Characterization of the Arabidopsis compact inflorescence (cif) mutant and isolation of CIF1 to ACA10, a P-type IIB CAp2p+s-ATPase gene
by Lynn DeAnn George

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biological Sciences
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Abstract:
A mutant in Arabidopsis, called compact inflorescence (cif), was identified among the T2 progeny of an Agrobacterium transformant. The most apparent aspect of the cif phenotype was a strong reduction in the elongation of internodes in the inflorescence. Elongation and expansion of adult vegetative rosette leaves were also compromised in mutant plants while juvenile vegetative and reproductive organs developed normally.

Thus, cif mutant plants appeared to exhibit a novel, adult vegetative phase-specific phenotype. In hopes of gaining insight into the genetic mechanisms that underlie inflorescence architecture and vegetative phase specific identities, and regulate vegetative phase change, a study of this mutant was undertaken. The specific goals of this project included the following: 1) Characterize the altered development of the compact inflorescence mutant and its relation to plant developmental growth phases; 2) determine the genetic basis for inheritance of the cif trait; 3) through map-based cloning, determine the identity of genes whose altered alleles confer development of the cif phenotype; 4) determine the relationship of the CIF genes to developmental pathways that are known to influence vegetative phase change in flowering plants.

Results of the study confirm the restriction of the cif phenotype to a single plant developmental growth phase and describe the impact on the cif trait of exogenous hormone applications, changes in light quality, and variable photoperiods, compact inflorescence is inherited as a two-gene trait involving the action of a recessive locus, and a naturally occurring dominant locus. These two cif genes appear to be key components of a growth regulatory pathway that is closely linked to phase change. Recombinant frequency mapping of the CIF genes, and final isolation of the cif1-1 and cif1-2 alleles to mutations in ACAl 0, a gene encoding a P-type IIB Ca^2+ ATPase are described, as well as cloning of the ACAl0 gene and transgenic complementation of the cif1-1 mutation. Implications of the cif mutant phenotype in plant calcium signaling are also discussed.

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ABSTRACT

A mutant in *Arabidopsis*, called *compact inflorescence* (*cif*), was identified among the T_2 progeny of an *Agrobacterium* transformant. The most apparent aspect of the *cif* phenotype was a strong reduction in the elongation of internodes in the inflorescence. Elongation and expansion of adult vegetative rosette leaves were also compromised in mutant plants while juvenile vegetative and reproductive organs developed normally. Thus, *cif* mutant plants appeared to exhibit a novel, adult vegetative phase-specific phenotype. In hopes of gaining insight into the genetic mechanisms that underlie inflorescence architecture and vegetative phase specific identities, and regulate vegetative phase change, a study of this mutant was undertaken. The specific goals of this project included the following: 1) Characterize the altered development of the *compact inflorescence* mutant and its relation to plant developmental growth phases; 2) determine the genetic basis for inheritance of the *cif* trait; 3) through map-based cloning, determine the identity of genes whose altered alleles confer development of the *cif* phenotype; 4) determine the relationship of the *CIF* genes to developmental pathways that are known to influence vegetative phase change in flowering plants.

Results of the study confirm the restriction of the *cif* phenotype to a single plant developmental growth phase and describe the impact on the *cif* trait of exogenous hormone applications, changes in light quality, and variable photoperiods. *compact inflorescence* is inherited as a two-gene trait involving the action of a recessive locus, and a naturally occurring dominant locus. These two *cif* genes appear to be key components of a growth regulatory pathway that is closely linked to phase change. Recombinant frequency mapping of the *CIF* genes, and final isolation of the *cif1-1* and *cif1-2* alleles to mutations in ACA10, a gene encoding a P-type IIB Ca^{2+} ATPase are described, as well as cloning of the ACA10 gene and transgenic complementation of the *cif1-1* mutation. Implications of the *cif* mutant phenotype in plant calcium signaling are also discussed.

CHAPTER 1

A REVIEW OF *Arabidopsis thaliana*, PLANT DEVELOPMENTAL GROWTH PHASES, THE GENETIC REGULATION OF INFLORESCENCE ARCHITECTURE, AND PLANT CALCIUM SIGNALING

Introduction

A mutant in the plant *Arabidopsis thaliana* was identified among the T_2 progeny of an *Agrobacterium* transformant. This mutant, called *compact inflorescence (cif)*, exhibited an altered structure of the flowering bolt, or inflorescence, and onset of the mutant trait appeared to coincide with the transition between two plant developmental growth phases. A detailed characterization of the *cif* phenotype was initiated, as well as recombinant frequency mapping of the genes whose altered alleles confer inheritance of the mutant trait. The study confirmed the association of the *cif* phenotype with a single plant developmental growth phase, and culminated in the molecular isolation of a mutated Ca^{2+} ATPase gene. The following chapter comprises reviews of the model organism used for this study, plant developmental growth phases, the genetic regulation of inflorescence architecture, and plant Ca^{2+} signaling.

Arabidopsis thaliana – A Model Organism for Molecular Genetics

Biology of *Arabidopsis*

Arabidopsis thaliana, a small herbaceous annual of the Brassicaceae or mustard family, was named in 1842. The species name, *thaliana*, honors Johannes Thal, a XVIth-century German physician of Nordausen Thüringen who described *Arabidopsis* in 1577

in his book detailing the flora of the Harz Mountains (Rédei, 1992). Common names are mouse-ear cress and wall cress. In the wild, *Arabidopsis* grows in the moderate temperate climates of the northern hemisphere including Europe, Asia and North America. *Arabidopsis* is not likely indigenous to America though as its distribution coincides with the shipping routes of European grains imported by the settlers (Rédei, 1992). The most likely origination is the Central Asian highlands of the Western Himalayas (Berger, 1965; Rédei, 1970). Wild ecotypes of *Arabidopsis* are most commonly winter annuals with seed germination in the fall, survival through the winter as a vegetative rosette, and transition to flowering in early spring. Many different accessions of *Arabidopsis* have been collected from natural populations and are presently available for experimental analysis. The Columbia and Landsberg ecotypes are the accepted standards for genetic and molecular studies.

In greenhouse conditions, the entire life cycle of *Arabidopsis* is completed in approximately six weeks, and includes seed germination, formation of a vegetative rosette, bolting of the reproductive shoot, flowering, and maturations of seeds. The flowers of *Arabidopsis* are 2-4 mm long, self-pollinate, and can be easily crossed by applying pollen to the surface of the stigma. Each flower has an outer whorl with 4 green sepals, and inner whorls containing four white petals, six stamens bearing pollen, and a central gynoecium. The ovary within the gynoecium develops into a silique that contains 30 to 60 seeds. A single plant may have more than 200 flowers during its life and thus may produce more than 10,000 seeds. Mature plants reach 15 to 20cm in height.

A History of *Arabidopsis* Research

Friedrich Laibach initiated the use of *Arabidopsis* as an experimental organism in 1907 (Rédei, 1992). He conducted the first deductive experiments in *Arabidopsis* including accurately counting the 10 somatic and 5 meiotic chromosomes. Laibach was also first to note that the amount of chromatin in *Arabidopsis* was only about one third that of other members of the Brassicaceae. Laibach's work that most significantly impacted the use of *Arabidopsis* as an experimental organism was published in Botanisches Archiv in 1943 wherein he notes the features of *Arabidopsis* that make it particularly well suited to experimental classical genetics (Rédei, 1992). Some of these features include: 1) small size; 2) rapid generation time (5-6 weeks under optimal growth conditions); 3) ability to grow well in controlled conditions (either on soil or defined media); 4) ease of cross- and self-fertilization; 5) fecundity (up to 10,000 seeds per plant); 6) small chromosome number; and 7) ease of mutagenesis.

George P. Rédei introduced *Arabidopsis* as a model system for plant genetics to the United States in 1965 (Rédei, 1992). Heslot et al. first described the use of ethylmethane sulfonate as a chemical mutagen in 1959, and its great efficiency in *Arabidopsis* was demonstrated by Röbbelen in 1962 and McKelvie in 1963 (Rédei, 1992). *Arabidopsis* research became more prevalent in the early 1980's with the release of a detailed genetic map and publications outlining the value of *Arabidopsis* for research in plant physiology, biochemistry, and development. This was followed by a subsequent advance, the establishment of a transformation system using co-cultivation with disarmed cointegrate agrobacterial vectors by Lloyd *et al.* in 1986 (Rédei, 1992). The discovery

that the size of the *Arabidopsis* nuclear genome is the smallest known among flowering plants (Sparrow et al., 1972; Leutwiler et al., 1984), and that it contains a very low level of dispersed repetitive DNA (Pruitt and Meyerowitz, 1986) prompted the development of *Arabidopsis* as a model system for molecular as well as classical genetics.

The modern era of *Arabidopsis* research began in 1987 with the opening of the Third International *Arabidopsis* Conference at Michigan State University and the subsequent formation of an electronic *Arabidopsis* newsgroup (Meinke, 1998). Numerous researchers experienced in the analysis of other model organisms began to study *Arabidopsis* as a promising model for basic research. An important outgrowth of this increased enthusiasm for *Arabidopsis* research was the drafting in 1990 of a vision statement outlining long-term research goals for the *Arabidopsis* community. These included saturating the genome with mutations, identifying every essential gene, and sequencing the entire genome by the end of the decade (Meinke, 1998). The importance of applying advances with *Arabidopsis* to other plants and to solving practical problems in agriculture, industry, and human health was also stressed. In 1994, several European labs cooperatively began a pilot-sequencing project of the *Arabidopsis* genome (Pennisi, 2000). The project quickly grew to 17 European groups under the coordination of Michael Bevan, a geneticist at the John Innes Centre in Norwich, United Kingdom. In 1996, the sequencing effort expanded to form an international consortium known as the *Arabidopsis* Genome Initiative and included the Institute for Genomic Research (TIGR) in Rockville, Maryland, Cold Spring Harbor Laboratory, Washington University, Stanford University, the University of Pennsylvania, the University of California,

Berkeley, and Kazusa DNA Research Institute in Kisarazu, Japan. This initiative became a model for multinational cooperation and resulted in the sequencing of 118.7 million base pairs of the 125 million base pair *Arabidopsis* genome.

Genetic Analysis

The *Arabidopsis* research community has developed most of the methods and resource materials expected of a model genetic organism. These include simple procedures for chemical and insertional mutagenesis, efficient methods for performing crosses and introducing DNA through plant transformation, extensive collections of mutants with diverse phenotypes, and a variety of chromosome maps of mutant genes and molecular markers. The absence of an efficient system for homologous recombination in *Arabidopsis* is a limitation shared by some other model organisms including *Drosophila melanogaster* and *Caenorhabditis elegans* (*C. elegans*).

Integration of cloned DNA into the *Arabidopsis* genome, or plant transformation, is routinely performed through the use of the pathogenic bacterium *Agrobacterium tumefaciens*, a technique that was pioneered during the late 1980's (Schell, 1987; Horsch et al., 1986; Weising et al., 1988; Zambryski, 1988). *Agrobacterium* is a soil organism that, in nature, induces the tumorous growths of crown-gall disease on susceptible host plants. During the initial stages of tumorigenesis, a defined region of *Agrobacterium* DNA, the T-DNA, is physically transferred from a bacterial plasmid (the Ti plasmid) into the nuclear genome of the infected plant cell. The T-DNA is flanked by 24-bp imperfect direct repeats which are required in *cis* for transfer. A set of genes outside the T-DNA, called the virulence (*vir*) genes, act in trans to promote T-DNA transfer. The T-DNA

contains genes that direct the synthesis of the phytohormones auxin and cytokinin and through the overproduction of these compounds within the transformed plant, uncontrolled cell division characteristic of a tumor results.

Genetic researchers have disarmed *Agrobacterial* strains by deleting the tumor-inducing genes. These disarmed strains are no longer tumorigenic, but are still fully transfer proficient and DNA inserted between the 24-bp repeats will be randomly and stably integrated into the plant genome. Currently, a binary-plasmid system is generally used for *Agrobacterium*-mediated plant transformation. The *vir* genes have been maintained on the Ti plasmid, while the *cis*-acting repeat sequences have been placed on relatively small plant transformation vectors capable of replicating both in *E. coli* and *Agrobacterium.* Between the *cis*-acting repeat sequences are multiple cloning sites that allow the insertion of gene constructs of interest, and a selectable marker gene that confers resistance to specific antibiotics upon transformed plant cells. The most widely used antibiotic resistance gene product is the bacterial neomycin phosphotransferase type II (NPTII) enzyme that detoxifies compounds such as Kanamycin and G418 by phosphorylation. Initially, *Arabidopsis* researchers used wounded tissue or entire seeds as the starting plant material to be transformed. More recently, floral dipping methods, that avoid plant regeneration through tissue culture, have made plant transformation routine (Clough and Bent, 1998). It has been shown that the ovule is the target of T-DNA integration in the floral dipping method (Desfeux et al., 2000). The main drawbacks of T-DNA transformation are the often complex integration patterns of T-DNA, including transfer of vector sequences that flank the T-DNA, multiple insertions,

and the high frequency of concatemeric insertions, which in T-DNA mutagenesis (see below) can complicate the identification of flanking sequences.

Agrobacterium-mediated plant transformation has been used extensively in T-DNA mutagenesis of *Arabidopsis*. Thousands of transgenic lines carrying random T-DNA insertions throughout the genome have been deposited in public stock centers. Many additional lines are being produced at private companies interested in functional genomics. Maize transposable elements introduced through *Agrobacterium*-mediated transformation have also been used extensively for gene disruption in *Arabidopsis*. Chemical mutagenesis, with alkylating agents such as ethylmethane sulfonate (EMS), has also been used extensively for genetic analysis. Mature seeds are the preferred targets for chemical mutagenesis as selfing M1 plants derived from a single experiment can produce millions of progeny seeds homozygous for recessive mutations. Bulk collection and screening of M_2 seeds requires only a small area: 10,000 seeds can be germinated in a single Petri dish, and selection for a particular phenotype among hundreds of thousands of newly germinated seedlings is possible.

The success of *Arabidopsis* as a model organism is primarily due to its amenability to forward genetic screens wherein mutagenized plants are screened for phenotypes of interest. Through insertional and chemical mutagenesis techniques, several thousand mutants of *Arabidopsis* defective in almost every aspect of plant growth including gametogenesis, seed formation, leaf and root development, flowering, senescence, metabolic and signal transduction pathways, responses to hormones, pathogens, and environmental signals, and many cellular and physiological processes

have been identified. Forward genetic screens culminate in the molecular isolation of the mutated gene, allowing the assignment of a developmental function to the wild-type allele. Once a mutation that affects a particular developmental or metabolic pathway has been identified, secondary screens allow further dissection of the pathway that is affected by the mutation. A screen for second-site mutations that enhance (worsen) the phenotype, generally identify genes that act redundantly with the primary mutation or possibly interact physically with the mutant gene product. Alternatively, enhancer mutations may uncover a partially redundant parallel pathway. Suppressor mutations identify interacting proteins, or downstream pathways that become activated by the second-site suppressor.

Genetic screens have also been developed to identify genes that affect epigenetic information including methylation profiles that contribute information for controlling developmental pathways. Unlike other model systems including mouse, *Arabidopsis* tolerates large defects in epigenetic regulation. The *Arabidopsis ddm1* mutant (*decrease in DNA methylation1*) exhibits a reduction in genome-wide cytosine methylation by 70%, but is viable and fertile (Vongs et al., 1993). Thus *Arabidopsis* is particularly well suited for studying epigenetic phenomena.

The availability of reporter genes, in combination with efficient transformation methods in *Arabidopsis,* make biological processes with subtle or hidden phenotypes accessible to forward genetic approaches. Typically, a reporter gene encoding firefly luciferase (LUC), β-glucuronidase (GUS) or green fluorescent protein (GFP) is fused to the promoter of a specifically regulated inducible gene. This allows for observation of

the wild-type expression pattern of the inducible gene. Subsequent mutagenesis of such lines allows screening for a deviation from the wild-type expression pattern and the identification of genetically interacting loci. This type of screen has been used since the late 1980s and has allowed the identification of important genes in the circadian system (Millar, 1995), hormone-signaling pathways (Meier et al., 2001), and in plant responses to biotic (Bowling et al., 1994) and abiotic stresses (Xiong et al., 2001). All of these are extremely difficult to detect in conventional phenotypic screens.

Plants were the first organisms in which the silencing of introduced DNA was observed (Napoli, 1990). Since then, numerous examples have confirmed the specific inactivation of a transgene either at the transcriptional level (transcriptional gene silencing, TGS) or after transcription (post-transcriptional gene silencing, PTGS) (Matzke et al., 2001). In PTGS, the mRNA of a transgene is specifically degraded. If endogenous mRNA, homologous to the transgene RNA, is also present, expression of the endogenous gene will also be silenced, (Matzke et al., 2001). Although gene silencing was initially perceived as an uncontrollable and unwanted source of instability of transgene expression, it offered an entry point for the genetic analysis of silencing mechanisms (Page and Grossniklaus, 2002). Studies of PTGS in *Arabidopsis* led to the establishment of a molecular link between PTGS in plants, Quelling in *Neurospora crassa* and RNA Interference in *C. elegans* (Page and Grossniklaus, 2002).

Reverse genetic research proceeds from genotype to phenotype; different mechanisms are used to silence or alter the expression of a particular gene of interest and the effects of this genetic change on the development of the organism indicate gene

function. Reverse genetic screens depend on the researcher's judgment of the candidacy of a gene by sequence alone and thus are biased and perhaps less likely to lead to new insights than unbiased forward screens. However, the *Arabidopsis* researcher currently has access to very powerful tools for taking the reverse genetics approach. Disruption of a gene itself is generally the most direct way to understand its function. Unlike other model systems, such as yeast or mouse, no efficient methods for targeted gene disruption exist in flowering plants. Transposons or T-DNA are used as more or less random mutagens to create loss-of-function mutations. Because the sequence of the inserted element is known, the gene in which it is inserted can be recovered using various cloning or PCR-based strategies. The Nottingham *Arabidopsis* Stock Centre (NASC), the Salk Institute, the Flanking Sequence Tags (FST) project at Versailles and the Torre Mesa Research Institute (TMRI) all maintain insertion databases that can be searched for known insertions in a gene of interest. The Knockout Facility at the University of Wisconsin, Madison maintains large populations of T-DNA insertion lines and offers a service whereby pools of independent T-DNA lines are screened to obtain a 'knockout' mutation in a gene of interest. Anti-sense technology utilizing PTGS can also be used to effectively "knockout" the expression of a chosen gene (Ruban et al., 2003; Laval et al., 2003).

A powerful tool in reverse genetics, called TILLING (Targeting, Induced Local Lesions IN Genomes), has recently become available to the *Arabidopsis* researcher. TILLING is a high-throughput, PCR-based screen for EMS-induced point mutations within a specific gene (McCallum et al., 2000). The technology is based on an

endonuclease that preferentially cleaves mismatches in heteroduplexes between wild-type and mutant DNAs. Subsequent analysis of cleavage products on a sequencing gel allows the rapid identification of induced point mutations. Unlike insertional mutagenic approaches that generally result in knockout mutations, TILLING provides an allelic series of point mutations in a gene of interest. This approach is especially valuable for analyzing the function of essential genes in which knockout mutations would be lethal.

The *Arabidopsis* Genome

Through an international consortium known as the *Arabidopsis* Genome Initiative, 118.7 megabases of the *Arabidopsis* 125 megabase genome sequence was released in the year 2000, and represents the first complete sequence of a plant nuclear genome (The *Arabidopsis* Genome Initiative, 2000). With the fewest gaps and most substantial extension into centromeric regions, the sequence obtained was also the most accurate sequence available for any organism, over that of *Saccharomyces cerevisiae, C. elegans,* and *Drosophila.* An extensive analysis of the *Arabidopsis* genome sequence was published by The *Arabidopsis* Genome Initiative in the journal *Nature* (2000) **408:** 796-813. A brief summary of that analysis follows.

The *Arabidopsis* genome contains approximately 25,500 genes encoding proteins from 11,000 different families. This functional diversity is similar to that of two other multicellular eukaryotes, *Drosophila* and *C. elegans,* indicating that a proteome of 11,000-15,000 protein types is sufficient for a wide diversity of multicellular life. Only 9% of the proposed 25,500 *Arabidopsis* genes have been characterized experimentally. However, from their similarity to proteins of known function in other organisms, 69% of

Arabidopsis proteins have been assigned a putative function. The *Arabidopsis* genome contains roughly 150 protein families that appear to be unique to plants. *Arabidopsis* genes tend to be compact, with an average gene length of approximately 2,000 kb per gene. *Arabidopsis* genes typically contain coding regions of about 250 base pairs each, punctuated by short introns. The genes are closely spaced, about 4.6 kb apart, indicating that their regulatory regions are also short. The exons in *Arabidopsis* are richer in guanosine and cytosine bases (44%) than are the introns (32%), a feature that is unique to plant genes. Approximately 60% of the *Arabidopsis* genome has been duplicated. The large-scale duplication, but not triplication, suggests that *Arabidopsis* had a tetraploid ancestor. Sequence comparisons between *Arabidopsis* and tomato indicate that this duplication event likely occurred around 112 million years ago. Gene loss and chromosomal rearrangements presumably generated the small genome and five chromosomes of the *Arabidopsis* we know today.

Approximately one-fifth of the intergenic DNA, or 10% of the genome is represented by transposons in *Arabidopsis*. In several cases, genes appear to have been included as 'passengers' in transposable units. *Arabidopsis* telomeres are composed of CCCTAAA repeats and average between 2-3 kb. The *Arabidopsis* genome project gave the first detailed picture of centromeric sequences in a higher eukaryote. *Arabidopsis* centromeres, like those of many higher organisms, contain numerous repetitive elements including retroelements, transposons, microsatellites and middle repetitive DNA. Such repetitive regions are very likely heterochromatic, and are generally viewed as poor environments for gene expression. However, 47 expressed genes were found to lie

within the centromeres of *Arabidopsis*. In several cases, the genes were found residing on islands of unique sequence flanked by repetitive arrays.

The *Arabidopsis* genome sequence also offers unique avenues for studying gene regulation and signal transduction. Consistent with its rather highly methylated genome, *Arabidopsis* possesses eight DNA methyltransferases (DMTs), including a chromomethyltransferase that is unique to plants. Transcription in *Arabidopsis* by RNA polymerases II and III appears to involve the same machinery as is used in other eukaryotes although most transcription factors for RNA polymerase I are not found. Only two polymerase I regulators (other than ploymerase subunits and TATA-binding protein) are found in *Arabidopsis*. Surprisingly, the *Arabidopsis* genome was found to encode atypical subunits for the largest and second largest subunits of polymerase I, II and III. These novelties may endow plant-specific functions. *Arabidopsis* possesses twice as many transcription factors as *Drosophila*, and more than three times as many transcription factors as *C. elegans*. The most widely used signaling pathways in vertebrates, flies, and worms, including Wingless/Wnt, Hedgehog, Nothch/lin12, JaK/Stat, TGF-β/SMADs, receptor tyrosine kinase/Ras and the nuclear steroid hormone receptors are all absent in *Arabidopsis*. However, 340 transmembrane receptor-like kinase (RLK) genes are found in the *Arabidopsis* genome, of which Ser/Thr kinases comprise the largest class. With the exception of CLV1, the ligands sensed by RLKs are essentially unknown, providing a compelling avenue for future research. While there are approximately twenty MAP kinases found in *Arabidopsis*, a higher number than in any other eukaryote, there is potentially only a single heterotrimeric G-protein complex.

With the full genome sequence in hand, the *Arabidopsis* community has recently taken on a more challenging goal yet. A new 10-year project, called the NSF 2010 Project, seeks to determine the function of every *Arabidopsis* gene by the year 2010 (Somerville and Dangl, 2000). To this end, every *Arabidopsis* gene will be inactivated or overexpressed. The resulting phenotypes will be examined by all available criteria, including full-genome mRNA expression profiling and metabolic profiling. Comprehensive information will be available about where and when every gene is expressed; where the protein is localized; how the protein is modified; and with what, if any, other proteins the gene product interacts. Additional goals of the 2010 project include determining the substrates for every protein kinase, and the promoters controlled by each transcription factor. In cases where genes have apparent functional redundancy, the phenotype of the multiple mutant will be determined. It is envisioned that this knowledge will facilitate the development of a virtual plant – a computer model that will use information about each gene product to simulate the growth and development of a plant under many environmental conditions (Somerville and Dangl, 2000).

Phase Change in Flowering Plants

Flowering plants pass through a succession of three major post-embryonic developmental stages, or phases, during their life cycle: juvenile vegetative, adult vegetative, and reproductive (Poethig, 1990; Lawson and Poethig, 1995; Kerstetter and Poethig, 1998). The juvenile vegetative phase begins when the shoot apical meristem generates a stem, true leaves and axillary buds. This phase may last for a few days or

many years, depending on the species, and is distinguished by unique morphological and physiological traits, including leaf shape, trichome distribution, and by the absence of reproductive capacity. The subsequent adult vegetative phase is characterized by changes in these morphological traits and by the development of a competency of the shoot to shift into reproductive growth. Reproductive phase is characterized by the production of flowers in place of leaves. In many flowering plants, including *Arabidopsis*, the transition to reproductive growth is accompanied by the generation of an inflorescence, an elongated scaffold of stem and leaf structures, which supports and presents the reproductive organs or flowers. Although the elongated internodes and small cauline leaves of the inflorescence develop after what is usually considered to be the initiation of reproductive phase, these organs are essentially "vegetative" tissues.

Although progression through two distinct phases of rosette development (i.e. juvenile and adult) is a recognized component of plant life cycles, little is known regarding the genetic basis for the regulation of vegetative phase change (Kerstetter and Poethig, 1998). Goebel (1900) originally proposed that a single morphogenetic program controls the development of all leaf types in flowering plants. He hypothesized that variation in leaf morphology arises because different parts of a leaf primordium are arrested at different points along this pathway (Kerstetter and Poethig, 1998). Much experimental evidence contradicts this early hypothesis however, and supports the modern view that juvenile and adult leaves are specified by distinct developmental programs (Kerstetter and Poethig, 1998). Strong evidence in support of this view comes from the study of phase change mutants in maize. The dominant gain-of-function *Teopod*

mutations result in the prolonged expression of juvenile traits (Evans and Poethig,1995; Poethig, 1988). The expression of adult traits however, including reproductive competence, is unaffected. The leaves of *Teopod2* mutants are morphologically and anatomically intermediate structures, combining features that are normally specific to either juvenile or adult leaves. The phenotypes of the *teopod* mutants suggest that the *TP* genes regulate a juvenile program of leaf identity that operates in parallel to, and to some extent independently of the program that specifies adult traits (Kerstetter and Poethig, 1998).

In recent years, large advancements have been made toward understanding the genetic mechanisms and developmental pathways that control the transition to reproductive growth. Meristem patterning, or the establishment of precise localities of gene expression, is very likely the primary process governing floral morphogenesis (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Thus, the specific meristem domain in which the organ originates determines floral organ identity. Like flower development, meristem patterning could also play a major role in determining the identities of juvenile and adult leaves and in vegetative phase change. However, there is strong evidence that argues against a meristem-patterning model in vegetative development. Clonal analyses in maize suggest that there is no cell lineage restriction between juvenile and adult organs of the shoot (Poethig et al., 1986). That adult phase maize shoots can be readily induced to revert to the juvenile phase in culture (Kerstetter and Poethig, 1998) further implies that the shoot meristem does not possess developmentally determined juvenile and adult domains. The development of leaves that

are transitional between the juvenile and adult vegetative phases argues perhaps most strongly against a meristem-patterning model for the specification of vegetative organ identity (Kerstetter and Poethig, 1998). In maize and in *Arabidopsis*, the distal region of transitional leaves often expresses juvenile traits while the basal region expresses adult traits (Goebel, 1900; Bongard-Pierce et al., 1996; Telfer et al., 1997). Such development suggests that upon induction, phase change occurs globally, in all cells of the meristem and in all undetermined cells in preexisting organs. Because leaves mature basipetally, regions at the tip of preexisting leaves are likely to have already become determined for the previous developmental state, whereas basal regions of the leaf will still have the capacity to switch to the new state. Hence, this model suggests that vegetative phase change comprises global changes in gene expression throughout the meristem and undetermined regions of preexisting organs such that organ identity is determined temporally depending on maturation of the shoot, rather than spatially (Kerstetter and Poethig, 1998).

To date, no *Arabidopsis* genes have been identified that are differentially expressed between the juvenile and adult phases of vegetative growth. Furthermore, little is known regarding the signaling mechanisms that trigger the global changes in gene expression that likely result in vegetative phase change (Kerstetter and Poethig, 1998). Relatively few phase-associated mutations have been described in *Arabidopsis*, all of which affect the timing of the phase transitions rather than the development of phase-specific organ morphologies. Most phase mutants in maize also exclusively affect the timing of phase transitions.

Genetic Regulation of Inflorescence Architecture in Flowering Plants and Mutants Affecting Internode Elongation and Inflorescence Development

As outlined above, *Arabidopsis* exhibits separate vegetative and reproductive growth phases. The vegetative phase, called the rosette, is characterized by the production of leaves in a spiral with very little stem elongation between leaves. A group of parental cells at the center of the rosette, termed the shoot apical meristem (SAM), provides the initials that differentiate into leaf and stem cells. Upon transition to the reproductive phase, the SAM begins to produce flowers in place of leaves. In most angiosperms, this transition is accompanied by dramatic elongation of internodes within the stem/shoot system resulting in the production of a showy, flower-bearing bolt called the inflorescence. Elongation of internodes is accomplished via numerous cell divisions in the apical region of the internode coupled with extensive cell enlargement. These processes occur after the SAM has moved slightly upward, and thus are considered a phase of development distinct from the generation of inflorescence initials by the SAM (Steeves and Sussex, 1989). *Arabidopsis* exhibits a high degree of internode elongation in both the primary inflorescence stem and in lateral branches such that an extended bolt called a compound raceme is produced.

Elongation of inflorescence internodes is a trait of particular significance in determining plant reproductive architecture and, hence, in the evolution of flowering plants. Long internodes generate a more conspicuous reproductive structure that functions in the crucial roles of presenting reproductive organs to pollinators and dispersal of seeds. Moreover, the vast array of inflorescence designs observed among

flowering plants results in large part from the differential elongation of inflorescence internodes. Bradley et al. (1996b) have proposed the existence of at least three pathways in *Antirrhinum* that determine inflorescence traits, including internode length, cauline leaf morphology, hairiness of the stem, and phylotaxy. Two of these pathways were shown to be independent of genes that control floral meristem identity, such as *Floricaula*, the homolog of *Leafy* in Arabidopsis (Bradley et al., 1996a), drawing a distinction between inflorescence and floral development.

Different inflorescence traits have also been strongly selected for during the domestication of many crop plant species. For example, modification of inflorescence branching patterns and apical dominance by allelic variation at the *teosinte branched* locus has been shown to be a major component of the evolution of cultivated maize from its wild ancestor teosinte (Doebley et al., 1997). This finding in maize illustrates the importance of genetic variation in relatively few critical regulatory genes in the origins of cultivated plant architectures.

Despite their importance in plant morphogenesis, the mechanisms through which specific plant cells are signaled to divide and expand and how these mechanisms are regulated at developmental transitions is poorly understood. Attempts have previously been made to isolate mutants of *Arabidopsis* that maintain normal vegetative growth and normal floral meristem identity but exhibit altered inflorescence development. One candidate for such a mutation is *erecta*, found in the common Landsber *er* strain, which produces plants with reduced height, somewhat clustered flowers, and short petioles and siliques (Torii et al., 1996). The *ERECTA* locus has been cloned and encodes a receptor-

like protein kinase that is expressed in the shoot apical meristem and in organ primordia (Tori et al., 1996; Yokoyama et al., 1998). Recently, more loci similar to *erecta*, called *corymbosa*, have been identified (Komeda et al., 1998). A large screen of mutagenized *Arabidopsis* for mutants with short flower stalks resulted in the identification of the *acaulis* mutants (Tsukaya et al., 1993; Hanzawa et al., 1997). The *ACAULIS5* gene has recently been shown to encode a spermine synthase (Hanzawa et al., 2000). However, all of these mutants are quite pleiotropic in their effects, altering growth of juvenile and adult vegetative tissues, overall plant stature, and in some cases, the number of flowers produced and the development of fruits. Hence, the *ERECTA, CORYMBOSA,* and *ACAULIS* loci are likely to be important generally in signaling appropriate growth regulation during organ formation in the plant, but are not restricted in their activities to a specific phase of plant development.

Calcium Signaling in Higher Plants

Calcium as a Signaling Molecule in Plants

Plant cells use a variety of messengers in signaling pathways including Ca^{2+}, lipids, pH, and cyclic GMP. No single messenger however, has been demonstrated to respond to more stimuli, both biotic and abiotic, than has cytosolic free Ca^{2+} (Sanders et al., 1999). Biotic stimuli that alter Ca^{2+} levels include the hormones abscissic acid and gibberellin, fungal elicitors, and nodulation factors. Abiotic stimuli include light (with red, blue, and UV/B irradiation each acting via different receptors and leading to distinct developmental responses), low and high temperature, touch, hyperosmotic stress, and

oxidative stress (Sanders et al., 2002). Calcium signals are generated through the opening of ion channels that allow the flow of Ca^{2+} from an area in which the ion is present at a relatively high concentration to an area of lower concentration. Plant cells generally maintain a very low cytosolic level of calcium, on the order of micromolar concentrations, whereas the extracellular fluid contains relatively high levels, on the order of millimolar concentrations. Although the electrochemical potential across the membranes of organelles is likely to be less negative than that across the plasma membrane, a number of intracellular compartments also serve as important reservoirs of Ca^{2+} ions. While the endoplasmic reticulum, mitochondria, and chloroplasts have all been shown to sequester Ca^{2+}, the large lytic vacuole of mature plant cells is the principal intracellular Ca^{2+} store (Sanders et al., 1999; Sanders et al., 2002). Maintenance of low cytosolic Ca^{2+} ion concentrations is achieved by either the ATP-driven removal of Ca^{2+} through pumps, or by the proton motive force-driven removal by carriers. The interplay between influx of Ca^{2+} through channels, and efflux from pumps and carriers determines the profile of a Ca^{2+} spike.

To date, the best understood calcium signal transduction pathway in plants controls the opening and closing of stomata by guard cells within the epidermis (Geisler, 2000). Here, voltage-gated Ca^{2+} release channels, and inositol 1,4,5-triphospahate and cADP-ribose-sensitive calcium release pathways function in the generation of calcium signatures (Geisler et al., 2000). The specific transduction events involved in stomatal closure that occur downstream from calcium however are not known. In general, the initial perception of a calcium signal occurs through the binding of calcium to many

different calcium sensors. Ca^{2+} sensors in plant systems can be divided into two types, sensor relays and sensor responders (Sanders et al., 2002). Sensor relays, such as calmodulin, undergo a calcium-induced conformational change (sensing) that is relayed to an interacting partner. The interacting partner then responds with some change in its enzyme activity or structure (for example, calmodulin stimulation of a calcium pump). In contrast, sensor responders undergo a calcium-induced conformational change (sensing) that alters the protein's own activity or structure. A primary class of Ca^{2+} sensor responders is the calcium-dependent protein kinase (CDPK) family. Most of the known calcium-stimulated protein kinase activities in plants are associated with CDPKs. Despite their major role in plant Ca^{2+}-signaling, CDPKs are found only in some protozoans and are absent from the genomes of *Saccharomyces cerevisiae*, *C. elegans*, *Drosophila*, and humans (Cheng et al., 2002). Four distinct domains typify CDPK family members: an N-terminal variable domain, a protein kinase domain, an autoinhibitory domain, and a calmodulin-like domain. The calmodulin-like domain contains Ca^{2+}-binding EF hands that allow the protein to function as a Ca^{2+} sensor (Cheng et al., 2002). Under the basal condition of low free Ca^{2+}, the autoinhibitory domain acts as a pseudosubstrate and is bound by the kinase domain, keeping substrate phosphorylation activity low (Harmon et al., 1994). Upon binding Ca^{2+} via the EF hand motifs, CDPKs undergo conformational changes that release the pseudosubstrate from the catalytic site, activating the protein. Little is known about the function of the N-terminal variable domain. It has been proposed that this region contains subcellular targeting information (Harper et al., 1994). Many plant CDPKs have a Gly residue at the

second position (Cheng et al., 2002). This N-terminal residue can be modified by covalent attachment of myristic acid, which promotes protein-membrane and protein-protein interactions. The *Arabidopsis* genome is predicted to encode 34 different CDPKs and the precise biological function(s) of most of these enzymes is not known (Cheng, 2002). By definition, sensor responders function through intramolecular interactions, whereas sensor relays function through bimolecular interactions. These two different modes of decoding calcium signals are used extensively in plants to provide many pathways by which calcium can trigger a diverse number of responses (Sanders et al., 2002).

Why Calcium?

Metabolism in all cells requires the presence of orthophosphate (P_i) and phosphorylated organic compounds, particularly for cytosolic reactions associated with the transduction of free energy (Sanders et al., 1999). Together, Ca^{2+} and P_i have a low solubility product, which indicates that at relatively high calcium concentrations, the availability of soluble P_i decreases drastically. For this reason, mechanisms for reducing cytosolic Ca^{2+} concentrations to a level well below the millimolar concentrations present in seawater would have arisen early in evolution (Sanders et al., 1999). Indeed, transport systems that export Ca^{2+} from the cytosol are present in all cells to sustain steady state values of cytosolic Ca^{2+} in the submicromolar range. Such mechanisms for maintaining Ca^{2+} homeostasis would have been ideal for subsequent evolution of Ca^{2+} based signaling pathways (Sanders et al., 1999). Specifically, the elevation of cytosolic Ca^{2+} levels by a

factor of 10 or 20 can occur more rapidly than would be possible for ions or solutes that are maintained at millimolar levels (Sanders et al., 1999).

The chemistry of Ca^{2+} also lends itself to signal transduction (Sanders et al., 1999). The ability of a Ca^{2+} ion to coordinate numerous uncharged oxygen atoms enables the evolution of protein conformations in which remote domains can participate in calcium binding. It has been shown that calcium-induced conformational changes can elicit downstream events in signaling pathways (McPhalen et al., 1991).

Specificity in Calcium Signaling

As stated previously, changes in cytosolic Ca^{2+} occur during the transduction of a wide variety of abiotic and biotic signals. During the last ten years of calcium signaling research, a single question has pervaded the field (McAinsh and Hetherington, 1998). How can a simple non-protein messenger be involved in a multitude of signal transduction pathways and yet convey stimulus specificity within these pathways? Growing evidence indicates that signal specificity is inherent in the amplitude and frequency of Ca^{2+} waves within the cytosol. The dynamic profile of a calcium wave is determined by the combinatory effects of influx through channels and efflux patterns through pumps and carriers. Research in two nonplant systems (Xenopus oocytes and Dictyostelium) has demonstrated that increasing the abundance or activity of a Ca^{2+} pump can indeed alter signal transduction (Lechleiter et al., 1998; Roderick et al., 2000). Thus, in addition to their housekeeping functions, efflux pathways play an integral role in defining the information encoded in a calcium signal.

Statement of the Problem

A mutant in *Arabidopsis*, called *compact inflorescence* (*cif*), was identified among the T_2 progeny of an *Agrobacterium* transformant. The most apparent aspect of the *cif* phenotype was a strong reduction in the elongation of internodes in the inflorescence. Elongation and expansion of adult vegetative rosette leaves were also compromised in mutant plants while juvenile vegetative and reproductive organs developed normally. Thus, *cif* mutant plants appeared to exhibit a novel, adult vegetative phase-specific phenotype. In hopes of gaining insight into the genetic mechanisms that underlie inflorescence architecture and vegetative phase specific identities, and regulate vegetative phase change, a study of this mutant was undertaken. Results of the study implicate alterations in Ca^{2+} signaling. The specific goals of this project included the following:

1) Characterize the altered development of the *compact inflorescence* mutant and its relation to plant developmental growth phases.
2) Determine the genetic basis for inheritance of the *cif* trait.
3) Through map-based cloning, determine the identity of genes whose altered alleles confer development of the *cif* phenotype.
4) Determine the relationship of the *CIF* genes to developmental pathways that are known to influence vegetative phase change in flowering plants.

CHAPTER 2

THE *Arabidopsis compact inflorescence* GENES: PHASE-SPECIFIC GROWTH REGULATION AND THE DETERMINATION OF INFLORESCENCE ARCHITECTURE

Introduction

Flowering plants go through a succession of developmental stages, or phases, during their life cycle. Three major phases have been described: juvenile vegetative, adult vegetative, and reproductive (Poethig, 1990). The juvenile phase begins when the shoot apical meristem generates a stem, true leaves and axillary buds. This phase may last for a few days or many years, depending on the species, and is distinguished by a variety of unique vegetative traits including leaf shape and trichome distribution, and by the absence of reproductive capacity. The subsequent adult vegetative phase is characterized by a different set of vegetative traits and by a competence of the shoot to shift into reproductive growth. For many plants, reproduction is the last phase in the life of the shoot and is characterized by the production of flowers in place of leaves.

In *Arabidopsis*, the juvenile vegetative phase is marked by the production of small, simple leaves with little or no internode elongation between them (Telfer and Poethig, 1998). The leaf blade and petiole of juvenile leaves are easily distinguished, and the lamina is round in shape with a smooth edge (Telfer and Poethig, 1994). Trichomes are evenly distributed on the adaxial (upper) surface and absent on the abaxial (lower) surface (Telfer et al., 1997). As the shoot shifts into adult phase growth, later leaves gradually take on adult characteristics including an elongated shape with the lamina and

petiole becoming less distinct, and the outer edge of the leaf blade becoming serrate (Telfer and Poethig, 1994). Adult vegetative leaves also bear trichomes on the abaxial surface, as well as the adaxial surface (Telfer et al., 1997). These differences in leaf anatomy are accompanied by a competence of the shoot to respond to floral inducers (McDaniel et al., 1992; Shannon and Meeks-Wagner, 1991). Although distinct organ types are generated during the juvenile and adult phases of vegetative development, intermediate organs that exhibit traits of both phases are typical during the transition from juvenile to adult growth. This suggests that expression of the juvenile and adult developmental programs overlaps during the progressive maturation of the shoot, and that the two programs are not mutually exclusive.

The reproductive phase in *Arabidopsis* is characterized by the production of flowers in place of leaves. The flowers are displayed on a highly elongated and branched bolt. Together the elongated bolt and the flowers that it supports are collectively known as the inflorescence. During production of the inflorescence, the shoot apical meristem initially generates nodes bearing small cauline leaves with secondary inflorescence meristems in the leaf axils. Later, the meristem generates leafless nodes with floral meristems that produce individual flowers. The lower internodes of the inflorescence that subtend cauline leaves elongate dramatically as the inflorescence grows. Although they develop after what is usually considered to be the initiation of reproductive phase, the elongated stem internodes and the small cauline leaves of the inflorescence are essentially "vegetative" tissues.

This report describes a mutant of *Arabidopsis* called *compact inflorescence.* The *cif* trait is specific to the growth pattern of adult vegetative leaves and vegetative portions of the inflorescence with the most striking aspect being a lack of internode elongation in apical inflorescence internodes such that a cluster of flowers is produced, rather than the wild-type raceme. Elongation and expansion of adult vegetative leaves is also compromised in *cif* plants. The *cif* phenotype is inherited as a two-gene trait requiring homozygosity for a recessive mutant allele of one gene, *CIF1*, and either heterozygosity or homozygosity for a dominant allele of a second unlinked gene, *CIF2.* Although the growth pattern of adult vegetative tissues is dramatically affected in mutant plants, the proper identity of organs is maintained, as well as the timing of organ production and of the phase transitions. Additionally, development of juvenile vegetative organs is indistinguishable from that of wild type, and *cif* plants exhibit normal floral meristem identity and are fully fertile. Thus, *cif* plants harbor mutations in two genes that appear to regulate the growth pattern of adult vegetative tissues, specifically in response to the juvenile to adult phase transition.

Results

Isolation of the *cif* Mutant

The *compact inflorescence* phenotype was identified among the T_2 progeny of an *Agrobacterium* transformant in the ecotype No-0. Figure 2.1 shows the phenotype of *cif* mutant plants, the most striking feature of which is the generation of a cluster of flowers at the apex of the inflorescence, rather than the wild-type raceme. *cif* plants produce