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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APPROVAL

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Jeffrey Carlyle Cameron

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Jeffrey Carlyle Cameron

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TABLE OF CONTENTS

LIST OF FIGURES

TABLES

ABSTRACT

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* (*cif*1) mutant (Goosey and Sharrock, 2001). The *cif*1 trait was shown to require altered alleles of two genes; a recessive mutation at the *cif*1 gene and a naturally occurring unlinked dominant allele, *CIF2*. Although the phenotypes of *cif*1 and the new mutant are similar, complementation tests show that they are different genes, and the new mutant is designated *cif*3. The *cif*3 mutation is recessive and, unlike the *cif*1 mutation, does not require the presence of a dominant allele of the *CIF*2 gene to cause the inflorescence phenotype. Moreover, the *cif*1 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, *cif*3 does not show an effect on adult leaves and therefore does not show apparent phase-specific expression. The *cif*3 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 T_2 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 T$_2$ 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 F_3 #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'-TCTAAGCTTGAGCCATGAACGAAGAATCC-3' with

HindIII site) and (5'-AGAGGATCCGAACAAGATCACGCAGCAAA-3' with *BamHI*

site). The 3' probe was amplified from No-0 cDNA with primers (5'-

TCTGGATCCGCTCACCAAGGAGGAAACTG-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'-CTTGTCTCAAAGATTAAGCCATGC-

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}P using the Ready-To-Go™ kit (Amersham Bioscience, UK).

<div align="center">Cloning the CIF3 gene</div>

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 pBIΔGus #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 F_3 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). T_1 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. T_2 seeds were collected from complemented plants and

plated on MS Gent75, Carb200 (see Appendix A). T_2 plants were also germinated on

GM and transferred to pots under 16/24hr fluorescent light. Complemented T_1 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-

AATTTCGCACTCAGTCTTTCA-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.

<u>Characterization of *CIF3*</u>

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

<u>Identification and Characterization of *cif3*</u>

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 ß-glucuronidase (GUS) gene, a colorimetric reporter gene.
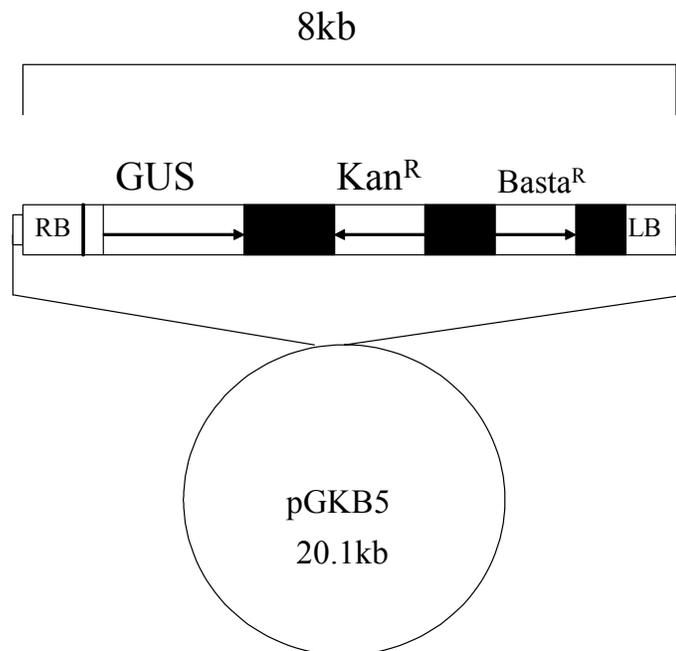
Figure 1. Diagram of the *Agrobacterium* T-DNA vector used originally for the promoter trap screen.

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
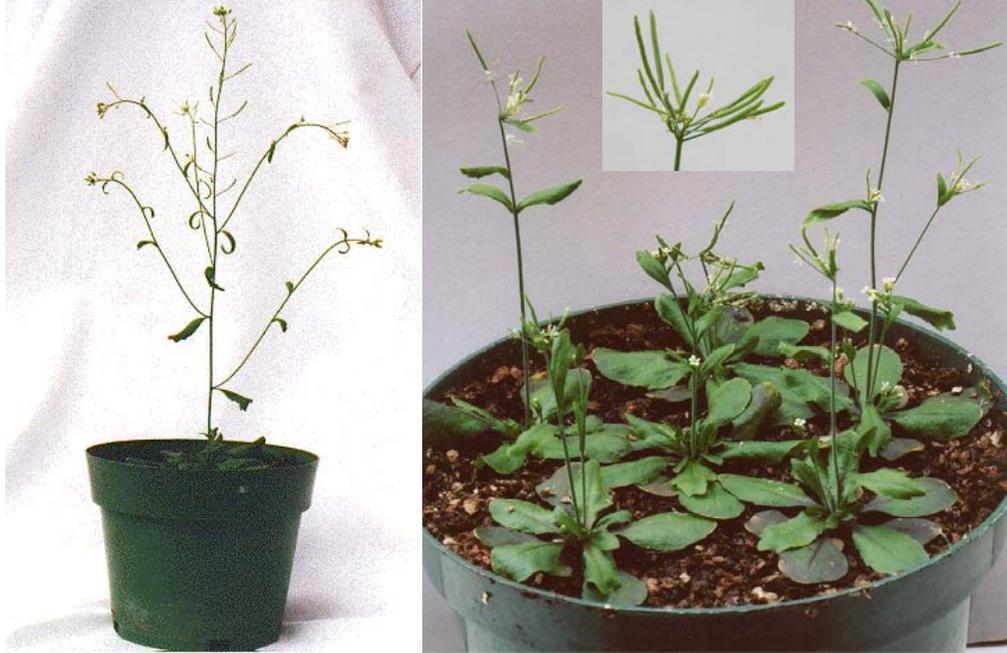
Figure 2. No-0 WT raceme (left) and the *cif3 compact inflorescence* phenotype showing floral clusters at the ends of primary inflorescence (right). A close-up of a *cif3* floral cluster is shown (inset).

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.
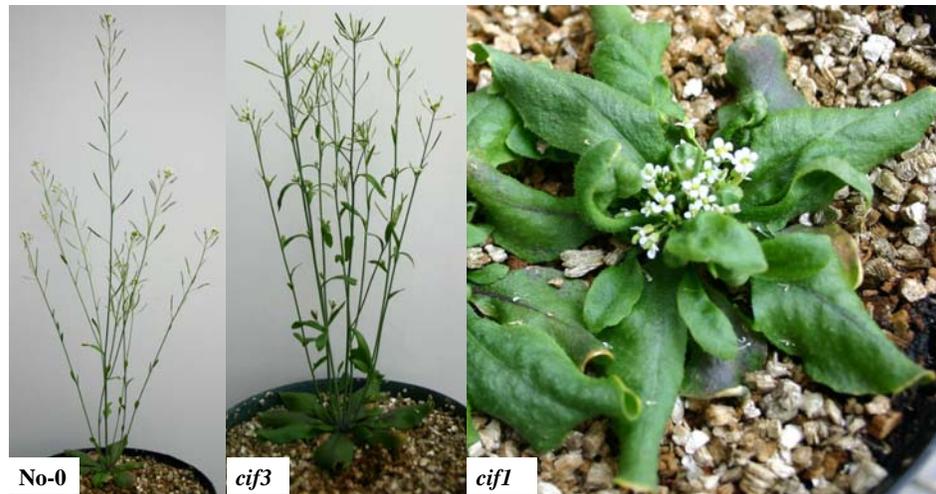

Figure 4B. Picture of 32 day old No-0 WT, *cif3* and *cif1* plants grown under 16/24hr fluorescent/incandescent light, showing a very strong *cif*

Fluorescent/Incandescent: 16 hr photoperiod

	Mean	Std. Dev.
No-0	18 days	0.0
cif1	19 days	0.0
cif3	18 days	0.0

Fluorescent: 16 hr photoperiod

	Mean	Std. Dev.
No-0	27.5 days	2.1
cif1	33.2 days	1.8
cif3	>46 days	N/A

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.

T-DNA tag was tightly-linked to the *cif3* phenotype, multiple *cif3* BCIII F$_3$ 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 *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 *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 F$_3$ plants showed the same banding

pattern on the Southern blot, suggesting that there were not multiple unlinked T-DNA insertion sites.
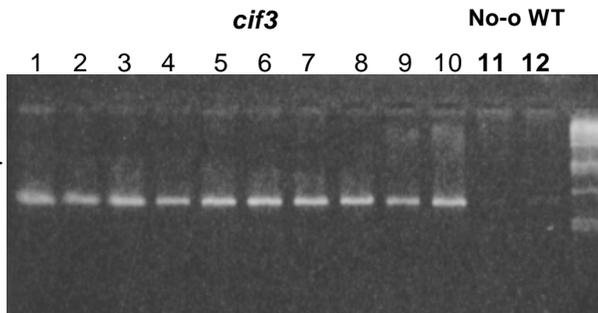
Figure 5. A 440bp Basta fragment was amplified from *cif3* genomic DNA of ten independent BCIII F_3 lines to show the presence of the T-DNA. No-0 WT was used as a negative control
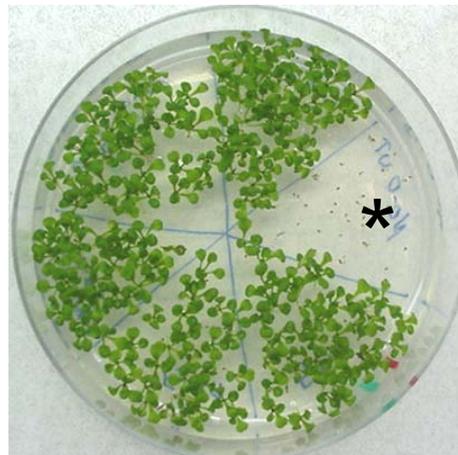
Figure 6. Five backcross III F_3 *cif3* lines and No-0 WT (asterisk) were planted on kanamycin plates. *cif3* lines co-segregate with the T-DNA carrying kanamycin resistance.

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

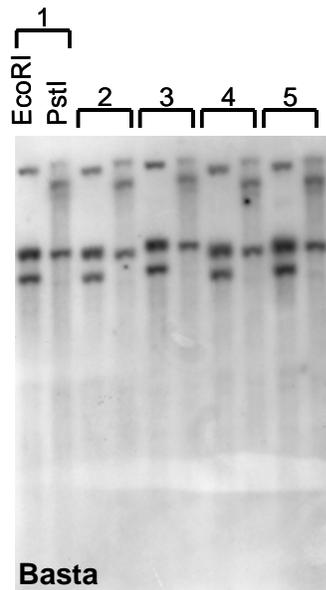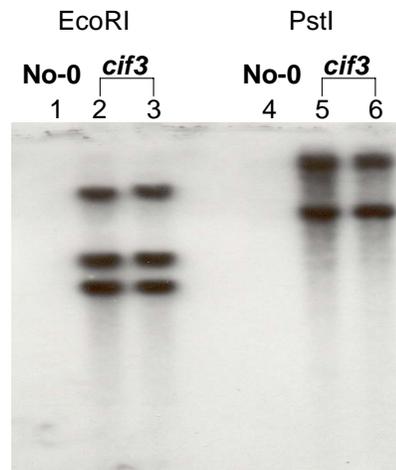Since co-segregation data was consistent with the *cif3* mutation being caused by a

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.
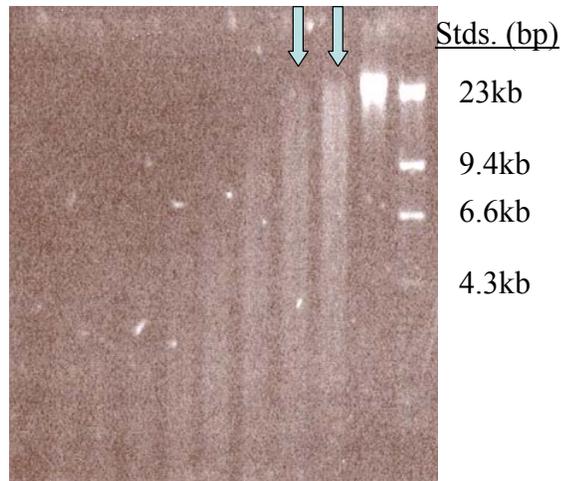
Figure 9. *cif3* genomic DNA was digested with dilutions of *Sau3AI* to obtain fragments approximately 15kb (fractions containing optimal size fragments are shown with arrows). A *HindIII* digest of λ was used as a size standard.

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.

T3   T7

T3   T7

4. Packaged as concatameric sequences into viral coats.

B
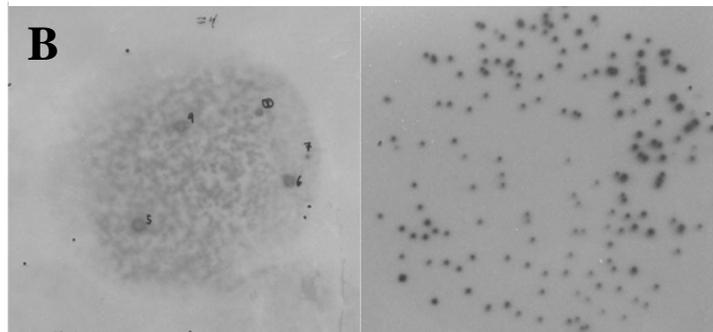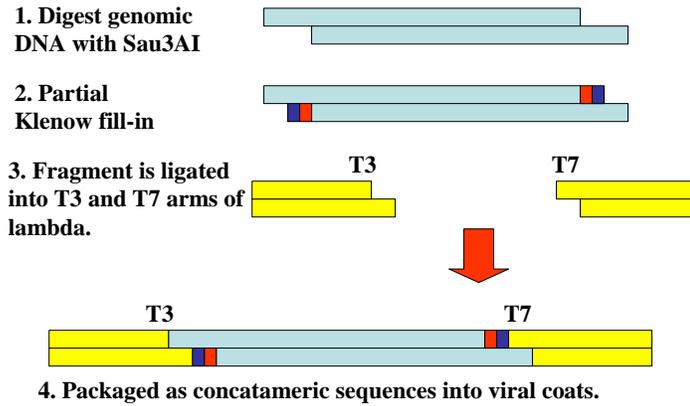
Figure 10. (A) Genomic DNA is cloned into Lambda Bacteriophage FIXII by cutting it into optimal size fragments (15kb), creating correct ends using Klenow fill-in reaction, and ligating it into the T7 and T3 arms of Lambda DNA. (B) The clone is then packaged into viral particles and grown to high titers 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.

A   TAIL-PCR
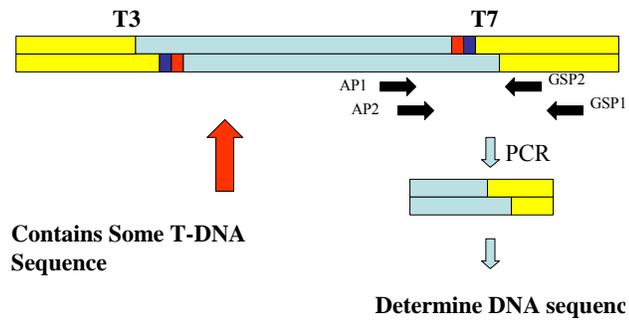
B
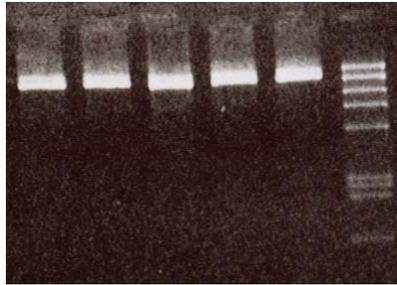
Figure 11**.** (A) TAIL-PCR is used to amplify a fragment from a known area into an unknown area. Adaptors are ligated onto the ends of blunt cut DNA, and used as templates for adaptor primers (AP1 and AP2). Gene specific primers (GSP1 and GSP2) are made from a known sequence. Nested PCR reactions are 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.
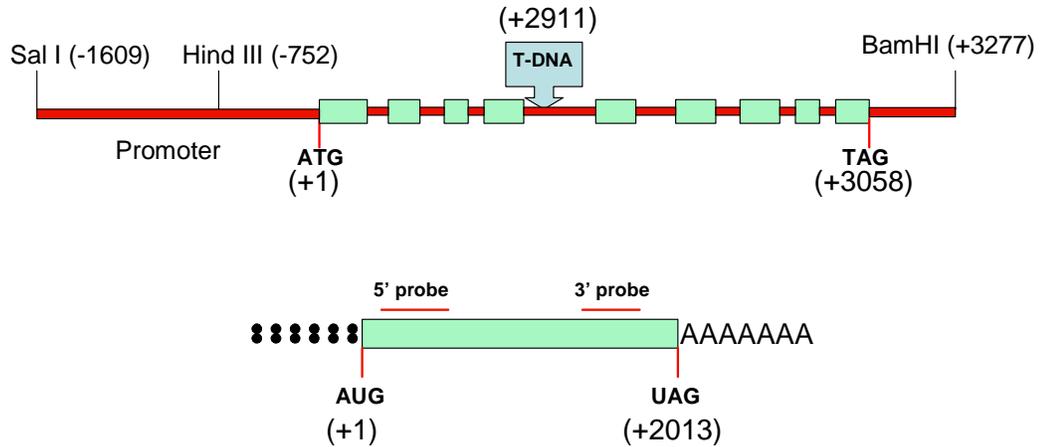
Figure 12. Diagram of the full-length *CIF3* gene with promoter and insertion site of T-DNA (top). The *CIF3* mRNA with the location of 5' and 3' probes (which flank the T-DNA) used in Northern blot analysis (bottom).

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. 1**2**). 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).
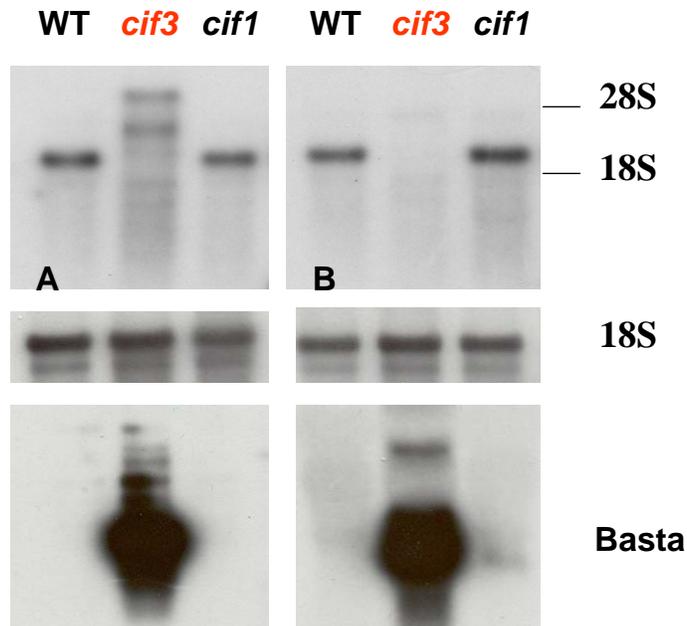
Figure 13. Poly(A)$^+$ Northern blots were probed with a 5' *CIF3* fragment (A) and a 3' *CIF3* fragment (B). An 18S rRNA probe was used as loading control. A 440bp Basta probe confirms the presence of this T-DNA encoded transcript in the *cif3* RNA sample.

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).

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 T_1 plants. T_2 seeds were collected from complemented T_1 plants and T_2 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 T_1 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).
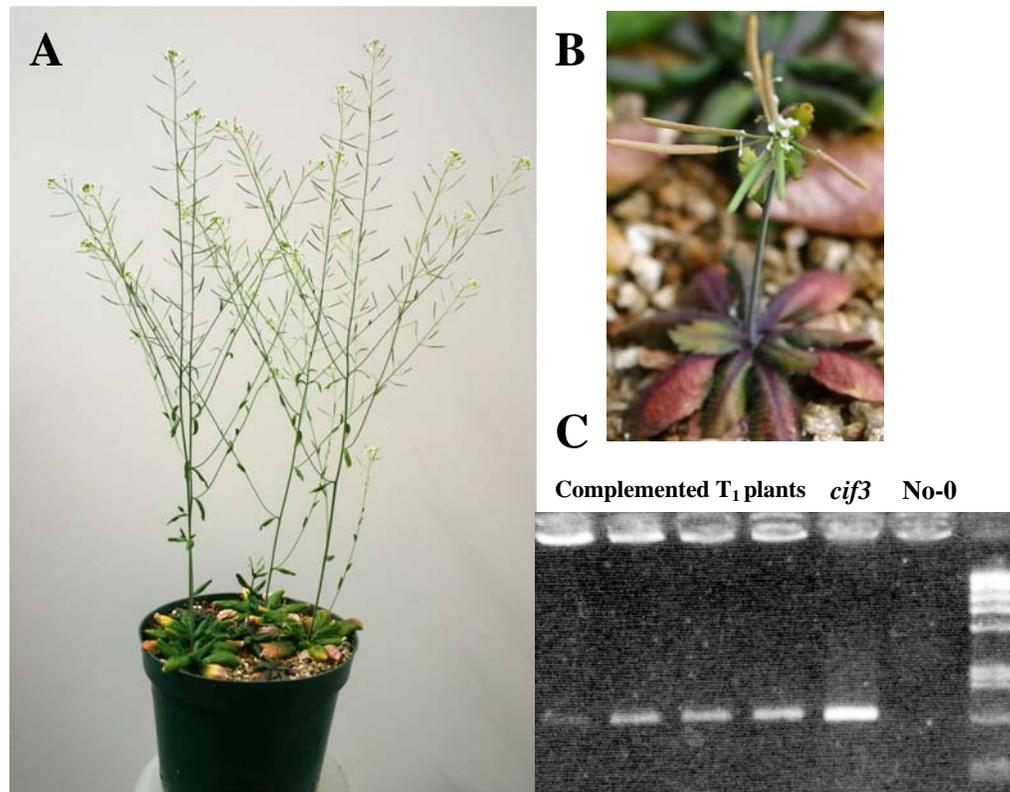
Figure 14. (A) Segregation of the complementary *CIF3* transgene in the T$_2$ progeny from T$_1$ complemented plants. Complemented transgene-containing T$_2$ plants show elongated inflorescences whereas T$_2$ plants lacking the transgene show strong *cif3* phenotypes. (B) Close-up of *cif3* phenotype seen in segregating T$_2$ population. (C) PCR screen for the *cif3* T-DNA in complemented T$_1$ plants. *cif3* and No-0 WT genomic DNA were used as positive and negative controls, respectively.
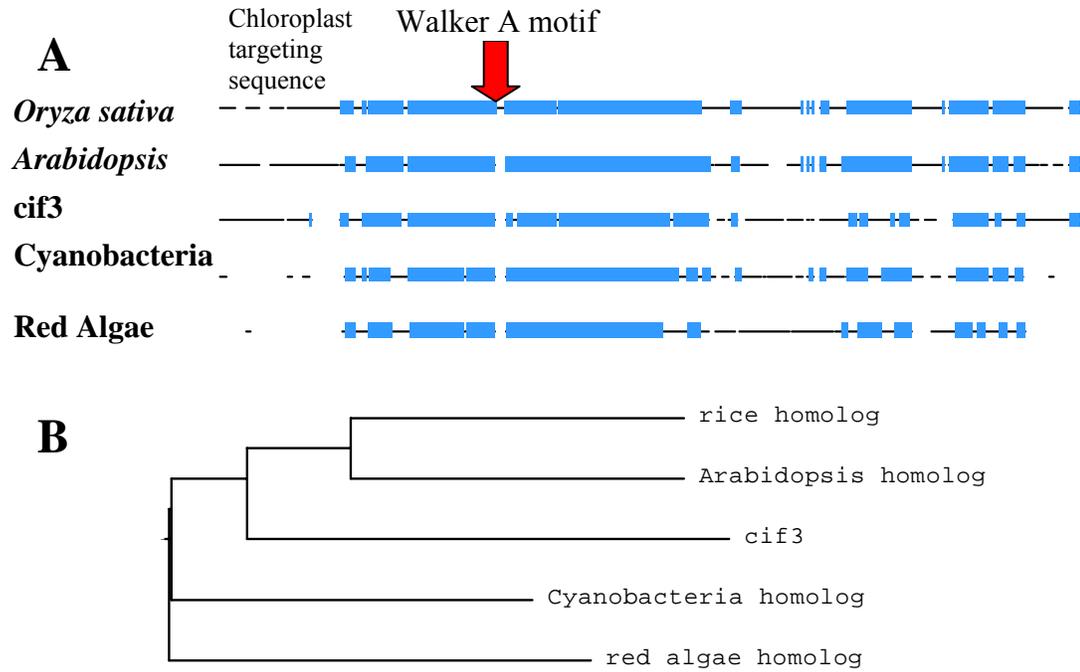
Figure 15. (A) A protein alignment shows amino acid similarities (box) between cif3 and homologous proteins. (B) A phylogenic tree shows similarity between cif3 and homologous proteins. Shorter lines indicate a closer relationship.

	cif3	Arabidopsis	Rice	Cyanobacteria	Red Algae
cif3	—				
Arabidopsis	40	—			
Rice	44	55	—		
Cyanobacteria	39	44	39	—	
Red Algae	35	39	37	53	—

Table 2. Percent amino acid identity between each cif3 homologue was calculated.

DISCUSSION

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 T_1 plants. T_2 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.

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APPENDICES

APPENDIX A

ADDITIONAL INFORMATION

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ADDITIONAL INFORMATION

<u>Media and Plant Growth Materials</u>

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 KNO_3
2.5 mls 1M K_2PO_4
2.0 mls 1M $MgSO_4$
2.0 mls 1M $Ca(NO_3)_2$
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.

<u>Northern Blot Analysis Solutions</u>

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

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PLASMID MAPS

SacI
EcolCRI
BanII
KpnI
Acc65I
XmaI
SmaI
BamHI

NOS RB

'lacZ''

12000

2000

ATPase

pGent-CIF3

10000

12862 bps

4000

8000

6000

35S

CmR

gentR

HindIII

LB

SacI
SalI
KpnI
SmaI
BamHI
XbaI

EcoRI

PstI
HindIII

NOS

RB

35S

35S

gentR

LB

pPZPY 122

9742 bps

8000

2000

6000

4000

Cm R

NheI

pBIdelta GUS

12081 bps

12000

10000

2000

8000

4000

6000

right border

NheI

NPT II

ApaI

NdeI

left border

Tn

HindIII

NheI

BamHI

SacI

EcoRI

SalI
XbaI
XhoI
EcoRI
KpnI
SmaI

NotI

RB

NPT II

ApaI

pBI-ClF3

16007 bps

16000

14000

2000

12000

4000

10000

6000

8000

HindIII

'ATPase'

LB

Tn

SacI
BamHI
SpeI

pBI101.1b

13914 bps