CHARACTERIZATION OF THE Arabidopsis compact inflorescence 3 (cif3) MUTANT AND IDENTIFICATION OF THE CIF3 GENE PRODUCT AS A CHLOROPLAST LOCALIZED PUTATIVE ATPASE

by

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Plant Science

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of a thesis submitted by

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Jeffrey Carlyle Cameron

April 18, 2005
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A new mutant of *Arabidopsis*, that exhibits very short inflorescence internodes in contrast to the wild-type raceme structure, was isolated from an *Agrobacterium tumifaciens* T-DNA insertion screen. This plant closely resembles the previously described *compact inflorescence* (*cif1*) mutant (Goosey and Sharrock, 2001). The *cif1* trait was shown to require altered alleles of two genes; a recessive mutation at the *cif1* gene and a naturally occurring unlinked dominant allele, *CIF2*. Although the phenotypes of *cif1* and the new mutant are similar, complementation tests show that they are different genes, and the new mutant is designated *cif3*. The *cif3* mutation is recessive and, unlike the *cif1* mutation, does not require the presence of a dominant allele of the *CIF2* gene to cause the inflorescence phenotype. Moreover, the *cif1* phenotype was previously shown to be restricted to the adult vegetative phase of growth and to strongly influence the morphology of adult rosette leaves. In contrast, *cif3* does not show an effect on adult leaves and therefore does not show apparent phase-specific expression. The *cif3* mutation is tagged with a T-DNA insertion and the *CIF3* gene has been cloned using forward genetics. Northern blot analysis shows expression of a disrupted transcript from the *CIF3* gene in the *cif3* mutant. A transgenic complementation test was performed and confirms the identity of the *CIF3* gene. The *CIF3* gene product has been shown to be a chloroplast localized putative ATPase. These studies provide insight into the genetic mechanisms controlling inflorescence development in *Arabidopsis* and may provide a foundation for understanding inflorescence architecture in agriculturally important crop plants.
INTRODUCTION

A novel mutant of *Arabidopsis thaliana* was isolated from an *Agrobacterium* T-DNA promoter trap screen. The mutant, called *compact inflorescence 3* (*cif3*), exhibits reduced inflorescence internode elongation, leading to formation of floral clusters in contrast to the extended wild-type raceme. This phenotype is similar to the previously described mutant, *cif1* (Goosey and Sharrock, 2001). The *cif3* mutant also has a darker green rosette and narrower leaves than the wild-type under certain light conditions. Using a forward genetics approach, the *CIF3* gene was determined to encode a chloroplast localized, putative ATPase. This thesis gives a brief review of *Arabidopsis thaliana* and inflorescence architecture, a description of the *cif3* mutant, a comparison of *cif3* to the previously described *cif1* mutant phenotype, and the identification of the *CIF3* gene product as a putative chloroplast-localized ATPase.

*Arabidopsis as a Model Organism for Genetics*

*Arabidopsis thaliana*, a small annual in the mustard family, is an ideal organism for molecular and classical genetics because of its: 1) small stature (15-20cm), 2) self compatibility (and ease of cross-pollination), 3) rapid generation time (approximately 36 days in growth chamber), 4) ease of mutagenesis (via *Agrobacterium*-mediated
transformation (Clough and Bent, 1998), 5) small genome (2n=10, 100Mbp) and 6) publicly available, sequenced genome (Arabidopsis Genome Initiative, 2000)

*Arabidopsis* passes through three distinct growth phases during its lifetime- the juvenile vegetative, adult vegetative and the reproductive phase (Poethig, 1990). The vegetative growth phases, or rosette, are characterized by the arrangement of leaves in a spiral phyllotaxy, and lack of internode elongation between the leaves. Under a 16 hr fluorescent light photoperiod, the juvenile vegetative growth phase is characterized as the first six true leaves formed after the cotyledons (Goosey and Sharrock, 2001). The adult vegetative leaves are formed after a transitional set of leaves (leaves 7 and 8), and continue to grow until the reproductive phase of development (Goosey and Sharrock, 2001). Only the adult vegetative growth phase is reproductively competent, meaning it has the ability to produce a flowering bolt. During the reproductive phase, the inflorescence internodes elongate, forming a flowering stalk called the inflorescence. The inflorescence is the structure that supports the flowers and seed pods. The inflorescence also develops lateral branches, which elongate and form a compound raceme.

*Arabidopsis* Inflorescence Architecture and Internode Elongation

Inflorescence architecture is important in the wild for seed dispersal and because pollinators must recognize and be attracted to flowers. Inflorescence architecture is also
important in agriculture because this is where the harvestable seeds are found. Short floral internodes will produce short, robust plants, making them less susceptible to environmental factors such as wind and rain. Many factors influence the overall architecture of the inflorescence, but I will focus on the elongation of floral internodes and the impact of this on inflorescence structure. Despite the importance of this trait in wild populations and in agricultural applications, very little is known about the genetics and regulation of inflorescence architecture and internode elongation.

The distance between the floral internodes determines the length and appearance of the inflorescence and may influence the out-breeding potential and overall yield of the plant. A number of Arabidopsis mutants that exhibit altered inflorescence phenotypes have been reported. An extensively characterized mutant in the ecotype Landsberg called erecta (er) has a reduced overall height, clustered flowers, short petioles, bluntly shaped siliques and round leaves (Torii et al., 1996). The ER gene product is a leucine rich repeat receptor-like protein kinase with an extracellular ligand binding domain that is expressed in the shoot apical meristem and regulates the shape of organs originating from the apical meristem (Torii et al., 1996). It has been suggested that the ER gene product is involved in cell-cell communication during plant morphogenesis. However, the ER gene product may also play a role in pathogen defense (Godiard et al., 2003). A second Arabidopsis mutant, acaulis1, affects the development of the inflorescence and the leaves due to a premature arrest of the reproductive meristem (Tsukaya et al., 1993). The inflorescence of acaulis1 mutants may be reduced in length
or even absent, leading to a reduction in the number of flowers. The phenotype extends to the rosette leaves of the mutant, which tend to curl downward (Tsukaya et al., 1993). A third mutant of *Arabidopsis, brevipedicellus (bp)*, also has a shortened inflorescence, short pedicels and downward pointing flowers (Venglat et al., 2002). The *BP* gene encodes the homeobox gene KNAT1, a member of the KNOX family, which is involved in shoot apical meristem maintenance and function (Venglat et al., 2002).

The *Arabidopsis compact inflorescence* mutant, *cif1*, was identified and characterized previously in this laboratory. The *cif1* mutant, which was isolated in the ecotype No-0, was identified among the T2 progeny of an *Agrobacterium* transformant but the *cif1* mutation is not associated with a T-DNA tag (Goosey and Sharrock, 2001). The *cif1* mutant has drastically reduced elongation of inflorescence internodes, leading to the formation of a cluster of flowers in contrast to the wild-type extended raceme. The *cif1* mutant also has an adult leaf expansion phenotype, resulting in small, crinkled adult leaves. Because the *cif1* phenotype influences the adult leaves and inflorescence, but not the juvenile leaves, the *cif1* mutant is thought to be adult vegetative phase-specific (Goosey and Sharrock, 2001). Expression of the *cif1* phenotype requires the presence of a naturally occurring dominant modifier allele specific to the ecotype No-0, *CIF2* (Goosey and Sharrock, 2001). Recently, the recessive *cif1* mutation was identified as a loss of function mutation in the ACA10 gene, which encodes a P-type IIB Ca$^{2+}$ ATPase (L. George, unpublished).
The *cif3* mutant was initially identified in the T₂ progeny of a T-DNA promoter trap screen performed in another laboratory. Because the *cif3* mutant closely resembled the *cif1* mutant, it was sent to our laboratory for further study. The *cif3* mutant has reduced elongation of inflorescence internodes, resulting in the formation of floral clusters that appear very similar to those seen in the *cif1* mutant. Both the *cif1* and *cif3* mutant phenotypes are strongly affected by light quality and photoperiod. Although the inflorescences of these mutants are similar in appearance, the *cif3* mutant does not exhibit the adult leaf expansion phenotype, and therefore does not appear to be growth phase specific. The *cif3* mutant does, however, have a darker colored rosette and has narrower leaves than the wild-type and the *cif1* mutant.

In order to better understand the molecular mechanisms that regulate inflorescence development and, more specifically, to determine whether the *Arabidopsis cif1* and *cif3* mutations affect the same signaling pathway, the *CIF3* gene has been identified and characterized. The *CIF3* gene product has been determined to be a soluble, chloroplast localized putative ATPase.
MATERIALS AND METHODS

Plant Materials and Growth Conditions

The cif3 mutant of Arabidopsis thaliana (ecotype Nossen (No-0)) was isolated from an Agrobacterium tumefaciens T-DNA insertion screen done at the Plant Gene Expression Center in Albany, California. The T-DNA insertion vector was pGKB5, which confers kanamycin and Basta resistance. Most physiological experiments and transgenic complementation analysis were performed using a back-cross III line (BCIII F3 #2-1) which was generated by backcrossing the cif3 allele into No-0. The cif1 mutant in the ecotype No-0 was isolated from a T-DNA transformation (Goosey and Sharrock, 2001). No-0 and/or Col-0 were used as Wild-Type (No-0 WT, Col-0 WT) controls as indicated in individual experiments.

Unless otherwise indicated, seeds were surface sterilized in 15% bleach for 25 minutes, rinsed five times in sterile water, and plated on 150x25mm Petri dishes containing GM media (see Appendix A). The seeds were dark treated at 4°C for three days before being placed under continuous fluorescent light. After about ten days the seedlings were transferred to potting soil drenched in nutrient solution (see Appendix A) and overlaid with vermiculite that was also drenched in nutrient solution. The plants were then grown in Conviron growth chambers under 16/24hr fluorescent and/or incandescent light at 20°C.
Liquid grown tissue used for genomic DNA preperations was produced by sterilizing the seeds (~15 mg) using the above protocol, transferring them to sterile 50ml flasks containing liquid GM media (see Appendix A), cold/dark treating them while shaking, and then placing them under continuous fluorescent light with continuous shaking at room temperature.

Seedling tissue was grown on sterilized filter paper in standard Petri dishes overlaid on GM agar media using the above protocol.

For physiology and flowering time experiments, seeds were dry sterilized in 70% EtOH, 1% Triton X-100 for four minutes, rinsed in 95% EtOH, and then dried on sterile filter paper in a sterile tissue culture hood. The seeds were then sprinkled on pots containing soil overlaid with vermiculite (both drenched in nutrient solution), given a three-day cold/dark treatment, and placed directly in the growth chamber. Plants used for Agrobacterium transformation were dry sterilized, sprinkled on mounded pots (see Appendix A), given a three-day cold/dark treatment and then placed directly in the growth chamber.

Selection media for plants are listed in Appendix A.

**Southern Blot Analysis**

Genomic DNA was extracted from 2.0 g liquid grown tissue using a standard protocol (Ausubel et al., 1989). Approximately 1µg of genomic DNA was digested with
EcoRI or Pst I and separated on a 50ml, 1% agarose gel. The fractionated DNA was transferred to a GeneScreen Nylon hybridization membrane (NEN™ Life Science Products, Inc., Boston, MA) and probed with $^{32}$P labeled DNA fragments derived from the GUS and Basta resistance genes on the T-DNA. The 2kb GUS fragment (SstI/BamHI) from pBI101.1 (see Appendix B) was gel purified. A 440bp Basta fragment (Fig. 5) was amplified from pGKB5 using upstream and downstream primers, respectively (5’-GTCTGCACCATCGTCAACC-3’, 5’-GTTTCTGGCAGCTGGACTTC-3’). All probes were labeled with $^{32}$P using Ready-To-Go™ DNA labeling beads (-dCTP) kit and protocol (Amersham Bioscience, UK).

**PCR Analysis**

For most PCR analyses, tissue was collected from seedling or rosette stage leaves with a paper punch. DNA was extracted and PCR was performed using REDExtract-N-Amp™Plant PCR Kit (Sigma-Aldrich, inc. St. Louis, MO). The Basta primers listed above were used to perform the PCR test for co-segregation of the T-DNA.

**Construction of a Genomic Library in Lambda Bacteriophage**

Genomic DNA isolated from cif3 mutant seedlings was serially digested with Sau3AI and fractionated on a 0.4% agarose gel to obtain an optimal size of 15kb. The
size fractionated DNA was packaged into lambda bacteriophage using Lambda FIX®II/Xho I Partial Fill-In Vector Kit (Stratagene, La Jolla, CA) and protocol. Lambda plaques were screened using a mixture of five $^{32}$P labeled probes made from the T-DNA. Individual positive plaques were isolated and purified by equilibrium centrifugation in cesium chloride as described (Yamamoto and Alberts, 1970).

Cloning the CIF3/T-DNA Junction

The CIF3/T-DNA junction was amplified from purified lambda DNA (described above) using Universal GenomeWalker™ Kit (Clontech laboratories, Inc., Palo Alto, CA) and protocol. Lambda DNA was cut with EcoRV and adaptors were ligated onto the ends according to the protocol. Adaptor primer AP1 (5’-GTAATACGACTCACTATAGGGC-3’) was used in a primary PCR reaction with a gene specific primer made from the T7 arm of lambda, GSP1 (5’-GCCGCGAGCTCTAATACGA-3’). Although the protocol called for a secondary PCR reaction using nested primers, the sequence from the primary PCR was adequate. The PCR product was commercially sequenced at Northwoods DNA, Inc. (Solway, MN).
Northern Blot Analysis

Total RNA was extracted from 2.0 g of cif3, cif1 and No-0 light grown, 11 day old seedling tissue as described (Sharrock and Quail, 1989). Poly(A)$^+$ RNA was purified from total RNA using Qiagen’s Oligotex® mRNA Mini Kit and protocol. RNA was quantified using a Spectronic® Genesys™5 spectrophotometer. Two sets of poly(A)$^+$ RNA (600ng and 440ng) and two sets of total RNA (35µg) from cif3, cif1 and No-0 were separated on a 1% FA gel (see Appendix A). Total and poly(A)$^+$ RNA blots were probed with $^{32}$P labeled fragments made from the 5’ and 3’ ends of the CIF3 cDNA, which are located on either side of the T-DNA insert. The 5’ probe was amplified from No-0 cDNA with primers (5’-TCTAAGCTTGGAGCCATGAACGAATCC-3’ with HindIII site) and (5’-AGAGGATCCGAACAAGATCACGCAGCAAA-3’ with BamHI site). The 3’ probe was amplified from No-0 cDNA with primers (5’-TCTGGATCCGCTCACCAGGAGGAACACTG-3’ with BamHI site) and (5’-AGAGGATCCGAACAAGATCACGCAGCAAA-3’ with HindIII site). The probe PCR fragments were first cloned into pUC18 and then used as templates to re-amplify the probe. This precaution was taken to reduce background from genomic DNA contamination. A 500bp 18S rRNA fragment was amplified from No-0 cDNA using upstream and downstream primers, respectively (5’-CTTGCTCTTAAAGGATTAAGCCATGCTGC-3’, 5’-ATACGCTATTGGAGCTGGAATTAC-3’). The Basta fragment described under Southern Analysis (above) was also used to re-probe the blot for the presence of this T-
DNA encoded transcript. All probes were labeled with \(^{32}\text{P}\) using the Ready-To-Go™ kit (Amersham Bioscience, UK).

**Cloning the CIF3 gene**

Col-0 WT genomic DNA was used as a template to PCR amplify the full-length CIF3 gene (Genbank accession # At1g73170) using the upstream and downstream primers respectively (5’-TCTGTCGACAAGTAAGCCATGGCACAACC-3’ with SalI site, 5’-TCTGGATCCGGAAGTCAAATTCCTACGAG-3’ with BamHI site). The PCR fragment was cut with BamHI and HindIII and ligated into pBI\(\Delta\)Gus #8, creating pBI-CIF3. pBI-CIF3 contains an insert of 4029bp comprising the full length CIF3 gene and 752bp of upstream promoter sequence. To make a gentamycin resistant binary vector for complementation analysis of the cif3 mutant (via *Agrobacterium* transformation) the polylinker from pUC18 was cloned into pPZPY122 (obtained from the Arabidopsis Biological Resource Center) with HindIII and SacI. The resulting vector, pGENT-NOS, contains the pUC18 polylinker in place of the pPZPY122 polylinker. The final transformation vector, pGENT-CIF3, was made by cloning the CIF3 gene from pBI-CIF3 into pGENT-NOS with BamHI/HindIII. This plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101.

See Appendix B for plasmid maps.
Complementation Analysis

Five mounded pots of the cif3 BCIII F₃ 2-1 line (8 plants/pot) were grown under 16/24hr fluorescent/incandescent light. They were transformed with pGENT-CIF3 in *Agrobacterium tumefaciens* strain GV3101 using the floral dip transformation method (Clough and Bent, 1998). T₁ seeds were selected on MS plates containing 100ug/ml gentamycin and 200ug/ml carbenicillin (see Appendix A), and then transferred to potting soil overlaid with vermiculite. T₂ seeds were collected from complemented plants and plated on MS Gent75, Carb200 (see Appendix A). T₂ plants were also germinated on GM and transferred to pots under 16/24hr fluorescent light. Complemented T₁ plants were PCR screened for the presence of the cif3 mutation using a primer in the cif3 gene (5’-CAGTTAGTCGCCACTGCTCA-3’) and a primer in the T-DNA (5’-AATTTCGCAGTCTTTCA-3’) to amplify a 568bp fragment.

Plant Physiology

No-0 WT, cif3 and cif1 lines were grown as described in growth conditions section. 24 plants each were placed into 16/24hr fluorescent and 24 each into 16/24hr fluorescent/incandescent light. Flowering times were calculated based on the number of days from placement of stratified seeds into the light at 20°C to when the first flower bud was observed.
Characterization of *CIF3*

The *CIF3* gene and gene product were characterized using The Arabidopsis Information Resource (TAIR) website. Phylogenic relationships and protein alignments were performed using CloneManager version 7.11 (Scientific and Educational Software).
RESULTS

Identification and Characterization of $cif3$

The $cif3$ mutant was isolated from an *Agrobacterium tumefaciens* T-DNA insertion screen performed in the laboratory of Dr. Sarah Hake at the USDA Plant Gene Expression Center (Albany, CA). The T-DNA region used for that screen (Fig. 1), from pGKB5, contains genes for Basta (herbicide) and Kanamycin (antibiotic) resistance and for the β-glucuronidase (GUS) gene, a colorimetric reporter gene.

![Diagram of the Agrobacterium T-DNA vector used originally for the promoter trap screen.](image)

Figure 1. Diagram of the *Agrobacterium* T-DNA vector used originally for the promoter trap screen.
The *cif3* mutant was identified initially as having reduced internode elongation between inflorescence internodes, leading to formation of flower clusters as opposed to the wild-type raceme. In this way, the *cif3* mutant is similar to the *cif1* mutant phenotype previously characterized in this laboratory (Goosey and Sharrock, 2001). Figure 2 shows examples of inflorescences of No-0 WT and *cif3*. The mutant has a very strong reduction in elongation of internodes both between co-florescence branches and between individual flowers on the primary and secondary stems, resulting in floral clusters. Nevertheless, flowers formed on the *cif3* mutant are phenotypically normal and are fully fertile. Figure 3 shows that *cif3* rosettes have a dark green color compared to No-0 WT control lines, however, they do not show the adult vegetative phase specific leaf expansion phenotype characteristic of the *cif1* mutant (Goosey and Sharrock, 2001).

The severity of the *cif3* phenotype is dependent on photoperiod and light quality. The *cif3* mutant flowers later and has a much stronger *compact inflorescence* phenotype under 16/24hr fluorescent light compared to growth under 16/24hr fluorescent/incandescent light (Fig. 4A, 4B and Table 1). A similar variation in the inflorescence elongation phenotype under different photoperiods and light conditions was observed for the *cif1* mutant (Goosey and Sharrock, 2001).

The original *cif3* mutant line, obtained from the Hake laboratory, was crossed to No-0 WT and the *cif3* phenotype segregated in a 3:1 ratio (WT:*cif*) in *F*₂ plants, indicating that *cif3* is a single, recessive mutation. Two further backcrosses to No-0 were performed and *cif3* BCIII *F*₃ progeny lines were identified. To investigate whether the
Figure 2. No-0 WT raceme (left) and the cif3 compact inflorescence phenotype showing floral clusters at the ends of primary inflorescence (right). A close-up of a cif3 floral cluster is shown (inset).

Figure 3. Rosette phenotypes of 36 day old No-0, cif3 and cif1 (left to right). Plants were grown under 16/24hr fluorescent light. The cif3 leaves are darker and narrower. The cif1 plants show an adult leaf expansion phenotype.
Figure 4A. Picture of cif3 grown under 16/24 hr fluorescent light showing a very strong cif3 phenotype.

Figure 4B. Picture of 32 day old No-0 WT, cif3 and cif1 plants grown under 16/24hr fluorescent/incandescent light, showing a very strong cif
T-DNA tag was tightly-linked to the \textit{cif3} phenotype, multiple \textit{cif3} BCIII F3 lines were tested for the presence of Basta sequence from the T-DNA (Fig. 5) and for resistance to kanamycin (Fig. 6). In all cases, complete co-segregation of the T-DNA with the \textit{cif3} phenotype was observed. To further analyze the structure of the T-DNA insert, Southern blot analysis was performed using GUS and Basta fragments from the T-DNA as probes. Figures 7 and 8 show that the T-DNA is associated with the \textit{cif3} phenotype and, moreover, that this insertion is likely not a single copy of the T-DNA but a complex multiple copy insert. Nevertheless, all of the BCIII F3 plants showed the same banding

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<tr>
<td>No-0</td>
<td>18 days</td>
<td>0.0</td>
</tr>
<tr>
<td>\textit{cif1}</td>
<td>19 days</td>
<td>0.0</td>
</tr>
<tr>
<td>\textit{cif3}</td>
<td>18 days</td>
<td>0.0</td>
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\textbf{Fluorescent/Incandescent: 16 hr photoperiod}

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<td>No-0</td>
<td>27.5 days</td>
<td>2.1</td>
</tr>
<tr>
<td>\textit{cif1}</td>
<td>33.2 days</td>
<td>1.8</td>
</tr>
<tr>
<td>\textit{cif3}</td>
<td>&gt;46 days</td>
<td>N/A</td>
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\textbf{Fluorescent: 16 hr photoperiod}

Table 1. Flowering time was calculated by counting the number of days between placing the seeds under light and the emergence of the first observable flower bud.
pattern on the Southern blot, suggesting that there were not multiple unlinked T-DNA insertion sites.

Figure 5. A 440bp Basta fragment was amplified from cif3 genomic DNA of ten independent BCIII F3 lines to show the presence of the T-DNA. No-0 WT was used as a negative control.

Figure 6. Five backcross III F3 cif3 lines and No-0 WT (asterisk) were planted on kanamycin plates. cif3 lines co-segregate with the T-DNA carrying kanamycin resistance.

Figure 7. Southern blot of five cif3 lines. Genomic DNA was cut with EcoRI or PstI (alternating) and probed with a 440bp Basta fragment (Fig.4A).

Figure 8. Southern blot of No-0 WT and cif3 lines. Genomic DNA was cut with EcoRI and PstI and probed with a 2kb GUS fragment.
Identification and Isolation of the CIF3 Gene

Since co-segregation data was consistent with the cif3 mutation being caused by a T-DNA insertion, efforts were directed toward cloning the T-DNA/genomic DNA border region in order to identify the CIF3 gene. A cif3 genomic library was constructed in lambda bacteriophage. Genomic DNA was extracted from cif3 plants and serially digested with Sau3AI to obtain an optimal insert size of 15kb (Fig. 9). The digested genomic DNA was treated with Klenow enzyme in the presence of dGTP and dATP to partially fill in the ends, then ligated into the T3 and T7 arms of lambda bacteriophage FIXII (Fig. 10A). The ligated fragments were packaged into viral coats. The titer of the primary library was determined on E. coli. Subsequently, the library was plated and screened by hybridization using a mixture of $^{32}$P labeled probes specific to the T-DNA. Positive plaques from this primary screen were re-screened by hybridization until every plaque on the plate was positive for the T-DNA (Fig. 10B). Pure plaques were grown to high titer in host cells and extracted. DNA was then isolated from the virus, cut with the blunt cutting enzyme EcoRV and subjected to TAIL-PCR (Thermal Asymmetric Interlaced PCR) (Fig. 11A). TAIL-PCR enables one to obtain DNA sequence beginning within a known region and extending into an unknown region. DNA is blunt cut and adaptors are ligated to the blunt ends. Adaptor primers (AP1 and AP2) anneal specifically to the adaptor, while gene specific primers (GSP1 and GSP2) were made from the known sequence of the T7 arm of lambda bacteriophage.
Nested sets of primers are used to re-amplify the PCR product to reduce background.

The sequence of the TAIL-PCR product was determined and captured the junction of the T-DNA and Arabidopsis genomic DNA (Fig. 11B). A Genbank BLAST search was used to find the Arabidopsis gene sequence flanking the T-DNA. The T-DNA was shown to be inserted in the fourth intron (2911bp downstream of the ATG) of a putative ATPase located on chromosome 1 of Arabidopsis thaliana (Fig. 12). The full-length wild-type CIF3 gene and promoter were PCR amplified and cloned.
A Packaging DNA into lambda phage to make a library

1. Digest genomic DNA with Sau3AI
2. Partial Klenow fill-in
3. Fragment is ligated into T3 and T7 arms of lambda.
4. Packaged as concatameric sequences into viral coats.

B

Figure 10. (A) Genomic DNA is cloned into Lambda Bacteriophage FIXII by cutting it into optimal size fragments (15kb), creating correct ends using Klenow fill-in reaction, and ligating it into the T7 and T3 arms of Lambda DNA. (B) The clone is then packaged into viral particles and grown to high titers on E. coli. A Primary screen (left) of the Lambda library and the final screen (right) show the purification of a single lambda plaque. Plaques were screened for the presence of the T-DNA using $^{32}$P labeled probes specific to the T-DNA.
Figure 11. (A) TAIL-PCR is used to amplify a fragment from a known area into an unknown area. Adaptors are ligated onto the ends of blunt cut DNA, and used as templates for adaptor primers (AP1 and AP2). Gene specific primers (GSP1 and GSP2) are made from a known sequence. Nested PCR reactions are used to re-amplify the original product, reducing background. (B) PCR products amplified from lambda DNA using AP1 and GSP1 were sequenced and revealed T-DNA insertion site.
Expression Analysis of *CIF3*

To characterize the expression levels of the wild-type *CIF3* gene and to determine the effect of the T-DNA insertion in the *cif3* mutant on expression of the gene, Northern blot analysis was performed on *cif3*, *cif1* and No-0 WT total and poly(A)\(^+\) RNA. Two hybridization probes were used: a 536bp 5’end probe (45-581 from the ATG) and a 632bp 3’ end probe (1279-1911 from the ATG). These two probes flank the T-DNA insertion site (Fig. 12). Poly(A)\(^+\) Northern blots show equal levels of expression of the *CIF3* mRNA in No-0 WT and in the *cif1* mutant with both probes (Fig. 13). Aberrant *CIF3* transcripts were detected in the *cif3* mutant using the 5’ probe, while *CIF3* transcripts were not detected at all in the *cif3* mutant using the 3’ probe (Fig. 13A and B).
These results demonstrate that the T-DNA insertion in the cif3 mutant disrupts the transcription and splicing of the gene and very likely causes a loss of function mutation. Control blots probed for 18S rRNA and for the Basta resistance gene transcript were included (Fig. 13).

Figure 13. Poly(A)$^\cdot$ Northern blots were probed with a 5’ CIF3 fragment (A) and a 3’ CIF3 fragment (B). An 18S rRNA probe was used as loading control. A 440bp Basta probe confirms the presence of this T-DNA encoded transcript in the cif3 RNA sample.
Description of the *CIF3* Gene Product

The protein product that is predicted to be encoded by the *CIF3* gene is a 666 amino acid long putative ATPase. This annotation is based upon the presence of sequence with high similarity to a Walker P-loop, a conserved region that can bind ATP/GTP. The protein is predicted to be soluble, as no trans-membrane domains are present, and it contains a 60 amino acid chloroplast localization sequence at its N-terminal end. Aside from these predicted functional domains, no further indications of the specific mechanisms or regulatory function of the protein are currently available.

Complementation Analysis

To confirm the identity of the *CIF3* gene, it was used to transgenically complement the *cif3* mutant phenotype. The full-length *CIF3* gene was cloned as described in the Methods section and inserted into *cif3* plants using *Agrobacterium* mediated transformation. Complementation (wild-type inflorescence) was seen in T₁ plants. T₂ seeds were collected from complemented T₁ plants and T₂ plants segregated in an approximate 3:1, wild-type:*cif3*, ratio (40/11) (Fig. 14A and B). PCR was used to confirm the presence of the *cif3* mutation in complemented T₁ plants (Fig. 14C).
Phylogenic Relationship of cif3

Homologues of the CIF3 gene were found in higher plants, algae and cyanobacteria. Arabidopsis contains a second gene (AT3g10420) that is related to CIF3 (AT1g73170). The two proteins can be aligned along their entire length, but share only 40% amino acid sequence identity (Fig. 15 and Table 2). Rice contains only a single CIF3-like gene (P0506C07.29). In higher plants, such as Arabidopsis and rice, these homologues are nuclear encoded and targeted to the chloroplast with a non-conserved signal peptide. CIF3-related genes are also found in more divergent organisms, including red algae, such as Porphyra purpurea (P51281) and cyanobacteria, such as Thermosynechococcus elongatus (ycf45). In red algae, the homologue is encoded in the chloroplast genome, whereas in cyanobacteria, the protein is likely cytosolic. A protein sequence alignment shows that the N-terminal regions of all of these phylogenetically diverse CIF3 homologues, which contain the Walker P-loop domain, are very highly conserved relative to the C-terminal halves of the proteins (Fig. 15A). The protein alignment was used to construct a phylogenic tree, showing relationships between cif3 and its homologues (Fig. 15B). Proteins were also aligned with each other to give percent similarity (Table 2).
Figure 14. (A) Segregation of the complementary CIF3 transgene in the T2 progeny from T1 complemented plants. Complemented transgene-containing T2 plants show elongated inflorescences whereas T2 plants lacking the transgene show strong cif3 phenotypes. (B) Close-up of cif3 phenotype seen in segregating T2 population. (C) PCR screen for the cif3 T-DNA in complemented T1 plants. cif3 and No-0 WT genomic DNA were used as positive and negative controls, respectively.
Table 2. Percent amino acid identity between each cif3 homologue was calculated.

Figure 15. (A) A protein alignment shows amino acid similarities (box) between cif3 and homologous proteins. (B) A phylogenetic tree shows similarity between cif3 and homologous proteins. Shorter lines indicate a closer relationship.
Inflorescence architecture plays an important role in natural populations of plants due to its integral role in pollination and plant mating, but it also plays a major role in agriculture because it can affect desirable harvesting traits and influence yield. However, little is known of the genetic mechanisms controlling inflorescence architecture. In the model organism *Arabidopsis thaliana*, two vegetative growth phases precede the reproductive phase. The vegetative phases, juvenile and adult, produce a rosette in which leaves are arranged in a spiral phyllotaxy with very little internode elongation between the leaves. The juvenile growth phase begins with the first true leaves formed after the cotyledons and continues through the fifth to eighth leaf, depending on the growth conditions. A transition from juvenile to adult vegetative growth is characterized by the competency of the rosette to produce an inflorescence meristem, which gives rise to floral meristems that have the ability to produce the reproductive organs, or flowers. In *Arabidopsis*, the inflorescence is described as a raceme, in which the flowers are produced on short pedicels attached to a long main stalk. In the raceme, internodes elongate to a much greater extent than in the rosette. In this thesis I describe a mutant of *Arabidopsis, compact inflorescence 3 (cif3)*, which exhibits reduced elongation of floral internodes, leading to a densely packed cluster of flowers compared to the wild-type raceme. The *CIF3* gene has been cloned using forward genetics and the *CIF3* gene product has been identified as a chloroplast localized putative ATPase.
The severity of the cif3 trait is dependent on light conditions. The most severe form of the phenotype is seen under 16/24hr fluorescent light, where very little elongation of any stem components occurs and the floral clusters form within the rosette. Under this condition, the rosette leaves are darker green and the leaves appear more narrow than the wild-type (Figs. 3 and 4). In contrast, when grown under 16/24hr fluorescent/incandescent light, the cif3 internodes elongate, leading to taller plants and a less compact inflorescence. Nonetheless, the terminal internodes between individual flowers do not expand and clusters of flowers form at the ends of the inflorescence stems. These results indicate that there may be a response to alterations in red/far red ratio associated with cif3. Flowering time of cif3 relative to wild-type is also affected by light conditions, with the mutant flowering at the same time as wild-type under fluorescent and incandescent but flowering later than wild-type under fluorescent only (Table 1).

The cif3 mutant has a similar phenotype to the previously described mutant, cif1 (Goosey and Sharrock, 2001). However, the cif1 mutation has been shown to have a growth-phase specific effect, resulting in altered adult vegetative leaves whereas cif3 does not have this phase-specific phenotype (Fig. 3). The rosette of cif1 resembles that of the wild-type rosette, with the exception of the adult leaf phenotype, and does not have a darker color or narrower leaves. cif1 has been shown to be a recessive mutation in the ACA10 gene, which encodes a P-type IIB Ca\(^{2+}\) ATPase (L. George, unpublished). In contrast to the single recessive mutation of cif3, expression of the cif1 phenotype requires the action of a specific allele of a naturally occurring dominant modifier gene CIF2,
which occurs in the *Arabidopsis* ecotype No-0, but not Col-0 (Goosey and Sharrock, 2001). It is possible, because of the similarities between the *cif1* and *cif3* phenotypes, that the *CIF3* encoded chloroplast localized ATPase and the *CIF1* encoded plasma membrane bound Ca\(^{2+}\) ATPase may be components of the same inflorescence architecture development pathway. Alternatively, they could be components of separate pathways which have parallel roles in plant development.

The *cif3* mutant was found in the T\(_2\) progeny of a T-DNA insertion screen. Problems can arise when cloning genes associated with a T-DNA insertion because mutations can occur that are not associated with the T-DNA and therefore it is important to show co-segregation of the T-DNA with the mutant phenotype. Two Southern blots probed with T-DNA sequences, PCR assays for the T-DNA, and a kanamycin resistance assay all indicated that the *cif3* gene is tagged. However, the insertion appears to be complex. An initial TAIL-PCR reaction using genomic *cif3* DNA only amplified T-DNA sequences, indicating a rearrangement or duplication of some kind during its integration into the genome. To solve this problem and clone the T-DNA insertion site, a *cif3* genomic library was constructed in lambda bacteriophage. Following isolation of lambda clones containing T-DNA sequences, TAIL-PCR was able to capture the junction of T-DNA and *Arabidopsis* genomic DNA. The T-DNA was shown to be inserted in an intron of a chloroplast localized putative ATPase gene with no known function.

Northern blot analysis was used to determine the expression levels of the *CIF3* gene in No-0 WT, *cif1* and *cif3* (Fig. 13). Probes were made to flank the T-DNA
insertion site to see if an altered message could be detected. The blot probed with the 5’ CIF3 fragment showed no difference between No-0 and cif1, but gave a number of large and small bands in the cif3 lane. These results indicate that the T-DNA is not correctly spliced from the intron and that an aberrant cif3 transcript is made. It is not known whether this mutant transcript is translated into a protein. The blot probed with the 3’ CIF3 fragment also showed equal expression in No-0 and cif1, but showed no expression in cif3. This result shows that the CIF3 gene is very likely functionally knocked out in the cif3 mutant.

To confirm the identity of cif3, a transgenic complementation test was done. The full-length wild-type CIF3 gene plus 752bp of promoter was transformed into cif3 plants and the phenotype was restored to wild-type in T1 plants. T2 plants segregated in an approximate 3:1 ratio, again confirming the recessive mutation in CIF3.

Annotation of the Arabidopsis genome identifies the CIF3 gene product as a chloroplast localized putative ATPase with no known function. The proposed ATPase function is based upon the presence of a glycine rich Walker P-loop ATP/GTP binding domain. A putative chloroplast localization domain is located on the N-terminal end and contains approximately 60 amino acids. The cif3 protein has not been directly shown to localize to the chloroplast, however there is evidence indicating likely plastid localization. CIF3 gene homologues have been found in Arabidopsis, rice, cyanobacteria and red algae. The cif3 protein was shown to be most closely related to the rice homologue (44% sequence identity), followed by Arabidopsis (40%), cyanobacteria
(39%) and red algae (35%). In the higher plants, *Arabidopsis* and rice, the homologues are nuclear encoded and predicted to be targeted to the chloroplast. In red algae, the homologue is encoded on the chloroplast genome and does not contain a targeting sequence. The cyanobacterium homologue is encoded in the genome and does not contain a targeting sequence. These data indicate that the progenitor *CIF3* gene started out being encoded in a prokaryotic genome and ended up in the chloroplast of algae through the endosymbiotic origin of these organelles. As higher plants evolved, the gene moved to the nucleus and the protein gained a chloroplast targeting sequence. The dark color of the *cif3* rosette and apparent increase of chloroplasts in *cif3* cells (J. Cameron, unpublished) may also be an effect of the loss of function *cif3* mutation. To confirm that the *CIF3* gene product is targeted to the chloroplast, a *CIF3::GFP* translational fusion will be constructed and monitored in transgenic plants.

The results presented in this thesis may provide insights into the pathways involved in inflorescence architecture development in *Arabidopsis*. Both the *Arabidopsis cif3* and *cif1* mutants exhibit similar clusters of flowers and lack of inflorescence internode elongation. The *CIF1* gene product is a plasma membrane Ca$^{2+}$ pump, whereas the *CIF3* gene product is predicted to be a soluble protein localized to the chloroplast but of unknown function. It is unclear at this time whether *CIF1* and *CIF3* act in the same regulatory pathway. If so, it would suggest that Ca$^{2+}$ ion signaling and chloroplast function may help to regulate plant reproductive development and the architecture of the inflorescence. Further studies may reveal the relation of the *CIF3* encoded protein with
the *CIF1* gene product and give insight into the genetic pathways involved in inflorescence development.
REFERENCES CITED
References Cited


APPENDIX A

ADDITIONAL INFORMATION
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Media and Plant Growth Materials

GM Medium:
900 mls deionized water
4.4 g M-S Basal Salts (Sigma)
20 g sucrose
0.5 g MES
Final volume: 1 liter
Add 8 g Agar Type E for solid medium

MS Solid Medium:
Same as GM, but without sucrose.

Selection Media:
Use GM or MS medium
Add Antibiotic to appropriate concentration.

Nutrient Solution:
5.0 mls 1M KNO₃
2.5 mls 1M K₂PO₄
2.0 mls 1M MgSO₄
2.0 mls 1M Ca(NO₃)₂
2.5 mls 20mM Fe EDTA
1.0 ml micronutrients
Water to final volume: 1 liter

Mounded Pots;
Fill 4 inch square pots with soil drenched in nutrient solution.
Overlay with handful nutrient soaked vermiculite.
Cover top with mesh.

Northern Blot Analysis Solutions

10X FA Gel Buffer:
200mM 3-[N-Morpholint]propanesulfonic acid (MOPS) (free acid)
50 mM Sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

Formaldehyde Gel Composition (FA Gel):
1% agarose
0.66 M formaldehyde
1X FA Buffer
APPENDIX B

PLASMID MAPS
APPENDIX B

PLASMID MAPS
pBldelta GUS

12081 bps

NheI

ApaI

HindIII

SalI

XbaI

XhoI

EcoRI

KpnI

SmaI

NheI

BamHI

SacI

NdeI

left border

Tn

right border

NPT II
pBI-CIF3
16007 bps

Apa I
HindIII
SpeI
BamHI
SacI
NotI
RB
NPT II
Apal
HindIII
LB
Tn
'ATPase'

ScaI
BamHI
SpeI
pGENT-NOS
8891 bps

ClaI
NcoI
SacI
KpnI
Smal
BamHI
XbaI
SalI
PstI
HindIII

EcOrI

NOS
'lacZ'

LB
35S
gentR

CmR

RB

Clal
pUC18
2686 bps