstream of ATG, while the 3’ terminus of the transcript lies between 123 and 168 base pairs downstream of the stop codon. This variability in the size of the 3’ UTR owes to variable results obtained from the 3’ rapid amplification of cDNA ends (RACE) procedure. These results differ from the genome annotation found on TAIR (www.arabidopsis.org), which suggest that this gene consists of a much smaller coding region containing only four exons.

Comparison of the conceptual translation of At2g28210 to the protein sequences contained within the NCBI database reveals that At2g28210 is similar to several α-type carbonic anhydrases (Figure 4-7). Two proteins that show significant similarity to At2g28210 are Nectarin III (E value of 4e-64) and Dioscorin A (E value of 8e-51). Nectarin III is a protein found in the nectar of
Figure 4-6 Correct annotation of At2g28210. 5' and 3' RACE were used to establish the composition of the At2g28210 transcript and genomic intron/exon junctions. The sequence of the seven exons are underlined and the three-letter amino acid translation appears above the codons.
1651 CGAAACCATA CTACCTTACT ATCTCTGAAC CATAAAACTT TGATAGATA
1701 GAAAACAAA TGTTCTTCTT TGTGTTGATA ATGGGACCCAA
1751 TTATATACCA CAAAGAAATC TATAAACATT AACATATACA
1801 CAACACATTT TGAGGCATTA CACAGTACCC GATGATTAGAA
1851 AGTAACTTCA CAAAGAAATC TAATATAAGTA TAATATAAGTA
1901 AATTATACCA AAAAAAATC CAAATTATCA CAAATTATCA
1951 ATTATTATAT ATTATCTATT TGATCTAAAT GTTTAATTGA CACATTATAG
2001 CTTAATCTTTG TTATCTTAAAA GTTATGCTAT TTGCTAGGAT TTTTAAAAA
2051 AAATTTATTTG TGAATATAAT ATGCCATAGTT ATCCTTATAT CACATAAAAA
2101 TGAATTTTAT CCAATTATTA CATTCCAGTT AATATATATA GATGTATAGG
2151 TAAATAATAA ATGGGCAATTTG TGCCTCTCGAG CTACACATGG TTCACGAAAA
2201 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2251 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2301 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2351 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2401 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2451 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2501 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2551 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2601 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2651 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2701 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2751 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2801 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2851 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2901 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
2951 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
3001 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
3051 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
3101 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
3151 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC
3201 CATTAACGGA AGTTTGGCTG TAGTCACAGT CCTCTACAAA ATCGGAAGGC

rgPhe AlaLeuGlu LeuHisMetVal HisGluAsnIleAsnGly SerLeuAlaVal ValThrVal LeuTyrLys IleGlyArgPheSerPheLeuGlyLeu
ThrAspGlnAsn GluAlaGlu LysTyrVal AspValIleAsp ProArgAspArgLys GlySerArgLys PheTyrArg TyrIleGly SerLeuThrT
hrProProCys ThrGlnAsn ValIleTrpThr ValValLys Lys
CTCCCTCCTTG TAGCCTAAATT GATATTTGGA CACGTCAATAA AAGAAGTAAAT
ACGATGCGTT ATTTCTTCTT TTTTTACAT TAATCAACCA TAGCATTTAT
AGATCATCTG AAGGTGACTA TAGGTGGAAT ATCCATATCC AAAAAAGTTA
TCCATCTACA TGTTAATTTAG GTCTATTTTT CCCAATTTTAA ATTGTGACTT
ValA
TTATATTTAA AATCATCTCTGTTAATTTAT TTGATGTTGTTT TTTTAGTAA
rgThrValThr LysAsnGln ValLysLeuLeu ArgValAla ValHisAsp
GGACTGTGAC GAAAAACCAA GTGAAGCTAC TCAGAGTGGC GGTTCACGAT
GTAAGTTTTA CTTAAATAT TTAATCTAGT AATTTGCAATA ATGACATTAAT
TCATAGAACT GTAAATTTTG TATATTTTAT TTATATTTAT TAATCTATCTA
CAACCTGGAATA AACAAATAGT CACAGCATAAT CGACAGACATG
GlnProThrAsn LysArgVal ValLysLeuVal TyrLysProLys SerLeu**
CAACCTGGAATA AACAAATAGT CACAGCATAAT CGACAGACATG

At2g28210 1 ----------------MDKISRCIFIRPLSFVTSTTVSCLSATDYREVEDEHEFSYE--
Nectarin 1 ---------------------KMAAINTKKPFLSLSLPPFSLSVFPG--EVDEGSCFVD--
DioA 1 ---------------------SSSTLLLPLSFLSCLPQ--QPSFSTTVI--
Cah3 1 MRSAVLQGRQQARRVCSVRADGSGQDSTFAASSSARPLIDKQTTGAASVIHTVC
CA2 1 2-----------------------------------------------MSHHFFHGG-

At2g28210 43 -------WNQENGPAKWGKLR--PEWK-MCGKGEMQSPIDLMNKR--VRLVTHLKKLTRH
Nectarin 38 -------EKSENGPANWGNIR--PDWK-ECS-GKLQSPIIDIFDLR--AEVVSNLRILQKD
DioA 36 -------EGSPNGPENWGNLK--KEWE-TCGKGMEQSPIQLRDNR--VIFDQTLGELRRN
Cah3 61 PCPLCKPGAEKAIADPELLAGSGAOSVOKKMPSNPRPLNTSAAYKLQEFVFA
CA2 9 2-----------------------------------------------MSHHFFHGG-

At2g28210 91 -------KPCN-ATLQKEGHDMGLQEGGSTIPV---GTEISGHLLNSMGSSEHTMN
Nectarin 85 -------KPSN-ATLQKEGHDLNLQKLFQSTPV---GTEISGHLLNSMGSSEHTMN
DioA 84 -------IAAE-ATLQKEGHNGLNQKLFQSTPV---GTEISGHLLNSMGSSEHTMN
Cah3 121 KFSFEDGALNARQWPLNHF-AALNAI-----NMELLPHHRANHRRHQS
PAH
CA2 51 ----KQATSLRILNTMEGQSVLQDFGQKGVPTSNGSLDDDQES

At2g28210 141 -------SFRAELVYIHESNKSLAVT----LAKQQ--EPQVLLENKSAIRDQNEA
Nectarin 133 -------SRFAELVYHESNKLSAVT----LAKQQ--EPQVLLENKSAIRDQNEA
DioA 133 -------SRFAELVYHESNKLSAVT----LAKQQ--EPQVLLENKSAIRDQNEA
Cah3 171 -------SRFAELVYHERKSTVQAV----LELQGLNFAVQPFLAPAPLS
CA2 111 -------RQATSLTAPPKLS-----PCRYQVQPGQCAVQFPVAKHKPVYDKSKK

At2g28210 193 -------KPDFLKEGRHSGLTIPCTQG----FSCQVLKQVNNVAVH
Nectarin 186 -------KPDFLPEGRHSGLTIPCTQG----FSCQVLKQVNNVAVH
DioA 185 -------INAG-SVPCQFDPCTQG----FSCQVLKQVNNVAVH
Cah3 227 ------KDPVFELKPSFKTPPPFLGSGLTIPCTQG---SFMQFIKPDSPDSIFDMPK
CA2 171 -------RAFAFPFAESLLPELFOGSLTIPCTQGJ--------LEPIQVSEYKFAKLNF

At2g28210 251 -------SDTNAPDFLPRLK-KLAPKL----
Nectarin 244 -------FTNAPDFLPRLK-KLAPKL----
DioA 243 -------AINTAPDFLPRLK-KLAPKL----
Cah3 287 --------YNK--------YNRFLKLYEL--
CA2 231 -------QGEPEPESLVMKGSFNLKNOHAKST

Figure 4-7 ClustalW alignment of genes related to At2g28210. The amino acid sequence of At2g28210 indicates that it is related to several α-type carbonic anhydrases. The genes with described function to which At2g28210 is most similar are Nectarin III (NectIII, accession number AAO85482), a gene of unknown function found in tobacco nectar and dioscorin.(DioA accession number CAA53781), the major storage protein found in the yam. Two other α-carbonic anhydrases with described function are also shown for reference, Cah3 from Chlamydomonas reinhardtii (accession number U40871), and CARBONIC ANHYDRASE II from human (CA2 accession number CAA28663). The histidine residues that coordinate the zinc ion in the active site of the human carbonic anhydrase II are marked below the protein sequence with an asterisk.
tobacco flowers (Carter and Thornburg, 2004), and dioscorins are the major root storage proteins of the yam (Conlan et al., 1998). Both proteins have been shown to be functional carbonic anhydrases (Hou et al., 2000; Carter and Thornburg, 2004). Plant α-carbonic anhydrases are the most similar to At2g28210, but α-carbonic anhydrases from both the algae Chlamydomonas reinhardtii and human show significant similarity to At2g28210. The three-dimensional structure of human CA2, an α-carbonic anhydrase, is known and the active site of this enzyme contains a zinc ion coordinated by three histidines and a water molecule (Hakansson et al., 1992). Three histidines are present in analogous locations in At2g28210 (Figure 4-7). This sequence similarity suggests that At2g28210 may have carbonic anhydrase activity.

4.2.6 Function of At2g28210

To determine the effect of inhibiting carbonic anhydrase activity on trichome phenotype, plants were exposed to ethoxyzolamide (EZ), a sulfonamide carbonic anhydrase inhibitor, during the time that trichome development occurs on the first leaf. Plants germinated on media containing EZ die before they are able to show a trichome phenotype. Therefore, the dex-inducible construct discussed in Chapter Three was used to provide a situation whereby trichome initiation could be coupled with exposure to EZ. Plants were grown for five days on MS media then transferred to a liquid solution containing MS salts, 1% sucrose, 10 µm dex (which will begin trichome development in these plants) and EZ in varying concentrations ranging from 1 µM to 1 mM. At five days of growth, the first leaves of these plants were very small, less than 200 µm, which
represents a stage in development when much of the leaf is competent to form trichomes (Larkin et al., 1996). The trichome phenotype of these plants was examined over the next four days.

Preliminary experiments revealed that there was no noticeable difference in trichome phenotype between control plants and plants exposed to 1 µM or 10 µM EZ (data not shown), but plants exposed to higher concentrations of EZ, either 100 µM or 1 mM, showed a significant retardation in trichome development (Figure 4-8 K and L). Plants exposed to EZ were also noticeably smaller than plants exposed to the control solution, so a subset of plants from each solution was removed for the first three days of the experiment and the length of their leaves was measured. Figure 4-10 shows that plants exposed to either 100 µM or 1 mM EZ displayed a clear retardation in leaf growth; however, this reduction is not as severe as the reduction in trichome growth seen in plants exposed to these concentrations of EZ. Trichomes on plants exposed to control conditions had expanded from the surface of the leaf and had developed their first branch from the main stalk by day three (Figure 4-8 G), while trichomes on plants exposed to 1 mM EZ could only be seen as bumps on the surface of the leaf (Figure 4-8 L). Trichomes on plants exposed to 100 µM EZ show a similar phenotype (Figure 4-8 K), but occasionally a trichome can be seen emerging from the plane of the leaf. After four days it is still extremely rare to find trichomes that have expanded from the plane of the leaf on plants exposed to 1 mM EZ, though plants that had been exposed to 100 µM EZ have trichomes that showed a greater degree of cell expansion (data not shown) and their leaves continued to
Figure 4-8 The carbonic anhydrase inhibitor ethoxyzolamide inhibits trichome development. *gl3 egl3* mutant plants with the GL3:GR construct were used to examine the effect of inhibition of carbonic anhydrase activity upon trichome growth. These plants were allowed to germinate on dex-free media, and then transferred to an aqueous solution containing dex and 100 µM or 1 mM ethoxyzolamide, a lipid-soluble carbonic anhydrase inhibitor, or a control solution containing DMSO instead of EZ. Trichome development was examined for four days using SEM. By day three the control plants had trichomes that had expanded from the surface of the leaf and begun branching, but this level of development was not seen on plants exposed to EZ even by day four. For figures A-L the white bar represents 1mm.
Figure 4-9 Close ups of GL3::GR leaves exposed to EZ and dex for two, three, and four days. A-C show close-ups of the trichomes on leaves of plants exposed to 1mM EZ for two (A), three (B), or four (C) days. The trichomes appear as unexpanded bumps on the surface of the leaf. Notice that though the number of trichomes increases in time, the amount the trichomes expand does not.
Figure 4-10 Effect of the carbonic anhydrase inhibitor EZ upon leaf growth. To make sure that the retardation in trichome expansion was not a by-product of a general retardation in leaf growth, leaf length was measured for a sub-set of plants over the course of the first three days of the experiment described in figure 4-8. These results show that there is a noticeable retardation in leaf growth in plants exposed to either concentration of EZ, however, this impediment to leaf growth does not correlate to the effects seen in trichomes.
increase in length, albeit more slowly than WT. No trichomes on plants exposed to these high amounts of EZ were found to have started branching. Figure 4-9 also shows that trichome formation begins on plants exposed to 1 mM EZ by day two, and trichome initiation continues until day four, but very little, if any, further development occurs. This experiment shows that inhibition of carbonic anhydrase activity by exposure to a pharmacological agent retards trichome development, which would suggest that the action of a carbonic anhydrase is necessary for the expansion of the trichome.

4.2.7 Identity of At2g28210 Homologs in Arabidopsis

A comparison of the amino acid sequence of At2g28210 to the known sequences in Arabidopsis revealed that there are sequences encoding seven other α–type carbonic anhydrases in the genome. An alignment of the sequences of these proteins shows that they have a very similar amino acid sequence (Figure 4-11). This similarity in sequence suggests that there could be redundancy of function among these proteins. To determine if any of the other α–type carbonic anhydrases in Arabidopsis could act redundantly with At2g28210, the expression of these homologous genes in the developing rosette was analyzed using RT-PCR. This assay used cDNA made from RNA from aerial organs of wild-type plants with three true leaves as template, similar to the template used to determine which gene flanking the E938 insert was affected by trichome development. As shown in Figure 4-12, At3g52720 is the only other α–type carbonic anhydrase to be strongly expressed in the rosette, though At1g08080, At4g21000, and At5g04180 also show very slight expression in the
rosette. This experiment shows that there are seven homologues of At2g28210 in Arabidopsis and that At3g52720 is the only one of these homologues to be expressed in a significant amount in the rosette.

4.3 Discussion

4.3.1 An Alpha Carbonic Anhydrase Is Upregulated During Trichome Development

This chapter describes the identification via an enhancer trap of a gene upregulated during trichome development that encodes an α-carbonic anhydrase. A carbonic anhydrase is an enzyme which catalyzes the interconversion of carbon dioxide and bicarbonate. There are four described families of carbonic anhydrases which appear to have arisen via convergent evolution: alpha, beta, gamma, and delta (Tripp et al., 2001). Arabidopsis and other plants contain α, β, and γ CAs, while vertabrates possess only α-CAs (Moroney et al., 2001). In plants the β-CAs are involved in photosynthesis and are the most abundant and best described CA family (Moroney et al., 2001). The β-CAs play an integral role in C4 plants and cyanobacteria in concentrating CO2 within the plant cell (Badger and Price, 1994). The β-CAs in the chloroplast stroma are responsible for the majority of the CA activity in C3 plants, such as Arabidopsis (Moroney et al., 2001). It is assumed that these β-CAs in the chloroplast stroma play a role in concentrating CO2 around rubisco (Moroney et al., 2001), however, reduction of the level of these CAs in tobacco had little effect on photosynthetic activity (Majeau et al., 1994; Price et al., 1994). A search of the Arabidopsis genome reveals six β-CAs, which include the previously described CA1, which
Figure 4-11 ClustalW alignment of all alpha carbonic anhydrases in Arabidopsis. There are seven other alpha-type carbonic anhydrases in Arabidopsis and they all are very similar at the amino acid level. The histidine residues that coordinate the zinc ion in the active site (based upon homology with human CA2) are marked below the protein sequence with an asterisk.
Figure 4-12 Analysis of expression of the other alpha CAs in Arabidopsis. To identify other α-CAs that might have redundant function with At2g28210, RT-PCR was performed for the other alpha CAs listed on the Arabidopsis database using the cDNA made from the aerial organs of plants with three true leaves as template. The only α–type carbonic anhydrase to be expressed in the rosette in any significant amount is At3g52720.
was found in the chloroplast, and CA2 which was found to be expressed in the cytoplasm of Arabidopsis (Fett and Coleman, 1994). Recently γ-CAs have been found targeted to the mitochondria and associated with complex I and IV of the electron transport chain (Parisi et al., 2004; Perales et al., 2004; Perales et al., 2005).

In comparison to the β- and γ-classes, the α-class of CA has received little attention in plants. Humans and other vertebrates have only α-class CAs and for many years this was the only class of CA known; consequently animal α-CAs are comparatively well-characterized at the physical level (Sly and Hu, 1995). Most of the knowledge about α-CAs in photosynthetic organisms comes from work done in *Chlamydomonas reinhardtii*, a unicellular green algae (Moroney et al., 2001). The α-CAs in *Chlamydomonas reinhardtii*, called Cah1, Cah2, and Cah3, are involved in a low-CO₂ response carbon concentrating mechanism. Cah1 and Cah2 are localized in the periplasmic space, while Cah3 is located in the thylakoid lumen (Karlsson et al., 1998). There are eight α-CA genes in Arabidopsis (Figure 4-11) with sequence similarity to α-CAs, but none of these have been described in detail in any published work. At4g20990 was identified in a study delimiting the plastid proteome (Friso et al., 2004). In a personal communication cited in Moroney et al (2001), the α-CA At3g52720 was reported to be localized to the chloroplast stroma using immunogold localization.

Several lines of evidence reveal that At2g28210 is expressed in trichomes. The assessment of At2g28210 expression (Figure 4-2) between glabrous and pubescent plants using qRT-PCR indicates that this gene is
expressed much less in plants lacking trichomes than in those possessing trichomes. This result indicates that though At2g28210 may be expressed in other tissues and cell types in the shoot, its principal expression is in trichomes. Quantitative RT-PCR analysis of At2g28210 expression in different tissues of the plant (Figure 4-4) also indicates that this gene is expressed predominantly in trichome-bearing tissues in the shoot. In situ hybridization shows that At2g28120 is expressed in trichomes (Figure 4-3), though this technique indicates that At2g28210 is also expressed within the leaf vasculature and the shoot apical meristem as well. This discrepancy between the trichome-specific expression shown by the qRT-PCR techniques and the more broad expression depicted in situ hybridization technique is difficult to reconcile. The GPF expression pattern of the E938 enhancer trap line offers little help in resolving this matter, because sub-epidermal GFP expression would be obscured owing to chlorophyll autofluorescence. Regardless of this dilemma, all of these methods do indicate that At2g28210 is strongly expressed in trichomes.

To determine the relationship between At2g28210 expression and trichome initiation, At2g28210 expression was measured in plants containing the inducible GL3:GR construct described in Chapter Three (Figure 4-5). After four hours, a clear increase in expression of this gene was seen in plants exposed to dex, which had thereby begun trichome production, as compared to plants exposed to a control solution lacking dex. This result indicates that At2g28210 is at least indirectly regulated by the trichome initiation machinery. Unfortunately, due to the increase in transcripts after exposure to chx alone, it is difficult to
determine whether At2g28210 is a direct transcriptional target of GL3, although the results are consistent with this possibility.

4.3.2 Carbonic Anhydrase Activity Is Required for Trichome Expansion

Inhibition of CA activity by the sulfonamide compound ethoxyzolamide, as shown in Figure 4-8, indicates that trichome expansion is inhibited by exposure to this pharmacological agent. The dex-inducible construct allowed us to initiate trichome production concurrently with EZ exposure. This was important because the toxicity of EZ at the concentrations necessary to produce a trichome phenotype prevented germination of plants in media containing this compound. Also, plants placed into liquid media containing EZ began to shown signs of severe stress, such as bleaching, after four to five days. Plants exposed to high levels of EZ had trichomes which stop development just after radial expansion of the trichome, which is referred to as stage one according to the nomenclature established by Szymanski et al (1998a). The first trichomes appear on both control and EZ-exposed plants by the second day of exposure, but the trichomes on plants exposed to EZ do not continue to expand and branch as do those in the control situation. By day four, many stage one trichomes can be seen on plants exposed to EZ, indicating that carbonic anhydrase activity is not required for initiation of trichome development. In the first days of the experiment the trichome phenotype was the same for plants exposed to either 100 µM or 1 mM EZ, though no phenotype could be easily seen on plants exposed to 10 µM or less of the inhibitor. However, some of the trichomes on plants exposed to 100 µM EZ could be seen beginning to expand very slightly from the plane of the leaf.
by day four, whereas plants exposed to 1 mM EZ had no trichomes at this stage of development. As Figure 4-8 and Figure 4-9 show, exposure to such high levels of EZ also had a deleterious effect upon the growth of the leaf; however, the effect of EZ on general leaf growth was not nearly as severe as its effect on trichome development. While trichome development on plants exposed to EZ was stopped at a very early stage, the leaves continued to grow at an appreciable rate, more than doubling in length over the course of two days. The fact that a carbonic anhydrase inhibitor can impede trichome expansion is consistent with the hypothesis that a carbonic anhydrase is involved in trichome expansion.

4.3.3 Possible Roles for an α-Carbonic Anhydrase in Trichome Development

Many different physiological processes have been found to require CA activity, including pH buffering, bicarbonate transport, lipogenesis, and gluconeogenesis (Sly and Hu, 1995; Sterling et al., 2001). It is difficult to ascribe At2g28210 a role in any of these processes without more biochemical data. The rapid increase in expression of At2g28210 after trichome initiation fits well with the idea that At2g28210 functions in trichome expansion, which is one of the first processes to occur in trichome development (Hulskamp et al., 1994). If At2g28210 does indeed play a role in cell expansion, then the possible role of this CA in lipid biosynthesis would be a good place to begin an investigation. In Hoang and Chapman (2002) inhibition of a CA in cotton was shown to decrease the incorporation of acetate into lipids. Lipid biosynthesis would be important in a rapidly expanding plant cell not only for synthesis of the plasma membrane of
this cell, but also for the synthesis of the vacuolar membrane. Zheng et al (2005) demonstrated that very long chain fatty acid synthesis is required for plant cell expansion. Perhaps At2g28210 is involved in very long chain fatty acid synthesis in a similar manner. Another process involved in cell expansion that could require the action of a carbonic anhydrase is cell wall expansion. Plant cell walls are more malleable and plant cell growth occurs more rapidly in an acidic pH environment owing to an effect called “acid growth” (Rayle and Cleland, 1992). An extracellular carbonic anhydrase could be used to lower the pH by making carbonic acid to facilitate cell expansion.

A key piece of evidence yet to be obtained that would give a clue as to the function of At2g28210 is its sub-cellular localization. C-terminal fusions of this gene with the GFP and YFP reporter genes have been made, but no transgenic lines have been generated. Introduction of these constructs into Arabidopsis and onion cells using particle bombardment did not yield any conclusive results. The programs SignalP and TargetP (http://www.cbs.dtu.dk/services/) predict that At2g28210 is targeted to a secretory pathway and has a signal peptide comprising its first 23 amino acids. These predictions were not confirmed by biochemical analysis, but these programs predict similar results for all of the other α-carbonic anhydrases in Arabidopsis, with the exception of At1g08065, which is targeted to a different, unspecified pathway according to this program. Two of these proteins which share a signal peptide with At2g28210 have been shown to be localized to the plastid or chloroplast. At3g52720 was shown to be localized to the chloroplast stroma (Moroney et al., 2001), and At4g20990, was
found in an analysis of the proteins found in the plastid proteome (Friso et al., 2004). If At2g28210 is also found to be localized to the plastid, then it would be suggestive of its involvement in lipid or fatty acid synthesis because these compounds are synthesized in the plastid in plants (Somerville et al., 2000).

4.3.4 Conclusion

In conclusion, the work in this chapter demonstrates that At2g28210, a gene encoding a carbonic anhydrase, is up-regulated in trichome development. We were led to investigate this gene because it was near the insertion site of an enhancer trap that showed expression in developing trichomes. While examination of trichome development in the presence of a carbonic anhydrase inhibitor leads us to posit that carbonic anhydrase activity is necessary for trichome expansion, there is insufficient evidence to make this claim with certainty. We await the results of several tests, which have been initiated, which will provide us with loss-of-function, tissue-level expression, and sub-cellular localization data. These data will provide a better picture of the nature of At2g28210 function. This work, while important in the context of trichome development, is also important in further delimiting the role of carbonic anhydrase function in Arabidopsis. Only β- and γ-carbonic anhydrases have been described in any detail in Arabidopsis. On the other hand, only passing reference has been made to Arabidopsis α-carbonic anhydrases in the literature and this is the most well described α-carbonic anhydrase to date. The fact that At2g28210 seems to be preferentially expressed in a particular cell type and not involved in photosynthetic carbon concentration or the mitochondrial electron
transport chain illustrates that carbonic anhydrases may play a novel role in plant physiology. Further research performed on this gene will give us a better understanding not only of trichome development, but also of the action of this class of enzyme and plant cell development as a whole.
5.1 Introduction

Cessation of cell division is typically a condition of terminal differentiation. When a cell has committed to the trichome fate it no longer divides, instead entering an alternate cell cycle in which its DNA is replicated without concomitant cell division (Hulskamp et al., 1994). This alternate cell cycle is called endoreplication, or endoreduplication (Edgar and Orr-Weaver, 2001). Wild-type trichomes make between 20-32 copies of their nuclear DNA through endoreplication (Melaragno et al., 1993; Hulskamp et al., 1994), which represents between three and four successive doublings of the genome of this cell. The cells in the leaf epidermis which do not adopt the trichome fate, the epidermal pavement cells, only contain 10-16 copies of their genomic DNA (Melaragno et al., 1993), which indicates that trichomes undergo more rounds of this altered cell cycle. This difference implies there is trichome-specific control of endoreplication.

Many mutations have been found that result in trichome nuclear DNA content either higher or lower than that found in wild-type trichomes. For example, the trichomes of gl3 mutants, which are smaller and less-branched than wild-type, have fewer copies of genomic DNA than do wild-type trichomes, while trichomes of try mutants, which are larger and more branched than wild-type, have a higher genomic DNA content as compared to wild-type. Additionally, mutants in KAKTUS (KAK) also have increased DNA content in trichomes (Perazza et al., 1999), though increased ploidy levels were also found in the
hypocotyls and cotyledons in *kak* mutants (El Refy et al., 2003), implying that *KAK* acts in a global rather than a trichome-specific manner. Several other less well characterized loci have also been found to increase DNA content in trichomes (Perazza et al., 1999), and *gl3-shapesifter*, a GL3 gain-of-function allele, was found to have greatly increased DNA content in trichomes (Esch et al., 2003). Arguably the most interesting discovery involving endoreplication in trichomes was made by my colleagues during my time of study in the Larkin lab. A screen of EMS mutagenized seeds revealed a mutant, named *siamese*, which possessed multi-cellular trichomes (Walker et al., 2000). This mutant represented not merely a means of modulating the intensity of the process of endoreplication, but rather a switch that directs a cell to adopt the alternate cell cycle of endoreplication rather than standard mitosis. Thus, study of this mutant became a primary focus of the lab ever since its discovery.

Upon cursory inspection, the *sim* mutation appears to form occasional clusters of trichomes (Walker et al., 2000), similar to *try*, which would imply that the *sim* gene would be involved in trichome patterning. However, closer inspection reveals that the trichomes in the clusters found in *sim* mutants were fused together at the base, rather than distinct cells as found in *try* mutants (Hulskamp et al., 1994). The formation of the trichome clusters in *sim* mutants were probably the result of a mitotic event early in trichome formation. Further inspection of *sim* trichomes revealed other multicellular trichome configurations, which consisted mainly of cell division events taking place at the branch points of trichomes. Most multicellular trichomes on the leaf contained only two to three
cells, but trichomes on the stem were found that consist of as many as fifteen cells. Strikingly, the gross morphology of multicellular leaf or stem trichomes remained unchanged in comparison to these structures on wild-type plants. The discovery of the sim mutant indicated that a single gene functioned as a control point between mitosis and endoreplication in trichomes. Therefore, it would be very informative to determine the identity SIAMESE gene and to determine if this gene is regulated by the trichome initiation signal. The focus of this chapter is the identification of the gene responsible for the sim phenotype, examination of its expression in relation to trichome development, and a characterization of its expression at both the sub-cellular and tissue levels.

5.2 Results

5.2.1 Identification of the SIAMESE Gene

The first step in identifying the SIM gene was to locate the site of the sim-1 mutation. In the work initially describing the mutation, sim-1 was mapped to a location six cM distal to the marker nga158 on chromosome V (Walker et al., 2000). Further fine mapping revealed that the sim-1 mutation was located between markers found on the T32M21 and T19N18 BAC clones on chromosome V. A second allele of sim, sim-2, donated to our lab by Martin Hülskamp, was used by Michelle Speckhart to identify a candidate gene for SIM. This allele was generated by the insertion of a T-DNA and adaptor PCR was used to identify the junctions between T-DNA and genomic DNA. These borders of the sim-2 T-DNA insertion are at chromosomal positions 1268466 and 1267004, indicating that 1462 base pairs have been deleted from the sim-2
genome by the T-DNA insertion event. This deletion removes a significant amount of the gene named by TAIR as At5g04470 (Figure 5-1). This gene lies within the region determined by mapping to contain the sim mutation. At5g04470 is projected by TAIR to have a single exon 384 base pairs in length. RT-PCR was performed using cDNA generated from the RNA of wild-type and sim-2 rosettes which indicated that sim-2 plants did not express the sim message.

Analysis of At5g04470 and HISTONE H4 expression using this method confirms that the sim-2 T-DNA insertion creates a deletion in the At5g04470 transcript (Figure 5-1). 5’ and 3’ RACE were performed using cDNA generated using RNA harvested from the rosette to determine the genomic organization of the At5g04470 gene. This method confirms the annotated coding region of At5g04470. 5’ RACE indicates that the 5’ terminus of the At5g04470 transcript lies at chromosomal position 1267369, 92 base pairs upstream of the start codon. 3’ RACE indicates that the 3’ terminus of this mRNA lies 217 base pairs downstream of the stop codon at chromosomal position 1266668 (Figure 5-2).

The area of the genome containing the At5g04470 transcript was sequenced in the sim-1 as well as sim-3, a second EMS generated allele obtained from David Marks. The sim-1 allele has a G to A transition in the ATG start codon, which should significantly disrupt translation of this gene in sim-1 plants. The sim-3 allele has a C to T transition that converts the proline residue at amino acid position 36 to a serine (Figure 5-2). These results strongly suggest that At5g04470 encodes the SIAMESE gene.
**Figure 5-1 Location of the siamese mutation.**

**A.** Schematic of the region of chromosome V containing *SIAMESE*, (At5g04470). The *SIM* coding region, which is comprised of 384 base pairs, is shown as a large black arrow. Dotted lines: 5' and 3' UTR of At5g04470 flanking either end of the coding region of the gene. Small black arrows: relative positions of the primers used to amplify the *SIM* message. Small grey arrows: primers used to amplify the region of the genome used for genetic complementation.

**B.** RT-PCR using cDNA generated from RNA from either wild-type (ecotype WS, the parent of the *sim-2* mutation) or *sim-2* rosettes. Left: RT-PCR using primers within the portion of the At5g04470 gene deleted in the *sim-2* allele, which are shown as small black arrows in A. Right: A control PCR using HISTONE H4 primers.
To confirm that At5g04470 is SIAMESE, a 3504 base pair fragment was amplified from genomic DNA by PCR. These primers flank a region 2870 base pairs upstream of the start of the coding region of the gene and 250 base pairs downstream of the end of coding region. The position of these primers relative to the coding region of the gene is shown in Figure 5-1. The coding region of the At5g04470 gene was sequenced and was found to be identical to wild-type. This fragment was cloned into a binary vector, creating the construct pSIM3500 and transferred to sim-1 plants via Agrobacterium-mediated transformation. Kanamycin-resistant progeny of this transformation had a wild-type phenotype, which indicates that the pSIM3500 construct was able to complement the sim-1 mutation. A plant with a wild-type phenotype and one with a sim phenotype, presumably representing the phenotypes of the transformed plants and their untransformed parents are shown in Figure 5-3. The complementation of the sim phenotype by a region of the genome containing the coding and regulatory regions of At5g04470 prove that this gene is SIAMESE.

5.2.2 SIAMESE Is Predicted to Encode a Novel Class of Protein

A primary question in our investigation of SIM is the biochemical function of the SIAMESE protein. This line of inquiry was begun by comparing the predicted amino acid sequence of SIM to known proteins in the NCBI database using the BLAST program. The results of this search did not immediately reveal a gene with similar sequence that had a described function, although it did reveal the presence of three similar genes in the Arabidopsis genome. A more thorough search of publicly available databases
Figure 5-2 The sequence of the At5g04470 transcript and the location of single-base-pair changes found in the \textit{sim-1} and \textit{sim-3} alleles. Above is the sequence of the At5g04470 transcript. The coding region is underlined and the corresponding amino acid sequence is given above. The location of the \textit{sim-1} mutation is shown in grey and consists of a guanine to adenine transition that changes the composition of the start codon. The location of the \textit{sim-3} mutation is shown in grey and changes the identity of a proline to a serine via a cytosine to thymine transition. These mutations were identified by Michelle Speckhart.
eventually led to the discovery of several proteins from other plant species that share four domains with SIM (Figure 5-4). Some of these shared domains are possibly motifs with described functions or properties. No function could be ascribed to the first motif, which is shown in Figure 5-4 shaded in blue, despite a diligent search of the literature and computer databases. The second domain, shown in red, is a “ZRXL” motif which functions as a cyclin-cdk2 binding domain in retinoblastoma protein, p21, and E2F1 (Adams et al., 1999). The third domain, shown in green, is similar to a motif found in a class of plant cyclin-dependent kinase inhibitors called the Kip-related proteins (KRP) (De Veylder et al., 2001). The fourth motif, shown in dark yellow, was shown to be a conserved site which targets the serine for phosphorylation (Pnueli et al., 2001). Residues 26-41 (shown underlined in Figure 5-4) are predicted to comprise a PEST domain by the PESTfinder program (http://bioweb.pasteur.fr/seqanal/interfaces/pestfind.html). Based upon these observations and the phenotypes of sim mutants and SIM over-expressers, SIM and its homologues are good candidates for a new class of cyclin-dependent kinase inhibitors.

5.2.3 Tissue Level Distribution of the SIAMESE Transcript

The tissue level expression of the SIM message was assessed using several methods. The T-DNA causing the sim-2 mutation contains an enhancer-trap which drives the expression of the GUS reporter gene when it is placed close to regulatory elements in the genome. The original sim-2 allele contained
Figure 5-3 A 3500 base pair genomic fragment containing At504470 rescues the sim phenotype. A 3504 base pair genomic fragment was introduced into sim-1 plants using Agrobacterium-mediated transformation. Plants that were transformed with this DNA lacked the trichome doublets normally found on sim-1 plants, indicating that this DNA was sufficient to complement the sim phenotype. The photograph on the left shows a leaf of an untransformed sim-1 plant. The white circles show the presence of a trichome pair. The photo on the left shows a plant transformed with the pSIM3500 construct, which lacked trichome doublets on all leaves surveyed. These plants came from a segregating line heterozygous for the pSIM3500 construct.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIM(At5g04470)</td>
<td>GGGCTTPTSSDH</td>
</tr>
<tr>
<td>At3g10525</td>
<td>DLRCSTPTSQEHKIP--EVETCPPPPRKRPRPPSAPSATAALMIRSCKRKLLVST----------CEIIMNREEIDRFFSSVYNETS---</td>
</tr>
<tr>
<td>At1g08180</td>
<td>LESLCTPTSSDHKIP--EVETCPPPPRKRPRP-------------LTKKTRLSKDLR-------FFEATDVGSQEVETLFVHEPNHVR---</td>
</tr>
<tr>
<td>At5g02420</td>
<td>EEGCKTPTSSDHKIPEVKYTLCPPAPRKPKPNRS-------S---GTKRKLTPVNVVN-------RIPIDLSREIEMFFEDLDRRI----</td>
</tr>
<tr>
<td>L.esculentum1</td>
<td>KDDCKTPKSSPFLIP--KILKCPAAPKKPRRVIS-----------SCKRKLQFVE------------I-VASKEVESFFKILDDDVVAS-</td>
</tr>
<tr>
<td>L.esculentum2</td>
<td>AVEIRTPGSPQNLIP--KILSCPPAPKKPKRG---------I---SCKRKLLSDL---------EFYDVTAREEVDSFFSSVDENSK---</td>
</tr>
<tr>
<td>LeSIP4</td>
<td>--ECKTPKSPSFRIP--KVVNCPGAPKKPKRAN-------RS---LCKRRLRFEV-----------IVMVDEEEIDSFFRNAEDVNNGG-</td>
</tr>
<tr>
<td>S.tuberosum</td>
<td>AVEIRTPGSPQNLIP--KILSCPPAPKKPKRG---------I---SCKRKLLSDL---------EFYDVAAREEVDSFFSSVDENSK---</td>
</tr>
<tr>
<td>Z.mays1</td>
<td>DYRCRTPTGGESQVR--PPGTCPPAPRKPRAAPAAPAAP-----------PCRKRLFEVE-----------VFSLRLEELERLFWRPRP------</td>
</tr>
<tr>
<td>Z.mays2</td>
<td>55 AVEIRTPGSPQNLIP--KILSCPPAPKKPKRG---------I---SCKRKLLSDL---------EFYDVAAREEVDSFFSSVDENSK---</td>
</tr>
<tr>
<td>O.sativa1</td>
<td>31 YSCCRTPTPGIKGAS----HLPAGAPEAQAGG--------------CRKLLFDPAQQQ----GKGKAISLRLDELERLFRPITNNAN---</td>
</tr>
<tr>
<td>O.sativa2</td>
<td>-GCCRTPTGGESNLK--APGTCPPAPRKPRAPAAP-----------CRKRLFEVE-----------VLSLRLEELERLFWRPPPPPPTTQ</td>
</tr>
<tr>
<td>P.tremula</td>
<td>52 DWPGTHSSLLFLPLLLLLASSRKQNRKKPMSTDLELLQDLPRIQVRPAVKIETLQEQQTOKHAI----------QKESSE</td>
</tr>
<tr>
<td>G.max</td>
<td>DESCRTPTSKESKIP--ATMTCPPAPRKPKFAS-------------CKRKLLEEFQ---------FFDVTNKEDMDAFFRSTFP------</td>
</tr>
</tbody>
</table>

**Figure 5-4** SIAMESE encodes a protein of a previously undescribed class. A sequence alignment of SIM-encoded protein and related proteins from *Arabidopsis thaliana* and other plants is shown here. No function could be ascribed to the first conserved domain found in these proteins (shown in blue). The second conserved domain (shown in red) is an “ZRXL” motif that functions as a cyclin-binding domain in CDKi’s from several species. The third conserved domain, shown in green, is similar to motif 3 found in plant KRP’s and is a putative cyclin-cdk binding domain. The fourth domain, shown in dark yellow is similar to a phosphorylation site found in SIP4, a SIM homolog found in tomato.

The accession numbers for each of the genes shown in this comparision: SIM = CAB85553; At3g10525 = BAC42937; At1g08180 = AAF18255; At5g02420 = CAB85979; L.esculentum1 = A1780963; L.esculentum2 = AW931119; LeSIP4 = AAG43410; S.tuberosum = BM110486; Z.mays1 = AZM4_61016; Z.mays2 = AZM4_26293 O.sativa1 = T03676; O.sativa2 = AAK20052; P.tremula = BU815024; G.max = AW704877. All of these accession numbers are from the NCBI database with the exception of Z.mays1 and Z.mays2 which are from TIGR. Courtesy of Michelle Speckhart.
Figure 5-5 GUS expression in the sim-2 plant. The T-DNA responsible for the sim-2 mutation contained an enhancer trap which shows an expression pattern that we presume to correspond to that of SIAMESE. The areas in which the regulatory regions near the sim-2 insertion direct expression are shown by GUS expression which is blue. A. GUS expression in the sim-2 line is strong in developing and mature trichomes. B. Strong expression can also be seen in stipules. C. While GUS expression is confined to trichomes in the epidermis, heavy GUS expression can be seen in the vasculature of all tissues, including this cotyledon. D. A whole young plant showing this vasculature expression. E. The root tip lacks GUS expression, save in some cases at the very terminus of the tip. F. Root hairs on the collette and elsewhere lack GUS expression.
two separate T-DNA inserts which were separated by outcrossing (Gwin, 2004). The line that co-segregated with the sim mutant phenotype showed GUS expression in developing and mature trichomes (Figure 5-5 A). Strong expression was also seen in stipules (Figure 5-5 B) and the vasculature (Figure 5-5 C) of both the aerial organs and the root. GUS expression in the root was absent in the root tips, though occasionally a slight bit of expression could be seen at the very tip of the root (Figure 5-5 D and E). GUS expression was absent from floral organs and siliques (Gwin, 2004). To corroborate this finding a second assessment of the distribution of SIM message was performed by David Oppenheimer using in situ hybridization. This technique indicates that the highest levels of expression of the SIM message are located in the trichomes, vasculature and meristem tissue (Figure 5-6), which is similar to the GUS expression seen in sim-2. These in situ results are consistent with the notion that the GUS expression pattern seen in sim-2 plants correlates to the actual expression of SIM in the shoot.

A comparison of the amount of SIM expression in different tissues in the plant was made using qRT-PCR in order to further corroborate the sim-2 GUS expression pattern in tissues unable to be visualized using in situ hybridization. RNA was harvested and cDNA was subsequently made from five tissues: whole root, whole three week old rosette, stems, siliques, and the shoot terminus, which contained both developing flowers and the shoot apical meristem. Absolute quantitation of SIM using the TaqMan® chemistry (Gibson et al., 1996) made it possible to determine the number of copies of the SIM message that were
Figure 5-6 Distribution of SIM message as reported by *in situ* hybridization. The distribution of the *SIM* message throughout the shoot was visualized using *in situ* hybridization by David Oppenheimer. **A.** Cross-section of the shoot probed with antisense SIM message. Red arrow = shoot apical meristem. Black arrow = vascular strand. Empty arrows = trichomes. **B.** Negative control.
Figure 5-7 Absolute quantitation reveals tissue-specific differences in expression in *SIM* and its homologs. The number of copies of SIM, At3g10525, At5g02420, or At1g08180 per ng of RNA was determined using quantitative RT-PCR. These results show that the level of SIM expression increases as the percentage of that tissue comprised of vascular tissue increases. At5g02420 and At1g08180 also show this pattern, though the message quantity is significantly less than that of SIM. At3g10525 is expressed most intensely in the stems, but the strong expression of this gene in the flowers and siliques as compared to other tissues shows a departure from the expression of the other genes.
present per nanogram of total RNA. These results, shown in Figure 5-7, show that among the tissues examined, the amount of SIM as compared to total RNA is highest in the root and stem of the plant. This is what would be expected if SIM is expressed in vasculature, because a significant portion of the stem and root is vasculature tissue. This tissue-by-tissue comparison of SIM expression supports the idea that the GUS expression in sim-2 plants is representative of the actual expression of the SIM message. Furthermore, all three methods indicate that SIM is expressed primarily in the vasculature, trichomes, and stipules.

5.2.4 Tissue Level Distribution of the Transcripts of the SIAMESE Homologues

As mentioned before, SIM and three other hypothetical proteins encoded by Arabidopsis genes, At3g10525, At5g02420, and At1g08180, share short stretches of amino-acid identity. The levels of the transcripts corresponding to these genes were assessed in conjunction with the qRT-PCR analysis of the SIM message in different tissues. The results of this assay are given in Figure 5-7. At1g08180 and At5g02420 both display similarities in expression between tissues to SIM in that the highest concentration of these messages are in the stem and root. However, it should be noted that the abundance of the At1g08180 and At5g02420 messages is much lower than SIM mRNA. The stem contains more copies of the At3g10525 message per nanogram of total RNA than any other tissue, which is akin to the expression of the other genes in this family. However, the expression of this gene is much higher in flowers as compared to the other genes in this family. The expression of At3g10525 is also lower in the root than would be expected based upon the results from other genes analyzed.
This would suggest that At3g10525 has a slightly different expression pattern than the other members of this family, though different methods will need to be utilized to confirm this.

To determine if any of the SIM homologs are expressed in trichomes, qRT-PCR was used to analyze the expression of these genes in glabrous gl3 egl3, col wild-type, and GL3OE (see Chapter 3) plants that were approximately two weeks old with three true leaves. Genes regulated by the GL1/GL3 trichome initiation complex should be expressed at higher levels in wild-type plants than in gl3 egl3 plants, and at even higher levels in GL3OE plants as compared to the glabrous gl3 egl3 plants. This experiment is similar to those conducted in Chapter Three, but the measurement of the abundance of SIM and its three homologs performed in this chapter used absolute rather than relative quantitation to measure the amount of message present. This method shows that SIM is expressed in a manner consistent with it being under the control of the trichome initiation signal (Figure 5-8). At5g02420 also displays this increased gene expression correlated with increased GL3 expression, indicating that this gene is either directly or indirectly regulated by the GL1/GL3 complex. The other two homologues, At3g10525 and At1g08180, display no significant change in expression with increased GL3 activity, which would indicate that these genes are not expressed in trichomes at a level detectable by this method. This quantitative RT-PCR data indicates that the SIM homologs are possibly expressed in a redundant fashion in trichomes. While this technique provides a
Figure 5-8 Quantitative RT-PCR determination of trichome-dependent expression of SIM and its homologs. Absolute quantitation was used to measure expression of SIM and its homologs in Arabidopsis. The levels of expression in these graphs represent numbers of copies of a particular message per ng of RNA. RNA was harvested from either gl3 egl3, wild-type or GL3\textsuperscript{OE} plants from the total shoots of plants possessing 3-4 true leaves. The results of two biological replicates are shown separately because differences in expression between the two replicates prohibited pooling the data.
good preliminary analysis of the expression of these genes, other methods must be used to more precisely describe the expression of these genes in the plant.

5.2.5 Sub-Cellular Localization of SIM

To determine where SIM is localized in the cell, a construct consisting of a GFP reporter gene attached to the 5′ end of the SIM coding region driven by the 35S promoter was made by John Larkin. This plasmid, called pK7WGF2/SIMC2-1, should create a protein consisting of GFP fused to the N-terminus of SIM. The pK7WGF2/SIMC2-1 plasmid was introduced into wild-type plants of the col ecotype by Michelle Speckhart and visualized using confocal microscopy. Plants containing this GFP::SIM fusion construct display GFP expression in the nucleus (Figure 5-9). This result suggests that the SIM protein is nuclear localized in vivo.

Because pK7WGF2/SIMC2-1 uses the essentially constitutive 35S promoter to drive expression, it would be expected that all the cells in plants containing this construct would show GFP expression. However, after examining several lines at different developmental stages no plant was found that had the universal GFP expression expected from the CaMV 35S promoter (Benfey and Chua, 1990). GFP expression has been seen in all the cells in the root, but in other instances GFP expression was confined to the atrichoblast cell file in the root (Figure 5-9 G-I). Some plants expressed nuclear localized GFP in all cells of the leaf (Figure 5-9 A-B), but this was extremely rare. Typically only one cell type on the leaf epidermis, either the trichomes, epidermal pavement cells, or the stomatal guard cells, could be seen expressing GFP in a given plant. The hypocotyls and cotyledons also showed this variable behavior regarding GFP
Figure 5-9 An N-terminal GFP-SIM fusion localizes to the nucleus. A construct encoding an N-terminal GFP-SIM fusion, called pK7WGF2, was introduced into wild-type plants. Nuclear-localized GFP expression was found in plants harboring this construct. Expression of the construct was sporadic throughout the plant despite the use of the ectopic 35S promoter to drive expression of this construct. Higher magnification revealed that the GFP:SIM fusion was distributed unevenly in the nucleus. All of the results reported here were observed using the same transgenic line, but similar behavior was observed using other lines as well.

A-B. GFP::SIM expression in the leaf showing GFP::SIM nuclear localization within a trichome and the rest of the leaf epidermis with (A) and without (B) DIC overlay.

C. GFP::SIM expression in a stomatal guard cell pair. Only stomatal guard cells displayed the GFP phenotype in the plant showing this expression.

D-E. Nuclear-localized GFP::SIM expression in the epidermis of a cotyledon. D includes the DIC overlay, while E does not.

F. Close-up of the nucleus shown in a white box in E. Notice the uneven distribution of GFP signal.

G-H. This plant displays GFP::SIM expression in most of the cells of the root, but lacks GFP expression in the hypocotyl. G includes the DIC overlay, while H does not.

I. Expression of GFP::SIM in the root. The blue coloration is caused by the nucleic acid staining dye DRAQ5 which also binds cell walls. Note the lack of GFP::SIM expression in trichoblast cell files.
expression. No correlation between GFP expression and growth conditions, developmental timing, or other factors was found. This suggests that epigenetic silencing was occurring, perhaps due to selection against GFP::SIM expression in some tissues.

5.2.6 The Effect of Ectopic Over-Expression of SIM

A 35S::SIM construct was made by John Larkin and introduced into plants so that the effects of increased and ectopic expression of SIM could be observed. This construct was introduced into wild-type and sim-1 plants by Michelle Speckhart. The epidermal cells of plants containing the 35S::SIM construct were enlarged as compared to wild-type and these plants had deficiencies in growth in all organs (Figure 5-10). Several lines of both parental genotypes were isolated, and there was much variation in the phenotype between these lines. Generally, the wild-type plants that contained 35S::SIM displayed more severe phenotypes than sim-1 plants containing this construct. Additionally, trichome clusters and an occasional multicellular trichome persist on sim-1 plants harboring the 35S::SIM construct.

A single wild-type line containing the 35S::SIM construct, called SIM\textsuperscript{OE}, was chosen to more thoroughly characterize the nature of SIM overexpression. SIM\textsuperscript{OE} plants display a very strong phenotype and this line is shown in Figure 5-10. The amount of SIM expression in wild-type, sim-1, and SIM\textsuperscript{OE} plants was determined using qRT-PCR (Figure 5-11). As would be expected based upon their phenotype, SIM\textsuperscript{OE} plants are found to have a much higher level of SIM expression than either wild-type or sim-1 plants. SIM\textsuperscript{OE} plants have isolated
groups of abnormally large epidermal pavement cells on the leaf and cotyledon, as well as having stunted growth and an abnormal leaf morphology. The other lines transformed with the 35S::SIM construct also show this phenotype to an identical or lesser degree. While some epidermal pavement cells in SIM\textsuperscript{OE} plants were obviously much larger than others on SIM\textsuperscript{OE} plants, a comparison between the epidermal cells in SIM\textsuperscript{OE} plants and wild-type epidermal cells revealed that, in fact, all of the epidermal pavement cells of SIM\textsuperscript{OE} plants are larger than the epidermal pavement cells of wild-type plants. As mentioned previously, Melaragno et al. (1993) found that the larger cells among the epidermal pavement cells of Arabidopsis have greater DNA content than smaller epidermal pavement cells, so these phenotypes are not unexpected for a construct that directs increased expression of a gene that is a positive regulator of endoreplication.

5.3 Discussion

The discovery of the SIAMESE mutation by Walker et al (2000) indicated that a single gene could control entry into the endoreplication cell cycle. Moreover, this gene appeared to be trichome specific, at least in the epidermis, which could provide a great opportunity to study both the process of endoreplication and its relationship to trichome development. SIAMESE serves as an excellent example of the power of the use of trichome development as a model system. If there was a gene with the same function as SIM that acted in the leaf epidermis or another vegetative tissue whose cells undergo
**Figure 5-10 Phenotypes of the SIM over-exresser.** Plants containing the 35S::SIM construct had many striking phenotypes. A. A wild-type (left) and SIM\textsuperscript{OE} (right) plant four weeks after germination. B. A close-up of a leaf from the SIM\textsuperscript{OE} plant in A. Note the deformed leaf morphology. C. SEM of the adaxial epidermis of a wild-type leaf. D. SEM of the adaxial epidermis of a SIMOE leaf displaying greatly enlarged epidermal cells. E. Cross-section of a wild-type leaf. F. Cross-section of a SIMOE leaf showing the greatly enlarged cells of the adaxial epidermis. G. DAPI staining of a wild-type leaf. DAPI stains DNA, which causes the nuclei of the cells in this picture to appear blue. H. DAPI stained cells from a SIMOE plant. Note the enlarged size of these nuclei as compared to wild-type in G. All of these images were provided by Michelle Speckhart. Size bars: A = 1 cm, B = 1 mm, C and D = 200 µm, E and F = 50 µm, G and H = 22 µm.
Figure 5-11 Expression of SIM in sim-1, wild-type and SIM\textsuperscript{OE} lines. Absolute quantitation (using qRT-PCR) of SIM expression in sim-1, wild-type (col), and SIM\textsuperscript{OE} plants.
endoreplication it is unlikely that a mutation in this gene would cause a phenotype that could be discerned from wild-type.

The work in this chapter provides evidence indicating that we have identified the SIM gene. Conceptual translation of the SIM coding region predicts a protein that is only 127 amino acids in length. SIM encodes a protein of a previously undescribed class, though there are three SIM homologues in Arabidopsis and several other similar proteins in other plant species.

Though no other protein like SIM has been thoroughly described, it is clear that SIM regulates the cell cycle in some way. Lack of SIM function causes a malfunction in the endoreplication cell cycle in trichomes, while over-expression of SIM causes an increase in endoreplication in epidermal cells (M. Speckhart, personal communication) with a concomitant increase in cell size (Figure 5-10). The fact that an N-terminal GFP::SIM fusion protein localizes to the nucleus (Figure 5-9) bolsters the idea that SIM interacts with the cell cycle in some way. The only protein that appears to be homologous to SIM that has been described in the literature is SIP4, a protein that was found to interact with the SELF PRUNING (SP) protein from tomato (Pnueli et al., 2001). SIP4 was shown by Pnueli et al (2001) to interact with a group of proteins including SP, a NIMA kinase, and a 14-3-3 protein. This same work showed via in situ hybridization that SIP4 has an expression pattern analogous to that of SIM. These interactions suggest that SIM may exert its control of the cell cycle through similar interactions if these interactions occur in Arabidopsis.
Sequence homology between regions of the putative SIM protein and other proteins involved in the cell cycle may also lend some insight into the function of SIM. Previous experiments showed that misexpression of either of two cyclins, CYCLIN B 1;2 or CYCLIN D 3;1, produced multicellular trichomes similar to sim mutants, but with a greater frequency of multicellular trichomes and more cells per trichome initiation site (Schnittger et al., 2002a; Schnittger et al., 2002b). Cyclins associate with proteins called Cyclin Dependent Kinases (CDK) to control a cell's progression through the cell cycle. Because SIM functions to alter this progression, it is likely that it interacts either directly or indirectly with CDKs, Cyclins, or Cyclin-CDK complexes. The second conserved domain of the predicted SIM protein appears to be a “ZRXL” motif which is required for stable association of certain proteins to cyclin-cdk2 complexes in human cell culture (Zhu et al., 1995; Adams et al., 1996; Chen et al., 1996; Adams et al., 1999). Also, the third conserved domain of SIM is similar to motif 3 from the KRP (De Veylder et al., 2001), which acts as a cyclin/cdk binding domain (L. DeVeylder, personal communication). Based upon these associations and the behavior of plants lacking a functional SIM protein or overexpressing the SIM protein, a reasonable hypothesis is that SIM represents a new class of cyclin-dependent kinase inhibitors. This hypothesis is currently being tested in the Larkin lab.

The SIM over-expression results may also shed light onto the link between DNA content and cell size. Rather than just demonstrating a correlation between DNA content and cell size within a given tissue as Melaragno (1993) did, SIM over-expression provides a more direct demonstration between ploidy and cell
volume. Assuming that the function of SIM is indeed to alter the cell cycle to increase the DNA content of the cell, SIM\textsuperscript{OE} plants demonstrate that increases in ploidy are not only related to, but rather, may cause increases in cell size.

Once the identity of the SIM gene was determined, I set out to determine the location of expression of the SIM message. The expression of the SIM was expected to be found in trichomes because of the trichome phenotype found in sim mutants. Indeed both the sim-2 enhancer trap expression pattern and the results of the in situ hybridization reveal that the SIM message is expressed strongly in trichomes. However, both the enhancer trap and the in situ hybridization experiments indicate that SIM is also strongly expressed in the vasculature of the plant. Analysis of the expression of SIM using qRT-PCR shows that the SIM message is most concentrated in roots and stems, which are tissues predominated by vasculature. While the fact that endoreplication is involved in trichome development has been known for some time (Hulskamp et al., 1994; Traas et al., 1998; Perazza et al., 1999) much less is known about the alteration of the cell cycle in the vascular tissue. This lack of knowledge probably owes to the fact that vascular tissue lays beneath other cell layers, which makes analysis difficult. There are two possible roles in vasculature formation that could be fulfilled by a cell cycle-modifying protein. The xylem and phloem of a plant both consist of elongated cells (Fahn, 1982), and it would be conceivable that an increase in endoreplication would be required to facilitate the development of these expanded cells in a manner similar to that found among epidermal pavement cells. Another possibility is that cell cycle modification is required to
induce cell death during vasculature formation (Fahn, 1982). Overexpression of KRP1, a separate class of CKIs, was shown to cause cell death in trichomes by Schnittger et al (2003) and this could be the mechanism by which cell death is induced during this process. However, too little is known about the role of the cell cycle in the vascular tissues to say that the role of SIM would be restricted to either of these two possibilities.

The SIM homologues appear to have a pattern of expression roughly similar to that of SIM, based upon the qRT-PCR results shown in Figure 5-7. All of the SIM homologues show the most concentrated expression in the stems, and all but At3g10525 also show a markedly higher expression in the roots as compared to other tissues. Perhaps these genes are expressed in vascular tissue because the stem and root are composed chiefly of vascular tissue. A comparison of the expression of SIM and its homologues in glabrous versus pubescent plants in Figure 5-8 showed that the expression of one of the SIM homologues, At5g02420, increased in a manner dependent upon trichome phenotype similar to SIM. This would indicate that At5g02420 may have a redundant role with SIM in regulating endoreplication in trichomes. The presence of other regulators of endoreplication in trichomes is predictable based upon the phenotype of sim mutants: not every trichome on a sim mutant plant is multicellular, but rather only a portion of them show this phenotype. If SIM were the only regulator of the entry into endoreplication in trichomes, then all trichomes should be multicellular. A SALK line containing an insertion in At5g02420 has been crossed to sim-1 by my colleague Michelle Speckhart and
we are awaiting the progeny of these plants for phenotypic analysis. In fact, double, triple, and quadruple mutants between all of the SIM homologues should produce very interesting results.

One of the more puzzling behaviors associated with the *SIAMESE* gene is its apparent uneven action among cells in a given tissue or between different tissues. As mentioned previously, not all of the trichomes in *sim* mutants are multicellular, and while there may be some redundancy in the switch between mitosis and endoreplication in trichomes there are other irregularities in the behavior of *SIM*. Figure 5-10 shows that SIMOE plants have much larger epidermal cells than wild type plants, which is expected based upon the predicted action of *SIM* and the link between cell size and endoreplication in the plant epidermis (Melaragno, 1993). However, not all of the cells of the epidermis are equally enlarged; some of the cells were so large that they could be observed without microscopy, while others appeared to be no bigger than wild-type epidermal pavement cells. Also, many multicellular trichomes were observed on *sim* plants, indicating that *SIM* was not acting in these trichomes in SIMOE plants. The basis behind this variability in action is mysterious at present. Based upon data presented in this chapter, either a mechanism controlling transcription or a post-translational control could be responsible for this variability of *SIM* action. In the analysis of SIM levels in wild-type, *sim-1* and SIMOE plants, higher *SIM* levels were found in *sim* mutant plants as compared to wild-type plants (Figure 5-11). This would suggest that there is an auto-regulatory mechanism controlling *SIM* expression. Despite this evidence, post-translational control of cell cycle actors is
a common theme in cell cycle regulators. Both phosphorylation (Morgan et al., 1998) as well as proteolytic degradation (Sheaff and Roberts, 1996) have been shown to regulate the action of various components of the cell cycle and SIM contains sequence domains similar to those for phosphorylation (the fourth conserved domain shown in dark yellow in Figure 4-4) and a putative PEST domain, which serves as a proteolytic signal (Rechsteiner and Rogers, 1996), is found in amino acids 26-41 according to the PESTfind program (http://bioweb.pasteur.fr/seqanal/interfaces/pestfind.html). A post-translational method of regulation is more likely to explain the variability in GFP expression seen in 35S::GFP::SIM plants. The 35S promoter should express the gene it controls in all tissues at all developmental timepoints (Benfey and Chua, 1990), however expression of GFP is highly erratic in these plants. Future investigations of the function of the SIM gene and its homologues are currently being undertaken by my colleagues in the Larkin lab and these investigations should shed light upon the more puzzling behaviors displayed by *SIAMESE*.

The identification and characterization of the gene responsible for the *SIM* mutation as described in this chapter represents an important first step in our understanding of a novel class of cell cycle regulator. Based upon the information available in various public databases the distribution of this new class of protein is widespread among various plant species and there appear to be at least three homologues of *SIM* in the Arabidopsis genome. This apparent redundancy may explain the sporadic nature of the *sim* trichome phenotype and may reveal the involvement of SIM-like proteins in other processes besides trichome and
vasculature development. Several lines of inquiry concerning SIAMESE are currently underway in the Larkin lab. The goal of all of these lines of inquiry is to further elaborate upon the mechanism of SIM function. Assays to determine if SIM interacts with a number of candidate proteins involved in the cell cycle have been initiated and these experiments should reveal if SIM interacts with particular candidate proteins. A screen of mutagenized sim-1 plants has revealed several mutants that exacerbate the SIM phenotype (Remmy Kasili, personal communication). Identifying the genes responsible for these mutations should reveal other actors in the pathway in which SIM is involved. Obviously, there is a great deal of work left to be done before the mechanism by which SIM controls the cell cycle is fully understood, but when that goal is achieved it will represent a significant advancement not only in our understanding of trichome development, but of the development of all plant cells.
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

Much has been learned about the development of trichomes since Marks et al (1991) suggested that they would be a good model for cell differentiation in plants. As of this writing, the genes that regulate the trichome cell fate decision appear to have been fully uncovered (Chapter 1). Work in this dissertation describes the discovery and behavior of GLABRA3 (GL3), which is one of the genes involved in the cell fate decision in trichomes. In fact, GL3 was a central theme of this dissertation because of the positive correlation between GL3 expression and trichome phenotype. In Chapter Three, a GL3-dosage gradient, consisting of gl3 egl3 plants (which lacked GL3 function), wild-type plants, and GL3OE plants (which expressed much more GL3 message than wild-type), was used to determine if a particular gene was expressed in a trichome dependent manner by examining its expression in these three genotypes of plants. Genes expressed in a trichome-dependent manner showed expression parallel to GL3 amount. This provided a system to rapidly determine if a gene was expressed in a trichome-dependent manner. This GL3 dosage gradient was used throughout this dissertation, and could be used in future work, to discriminate between genes that are controlled by the trichome initiation complex, and those that are not.

In Chapter Four, the expression of several genes near the T-DNA insertion site of an enhancer trap line that showed strong expression in developing trichomes was analyzed using the GL3 dosage gradient described in Chapter Three. This led to the discovery of the involvement of an α-carbonic
anhydrase, At2g28210, in trichome development. Studies using a carbonic anhydrase inhibitor, called ethoxyzolamide, indicated that pharmacological inhibition of CA function could impede trichome expansion. This work opens several other avenues of investigation. The work in Chapter Four provides a template for finding other genes controlled by the trichome initiation complex using the enhancer trap screen performed by Scott Poethig’s lab (http://enhancertraps.bio.upenn.edu). Also, genetic methods, such as a T-DNA insertion line or RNAi of At2g28210, would be preferable to inhibitor studies to determine the function of this carbonic anhydrase in trichome development. Subcellular localization of the protein encoded by At2g28210 will also be required to fully understand its function.

Chapter Five detailed the identification and characterization of SIAMESE, a gene involved in the regulation of endoreplication. SIM was found to encode a novel protein with motifs resembling those found in various cell-cycle regulating proteins. According to a GUS enhancer trap line and in situ hybridization, SIM is expressed in a trichome-specific manner in the epidermis, but it is also expressed in the vasculature throughout the plant. A protein consisting of GFP translationally fused to the N-terminus of SIM was shown to localize to the nucleus. A search of the Arabidopsis database revealed that there are three homologs of SIM in the Arabidopsis genome. Analysis of the expression of SIM and its three Arabidopsis homologs in gl3 egl3, col, and GL3OE plants revealed that SIM and At5g02420 were expressed in a trichome-dependent manner. Investigations concerning SIM are currently the primary focus of the Larkin lab.
The interaction of SIM with other cell-cycle participants, isolation genes affecting the sim phenotype, the effect of over-expressing the SIM homologs, and the effect of T-DNA knockouts of the SIM homologs are just a few of the directions of research currently being pursued.

Understanding how the action of a transcription factor, such as GL3, can ultimately bring about such a drastic shift in morphology as seen between trichomes and the epidermal pavement cells and stomata, which arise from the same precursors as trichomes, is a central question in developmental biology. This dissertation presents evidence that GL3 controls an α–carbonic anhydrase and a novel cell-cycle regulator. Hopefully, methods in this dissertation can be used to begin to tease apart the many other physiological changes that occur during trichome development. The steroid-inducible GL3 construct presented in Chapter Three presents a means to further resolve the level of GL3 control of a candidate gene, which should give an idea of the timing of expression of various genes involved in trichome development. This would create an even greater understanding of the physiological processes that take place in trichome development. The determination of the role of genes such as SIM, which is expressed in a trichome-dependent manner but is also expressed in other tissues, in trichome development will allow for a greater understanding of the role of these processes in the development of other tissues. Therefore, the study of the regulation of genes involved in trichome development is also the study of genes involved in the development of the plant as a whole.
WORKS CITED


Acid Synthesis in Cell Expansion during Plant Morphogenesis. Plant Cell 17, 1467-1481.


**APPENDIX: LIST OF PCR PRIMERS USED IN THIS WORK**

For primers that have a restriction enzyme recognition sequence, the restriction enzyme sequence is lower case, while the sequence that hybridizes to the gene in question is upper case. All primers that were used in taqman real-time PCR are denoted by a “TM” in their name.

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VITA

Matthew Lloyd Brown was born in West Monroe, Louisiana, on March 26, 1974. His education began within his family and a love of living things was instilled within him early in his life while gardening with his grandfather, F. L. Brown. He graduated from West Monroe High School in 1992. His first two years of collegiate education were taken at Centenary College in Shreveport, Louisiana, and he completed his undergraduate work at Louisiana State University in May of 1996. He began his graduate work in 1998 at Louisiana State University, and after a short rotation in another laboratory, he formally entered the Larkin Lab in the Spring of 1999. Matthew was heavily involved in the Biological Sciences Graduate Student Association, also known as BIOGRADS, where he served as vice-president from 2000-2001. He also organized the first BIOGRADS intramural flag football team in 2001. Graduate school has provided Matthew with the opportunity to travel extensively. He has gone to Wisconsin, Utah, and Berlin, Germany to present his work at scientific conferences. Matthew met an enchanting girl named Emily McMains in a Developmental Neurobiology class in 2001, and as of this writing, is engaged to be married to her. The degree of Doctor of Philosophy will be awarded to him in May of 2006.