

**THE MECHANISM OF CHLOROPLAST DIVISION IN HIGHER
PLANTS**

A Senior Honors Thesis

By

JOHN MICHAEL PROCTOR

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University

In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2000

Group: Cell Biology 2

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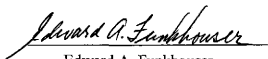
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April 2000

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ABSTRACT

The Mechanism of Chloroplast Division in
Higher Plants. (April 2000)

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The majority of plant cells contain plastids that are self-replicating, double membrane organelles with their own unique genetic component. The current investigation concerns the developmental events that strictly determine the number of chloroplasts found in a normal mesophyll cell. The process of chloroplast biogenesis has been well characterized at the cellular level, but the molecular basis of chloroplast division and the role of nuclear genes in the control of plastid division and maturation are poorly understood. In young developing leaf cells proplastids number approximately 10 per cell. As the leaf continues to develop these proplastids divide and develop concomitantly until roughly 65 to 100 chloroplasts are present in each mature mesophyll cell. A gene controlling chloroplast division has been identified in *Arabidopsis thaliana*, and its inactivation results in a large decrease in the number of chloroplasts per cell. Because of the complexity of leaf development in *Arabidopsis* and other dicots, chloroplast division studies are typically very difficult. Unlike *Arabidopsis*,

understanding the chloroplast division process in a grass species, such as rice, is greatly facilitated by the direct spatial and temporal relationship between chloroplast biogenesis and leaf development. To extend the body of knowledge of chloroplast division into the grass species, the *Arabidopsis* gene was used to isolate the corresponding gene in rice, *Oryza sativa*. To verify the function of this rice gene, it was introduced into the *Arabidopsis* mutant, characterized by a decreased number of chloroplasts, in order to reinstate chloroplast biogenesis in the mutant. A plasmid construct containing an antisense version of the gene and the hygromycin gene (used as a selectable marker) was then introduced into rice callus in order to knock-out production of the protein involved in chloroplast division. A phenotypic analysis of the resultant rice plants revealed that there was no significant reduction in chloroplast number. To determine gene expression patterns, mRNA was isolated from mature rice tissue and analyzed via the northern blot method. A Southern analysis of genomic DNA was performed to quantitate the number of integration sites of the antisense gene.

To Mom, Dad, Jackie, and Ryan: You are the best parents and friends a guy could have. Thank you for your comfort and support over the years and know that all of you fill me with inspiration and happiness. I love you all.

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INTRODUCTION

Chloroplasts are a member of a large family of plant cell organelles known as plastids. Plastids are important because they house a number of metabolic pathways crucial for plant growth and development. They are thought to have arisen after an endosymbiotic event in which a primitive eukaryotic host enveloped a photosynthetic prokaryote (Margulis, 1970; Gray 1992). Plastids have subsequently evolved to be an integral part of all plant cells. Their role consists of providing energy to the plant cell by capturing light from the sun and converting it to chemically usable forms of energy via carbon assimilation. Furthermore, chloroplasts have also been shown to be important in the synthesis of various amino acids, lipids, and plant growth regulators (Mullet, 1988).

Chloroplasts, as all plastids do, come from proplastids that are self-replicating, double membrane organelles. In young developing leaf cells proplastids number approximately 10 per cell. As the leaf develops, these proplastids divide by fission and develop until roughly 65 chloroplasts are present in each mature mesophyll cell (Mullet, 1988). Although chloroplasts have their own genetic material that codes for approximately 80 proteins along with ribosomal and translational RNA, it is only through the strict interplay with proteins coded by nuclear genes that insure proper development and biochemical function within the chloroplast.

This thesis follows the style and format of *The Plant Cell*. It should be noted that in this journal the methods section is the last section of the manuscript. However, formatting limitations imposed for this thesis require the conclusion section to be last.

The cellular process of chloroplast biogenesis has been well characterized by light and electron microscopy (Leech, 1981), but the molecular basis of chloroplast division and the role of nuclear genes in the control of chloroplast division and maturation are poorly understood. Mutants that show a variance in the number of chloroplasts per cell have allowed researchers to identify genes whose proteins are believed to play a role in the process of division. Much of the current research has been performed on the model dicot plant, *Arabidopsis thaliana* (Pyke and Leech, 1991, 1992, 1994; Pyke 1997). Characterization of the accumulation and replication of chloroplasts (*arc*) mutants has demonstrated that specific nuclear genes play specific roles in chloroplast division and development. Furthermore, homologs to genes involved in bacterial division such as the filamentous temperature-sensitive (*fts*) genes and the *min* genes found in the *E. coli* *minicell* locus have also been found in the nuclear genome of *Arabidopsis* and indicated in chloroplast division (Osteryoung et al., 1998; Osteryoung and Pyke 1998).

Chloroplast division studies are typically very difficult using members of the dicot family because the timing of the development of the chloroplast is not directly related to the timing of the development of the leaf. Conversely, studying chloroplast division in a monocot such as rice, *Oryza sativa*, is easier because chloroplast development is directly proportional to the temporal stage of leaf development. As the leaf proliferates and matures from the primordial shoot, the chloroplasts divide and mature at a rate necessary to meet the energy demands of the developing leaf. Thus, all stages of chloroplast maturation are strictly defined in a single leaf.

In order to study the role of a nuclear gene in chloroplast division, a method must be chosen that can elucidate the function of the gene in both wild-type plants and plants that have a malfunctioning gene hindering chloroplast division from proceeding normally. The obvious method of choice is transformation, the introduction of foreign DNA into the host's genome to effect a change in its phenotype, since it is the only direct method of over-expressing the product of a gene or silencing the gene using antisense transformation.

Multiple methods for introducing foreign DNA into the genome of plants include PEG treatment, electroporation, biolistics, *Agrobacterium*-mediated, and a variety of others (Tyagi et al., 1999). For many years *Agrobacterium*-mediated transformation has been the method of choice for dicotyledonous species since *Agrobacterium tumefaciens* is a normal parasite of those species and because the other above mentioned methods have been associated with integration of DNA of varying lengths and copy number (Dong et al., 1996). Only recently has *Agrobacterium*-mediated transformation been identified as an efficient method for introducing DNA into monocotyledonous tissues (Aldemita and Hodges, 1996; Hiei et al., 1994, 1997), which do not normally host *A. tumefaciens*. *Agrobacterium*-mediated transformation has the inherent advantages of random integration of transgenes into the host chromosomes, Mendelian segregation of transgenes in progeny, and simple technical procedures.

The current investigation seeks to characterize the function of a novel gene in *Oryza sativa* homologous to a gene, *cdm1* for chloroplast division mutant, recently found in *Arabidopsis* and known to function in chloroplast division (Morishige and

Mullet, unpublished data). After isolation of the genomic DNA from a BAC (bacterial artificial chromosome) library and retrieving the cDNA from a cDNA pool made from polyadenylated mRNA, the *Arabidopsis* mutant from which the gene was originally identified was transformed via *Agrobacterium*-mediated transformation with the cDNA of the gene from rice to reinstate chloroplast division. Visual analysis indicated that chloroplast division was reinstated signifying that this gene from rice also functions in chloroplast division. Subsequently, *Agrobacterium*-mediated transformation of rice callus was employed to knock-out normal gene function in wild-type *Oryza sativa* via an antisense construct of the rice gene. After verification that the transformation was successful, a phenotypic analysis of the transformed plants via light microscopy showed no change in chloroplast number per cell. A northern and Southern blot analysis of these transformed plants was employed to rationalize the observed phenotypic result.

METHODS

Plant Material

The *Arabidopsis thaliana* ecotype Wassilewskija chloroplast division mutant 1 (*cdm1*) was used for the initial investigation of the function of this novel gene from rice. Seeds were sown on MS medium containing 1% sucrose, 0.5g/L MES, 0.8% agar. Seeds were placed at 4 C for 36 hours in the dark and then placed in continuous light at 20 C in a controlled environment chamber. After the first leaves appeared, each seedling was transferred to Scotts MetroMix 360® soil.

Taipei 309 *Oryza sativa* seeds were used as experimental material for the rice transformation experiment. Following a modified protocol from Aldemita and Hodges

(1996), the seeds were dehusked and washed with 70% ethanol for 3 minutes. After removing the ethanol, the seeds were treated with 70% sodium hypochlorite and Tween-20 under vacuum for 5 minutes and then without vacuum for one hour. Lastly, the seeds were rinsed three times with distilled water before use.

Isolation of Full-Length cDNA From *Oryza sativa*

Total RNA was isolated according to the standard protocol. Poly(A)⁺ RNA was then isolated from the total RNA using the FastTrack® 2.0 mRNA Isolation Kit (Invitrogen). Subsequently, a pool of cDNA was synthesized using the SuperScript™ II RNase H⁻ Reverse Transcriptase (GibcoBRL). Primers flanking the region of homology to this novel gene in rice were designed using an expressed sequence tag (EST) (accession number D46985), generated using the *cdm1* cDNA sequence, and used to amplify the corresponding region of *Oryza sativa* cDNA. The primers had the following sequences: forward primer, 5'-TTTCTGTGACCGTCCAAAGGATCAGAGGCC-3'; reverse primer, 5'-CTCAATCTTCTTCTCCAGCGTCAATGGAGC-3'. The sequence of the reverse primer was derived by first sequencing a cDNA fragment, obtained with a non-specific reverse primer, with the forward primer so that a highly specific cDNA product could be obtained. The amplified cDNA fragment was isolated on a low-melting agarose gel, purified via the QIAquick Gel Extraction Kit protocol from QIAGEN, radiolabeled with ³²P-dCTP by the random primer method, and used as a probe to screen the Teqing rice BAC library from the Texas A&M University BAC Center. Prehybridization and hybridization were carried out in Dupont/NEN hybridization buffer at 65 C. BAC 21E24 was identified as a positive clone and isolated according to the standard protocol.

A Southern blot was prepared with the Dupont/NEN Salt Transfer protocol and probed with the cDNA probe described above to confirm that the identified BAC contained a genomic sequence of this novel rice gene and that the probe was not hybridizing to BAC DNA. The genomic DNA from BAC 21E24 was then sequenced and used to design primers to amplify the full-length cDNA from the cDNA pool described above. The primers had the following sequences: forward primer, 5'-CCTCGCGAATTCGCCGTGGAACACC-3'; reverse primer, 5'-TACACGGGGCTGAATTCTAAACGAAGGCAG-3'. The amplified cDNA PCR product of ~840bp was purified as described above and cloned into the EcoRI site of the pBluescript® SK+/- (Stratagene) vector, sequenced from both ends, and stored at -20 C for later use.

Transformation of *Arabidopsis thaliana cdm1* Mutant

The gene isolated from rice and cloned into the pBluescript® SK+/- (Stratagene) vector was cut out and sub-cloned into the EcoRI site of the pRT101 vector containing the CaMV 35-S promoter (Nucleic Acids Research Vol. 15, Num. 14, 1987). The promoter and the gene were removed via digestion with HindIII and subcloned into the pBin19 vector (Bevan, 1984) that contained the appropriate tDNA regions for the subsequent transformation by *Agrobacterium tumefaciens*. The ligation products were amplified in *Escherichia coli*, and the resulting plasmids were purified and transferred to *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986). Transformation of the *Arabidopsis thaliana cdm1* mutant was done by dunking the flowers of the plant into a

broth of *Agrobacterium* containing the sense construct of the rice gene. Phenotype analysis was done by visual inspection.

Construction of Antisense Gene and Transformation of *Oryza sativa*

The gene isolated from rice and cloned into the pBluescript® SK+/- (Stratagene) vector and the *pAct1-D* plasmid (McElroy et al., 1990, 1991), containing the constitutive rice actin 1 gene promoter, was digested with *Sma*I and *Eco*RV to nondirectionally fuse the actin promoter to the 3' end of the novel rice gene within the *pAct1-D* plasmid. The ligation products were amplified in *Escherichia coli* and the appropriate ligation product was identified via restriction digest analysis. The NOS terminator was removed from the *pAct1-D* plasmid using *Sac*I and blunt end ligated to a blunt ended *Not*I site on the 5' end of the ligation product described above. The ligation products were amplified in *Escherichia coli* and a restriction digest analysis of the resultant ligation products confirmed the antisense orientation of the gene between the rice actin promoter and the NOS terminator.

The antisense construct described above was further digested with *Ap*I and *Sac*II to nondirectionally clone the actin promoter/antisense rice gene/NOS terminator sequence into the *Sma*I site of the pCAMBIA 1300 vector from CAMBIA, which contains the hygromycin resistance gene that was used as a selectable marker for transformation of the *Oryza sativa* genome. Both the insert and vector fragments were purified using the PCR purification kit from QIAGEN before ligation. The ligation products were amplified in *Escherichia coli* and PCR analysis, using the primers used to obtain the full-length cDNA sequence of the gene described above, confirmed proper

cloning of the antisense construct. The resulting plasmids were purified and transferred to *Agrobacterium tumefaciens* EHA105 from CAMBIA. *Agrobacterium*-mediated transformation of *Oryza sativa* was done using the method described by Aldemita and Hodges (1996). Transformation of genomic DNA, isolated using the PEX DNA Isolation protocol (Williams and Ronald, 1994), was verified via PCR using primers to the hygromycin gene. The primers had the following sequences: forward primer, 5'-AAAGCCTGAACTCACCGC-3'; reverse primer, 5'-GGTTTCCACTATCGGCGAG-3'. The PCR parameters consisted of an annealing temperature of 52 C and an elongation temperature of 72 C.

Analysis of Transgenic Phenotypes by Microscopy

Young leaf tissue was harvested ~5cm from the base and cut into 5mm strips with a razor blade. These strips were processed at 60 C in 100mM EGTa pH 9 using the following modified procedure from Possingham and Smith (1972). After soaking at 60 C for three hours, the strips were placed at 4 C for 3-7 days. A scalpel was then used to scrape mesophyll cells onto a microscope slide and covered with a cover slip for analysis. Samples were analyzed with Nomarski differential interference contrast optics, using a Nikon DIAPHOT-TMD inverted microscope. Chloroplast numbers and mesophyll cell areas were analyzed by eye under the microscope. Cell area was quantitated using a micrometer ruler that fit into the eyepiece of the microscope.

Hybridization Analysis

The method used for DNA extraction from rice leaf tissue was an adaptation of a mini-prep DNA isolation procedure for small amounts of fresh plant tissue from

Dellaporta et al., (1983). Polysaccharides were removed by ethanol precipitation as described by Michaels et al., (1994). For the Southern (DNA) blot analysis, 2µg of genomic DNA was digested with EcoRI, electrophoresed through a 0.8% agarose gel, and processed according to the Dupont/NEN GeneScreen™ method. The hygromycin gene was isolated from the pCAMBIA 1300 plasmid and radiolabeled with ³²P-dCTP by the random primer method. The blot was washed at 65 C according to the Dupont/NEN GeneScreen™ protocol. The method for RNA isolation from rice leaf tissue was the standard phenol:chloroform method. For the northern (RNA) blot analysis, 10µg of total RNA was used and electrophoresed through a 1% agarose gel in 1×MOPS buffer. Sense and antisense riboprobes were synthesized using Promega's Riboprobe® In Vitro Transcription System. The blot was hybridized in the ULTRAhyb™ hybridization buffer containing 50% formamide at 68 C. The blot was then washed at 68 C according to the Dupont/NEN GeneScreen™ protocol.

RESULTS

***Oryza sativa* Contains a Homologous cDNA Sequence of *Arabidopsis thaliana* *cdm1* Gene**

The cDNA sequence of the *cdm1* gene from *Arabidopsis* was used to perform a BLAST search of the expressed sequence tag (EST) database at the National Center for Biological Information to find homologous sequences. The search returned an EST in rice with high homology, and was subsequently used for obtaining a cDNA fragment of this novel gene. The cDNA fragment was further used to probe the rice Teqing BAC library at the Texas A&M University BAC Center. The isolation of the ~2.25kb

genomic sequence of this rice gene permitted the acquisition of the cDNA sequence of this gene in rice of 829bp coding for a protein of 275 amino acids. This sequence and the proposed amino acid sequence, deduced from comparison to the *cdm1* amino acid sequence, are shown in Figure 1.

Base 001: ATGGGCTCCGGCGAGGACACCGGCGCGGGCGTCGCGGGAGGAGGGGAGGCG
M G S G E D T G A G V A G G G G G

Base 053: GAGGCGCTGGTGGGGTGGTGC GG GCGCGGTGCTGAAGGCGCTCGTGGTCGT
G G A G G V V R G A V L K A L V V V

Base 105: CGGCGCGGTGCTGCTGCTCCGGCGGGTGC GCGCGCTCCACCACCCGGTGGGAC
G G V L L L R R L R R S T T R W D

Base 157: CACGCCCGCGCCGTGCTGGACGCGCTCTCCGGTGAGAAGTTCTCGAGGGAGC
H A R A V V D A L S G E K F S R E

Base 209: AGGCGAGGAAGGATCCTGATAACTACTTTAATTTGAGGATGCTTACATGCCC
Q A R K D P D N Y F N L R M L T C P

Base 261: TGCAACAGAGATGGTGGATGGTTCTAGAGTGCTTTACTTTGAGCAAGCATT
A T E M V D G S R V L Y F E Q A F

Base 313: TGGAGAAGTCCAGAAAAACCTTCCGACAAAGATTCTACATGGTAAAGCCAT
W R S P E K P F R Q R F Y M V K P

Base 365: GCCCAAAGGATATGAAATGTGATGTTGAGTTGAGTTCATATGCAATTAGAGA
C P K D M K C D V E L S S Y A I R D

Base 417: TGTGTAAGAGTACAAGAATTTCTGTGACCGTCCAAAGGATCAGAGGCCACAA
V E E Y K N F C D R P K D Q R P Q

Base 469: CCAGAAGAAGTCATTGCGGACATTGCAGAGCACCTGACTACCATACACTTGT
P E E V I A D I A E H L T T I H L

Base 521: CGCGGTGTGAGCGTGGAAAGCGCTGCTTGTACAAAGGATCAACCCCTCCTGA
S R C E R G K R C L Y K G S T P P E

Base 573: AGGCTTTCCCAACAGCTGGAGCGGTGCGACATATTGTACATCGGATTTGTCC
 G F P N S W S G A T Y C T S D L S

Base 625: ATTCACAAGAATGGTGAGGTGCATATCTGGGACAAGGGTTTGGACGATGATG
 I H K N G E V H I W D K G F D D D

Base 677: GGAATCAGGTTTGGGGAACCAAAGCTGGCCCTTACGAGTTCAAGCCTGCCCC
 G N Q V W G T K A G P Y E F K P A P

Base 729: CAAGTCGAATTACGACGACATGTTCTCGCCGTTGAATTTTCTGCTCCATTG
 K S N Y D D M F S P L N F S A P L

Base 781: ACGCTGGAGAAGAAGATTGAGAGCTCGTTCCGAATCGATGATCAGTAG
 T L E K K I E S S F A I D D Q .

Figure 1. cDNA and Amino Acid Sequence of the Novel Rice Gene.

The cDNA sequence has 829bp in total. Base pair numbers are indicated on the left of each row of bases and the amino acid sequence is written below the cDNA sequence.

The dot at the end of the amino acid sequence indicates a stop codon.

Gene in *Oryza sativa* May Function in Chloroplast Division

As a preliminary test of function, the *Arabidopsis cdm1* mutant was transformed with a sense construct of the gene from rice in an effort to reinstate wild-type chloroplast division. Using *Agrobacterium tumefaciens*, the gene construct was introduced into the seeds of the *cdm1 Arabidopsis thaliana* mutant. Although chloroplast number was not quantitated, the first leaves of the transgenic plants appeared greener than the ones of the mutant when analyzed by visual inspection. This was taken as an indication that this gene from rice may function in chloroplast division and that transformation of rice was an appropriate method to ask questions about the function of this gene in rice.

Transformation of *Oryza sativa* was Successful but Chloroplast Division was Unaltered

In order to access the function of this gene in rice, *Agrobacterium*-mediated transformation was chosen to introduce an antisense construct of the gene intended to knock-out or reduce endogenous expression of this rice gene putatively involved in chloroplast division. It was hypothesized that if this gene was involved in chloroplast division, expression of the antisense gene would inhibit chloroplast division, yielding plants with reduced numbers of chloroplasts. The antisense gene was constructed in the binary vector pCAMBIA 1300 that contained a hygromycin resistance gene as a selectable marker and expressed using the constitutive actin promoter. Figure 2 shows a PCR analysis of a small group of plants tested for integration of the antisense construct. In total 46 hygromycin resistant plants from 19 independent lines of transformation clones were tested. Primers to the hygromycin gene were used to amplify a 1kb fragment of the gene. The result was positive. Thirty of the plants tested yielded a 1kb PCR product, indicating that the hygromycin gene had been integrated into the genome of those rice plants. This was taken as an indication that the gene hypothesized to function in chloroplast division was also integrated into the genome.

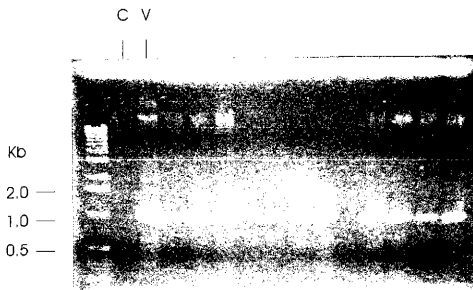


Figure 2. PCR Analysis of *Oryza sativa* Transformed with an Antisense Construct of this Novel Gene.

PCR products were electrophoresed on a 1% gel. Primers to the hygromycin gene were used to amplify a 1kb fragment of the hygromycin gene to verify integration of the antisense construct. The first lane is the DNA marker, the second lane, labeled C is a negative control lane in which wild-type rice genomic DNA was used as template DNA, and the third lane is a positive control lane in which the pCAMBIA 1300 plasmid DNA was used as template DNA.

The Southern blot shown in Figure 3 below was probed with the hygromycin gene radiolabeled with ^{32}P -dCTP. It confirms the PCR analysis shown above with the exception of the sample in lane number 9. It shows no band indicating that this clone was a false positive by PCR. Furthermore, the number of bands in each lane is equal to the number of integration events that occurred during the transformation process. It is

evident that anywhere from 1 to 3 integration events of the antisense construct into the rice genome took place when rice callus was subjected to infection with *Agrobacterium tumefaciens* containing the antisense construct.

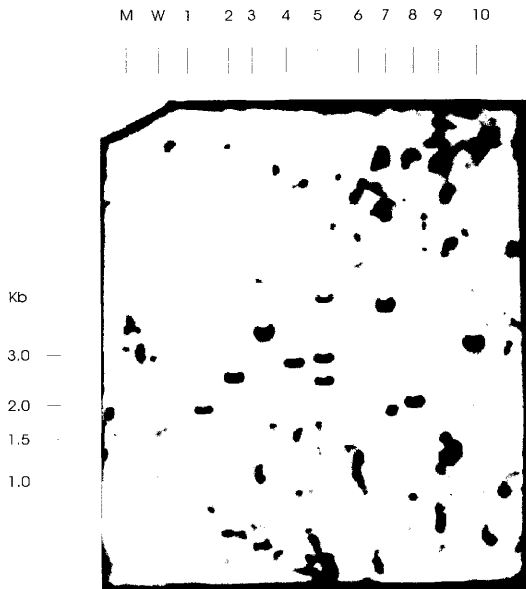


Figure 3. Genomic DNA Southern Blot of *Oryza sativa* Transformed with the Antisense Construct.

Genomic DNA (2 μ g) was electrophoresed on a 0.8% gel and transferred to a nylon membrane for hybridization. The hygromycin gene was cut from the pCAMBIA 1300 vector and radiolabeled with 32 P-dCTP and used as the probe. Kilobases are indicated to the left of the blot. M = Marker Lane, W = Wild-Type Lane, 1-10 = Randomly selected plants that tested positive by PCR for antisense construct lanes.

After verifying integration of the antisense construct by PCR, leaf tissue samples from a representative plant from each of the 19 independent lines, a group of plants all arising from a single piece of callus or transformation event, were prepared to allow visualization of individual leaf mesophyll cells under the microscope in order to analyze chloroplast number and cell area for approximately 50 cells per line. None of the plants analyzed showed a significant diminution in the number of chloroplasts per cell or cell area from wild-type or control plants, see Table 1.

Table 1. Summary of Phenotypic Analysis of *Oryza sativa* Plants Transformed with the Antisense Construct

Plant	Average Chloroplast Number \pm Standard Deviation	Average Cell Size \pm Standard Deviation
A8A-B	6.87 \pm 1.68	284.3 \pm 89.4
A15A	8.00 \pm 2.48	322.5 \pm 140.3

A22B	6.9±1.62	294.6±181.7
A22C	7.72±1.96	288.9±129.0
A32A	7.42±1.97	300.9±141.5
A32B	6.36±1.94	282.4±148.2
A36F	7.46±2.44	288.6±138.3
A37B	7.25±1.67	287.3±110.0
A40C	6.98±1.70	270.0±108.6
A40E	6.41±1.91	250.2±113.8
A41F	7.72±2.50	334.4±144.3
A42A	6.67±1.23	277.0±89.6
A45B	7.80±2.21	306.6±111.0
A46A	7.05±1.63	265.4±87.6
A50A	6.78±1.75	287.8±110.3
A51A-C	6.96±1.71	294.4±101.6
A52E	7.35±2.14	299.2±98.1
A56A	7.95±3.73	311.7±134.1
A58E	7.48±1.90	292.6±96.3
A59A	6.71±1.82	259.2±112.7
A62C	7.16±2.19	329.5±144.1
A63C	7.17±2.33	307.2±141.1
A66A-B	7.06±1.27	345.4±97.7

Control 1	7.00±1.99	325.3±276.7
Control 2	6.82±2.05	265.8±104.0
Control 3	6.83±1.72	297.2±99.5
Wild-Type 1	6.42±1.89	260.7±123.5
Wild-Type 2	7.13±2.03	306.8±110.4
Average for Antisense	7.20±1.99	293.5±120.4
Average for Control	6.88±1.92	295.4±160.1
Average for Wild-Type	6.78±1.96	283.8±117.0

Plant identification number are as follows: A#L = antisense (A), line number (#), clone letter (L). Control plants were plants that went through transformation but did not test positive for hygromycin via PCR.

To delineate the basis for this negative result a hybridization analysis of total RNA via northern blot, from some select plants that tested positive for integration by PCR, was performed. Unfortunately, the northern blot yielded no discernable information (blot not shown), which is needed to determine the expression levels of the endogenous rice gene, as well as the amount of expression of the antisense construct introduced by *Agrobacterium*-mediated transformation. This necessary data might indicate either that the construct is not being properly expressed, that the endogenous

gene expression is simply not being reduced enough, or that both are being expressed properly and that possibly this gene does not function in chloroplast division.

DISCUSSION

The experiments presented here suggest that this gene from rice may function in chloroplast division. Transformation of the *Arabidopsis cdm1* mutant reinstated chloroplast division to wild-type levels. Although PCR analysis and southern analysis of the transgenic rice plant DNA confirmed integration of the antisense construct, it remains undetermined as to whether this gene is involved in chloroplast division in rice since no phenotypic change was observed.

The results of the phenotypic characterization of the rice plants transformed with an antisense construct of the gene are surprising and unexpected since the homologous *cdm1* gene has been demonstrated to function in chloroplast division in *Arabidopsis thaliana*. However, transformation is a difficult technique to use. General problems associated with transformation include rearrangement of the constructs that are introduced into the plant, methylation and silencing of expression of the introduced construct, and integration of the constructs with varying copy number and length into the plant's genome (Dong et al., 1996). These various mechanisms of DNA contortion reflect the plant's ability to guard itself from the introduction of foreign DNA in the form of viruses. Their large genome size and repetitiveness gives them this evolutionary advantage. Therefore, although the antisense gene was successfully introduced into the genome, it is understandable that its expression was either hindered or completely quenched.

An alternative explanation of the results presented here is that perhaps only a small amount of the endogenous gene is required to allow proper chloroplast division to take place. Even if the antisense construct was being properly expressed, but only partially knocking out expression of the endogenous gene, there may have been enough RNA message, and therefore protein, left to allow chloroplast division to proceed normally. A definitive northern blot is necessary to answer that question.

There is still the possibility that this gene from rice is not involved in chloroplast division. The fact that the antisense gene was properly integrated into the genome but no significant reduction in chloroplast number was observed may indicate that although the homologous gene in *Arabidopsis* functions in chloroplast division, the gene in rice has a different function. However, based on the results of the transformation of the *Arabidopsis cdm1* mutant, this is probably not the case. Lastly, if these experiments were to be repeated the expected result of a reduction in chloroplast number might be difficult to obtain. Since only about seven chloroplasts are present in wild-type plants it may be difficult to claim that antisense plants having only four to five chloroplasts per cell show a significant reduction in chloroplast number.

CONCLUSION

Understanding chloroplast division is important for several reasons. Since chloroplasts provide energy to the plant cell by capturing light from the sun and converting it to chemically usable forms of energy, they could be manipulated to improve the growth and quality of any given plant. To fully exploit their role as the primary energy-transducing unit required for the production of food for the world,

chloroplasts have been identified as a model system for genetic engineering (Daniell et al., 1998). A chloroplast genome transformed with a foreign gene, such as for herbicide resistance, would allow farmers to spray their crops for weeds without reducing their crop yield. Due to the maternal inheritance of chloroplasts, unwanted effects such as the spreading of the herbicide resistance gene to problematic weeds through cross-pollination would be avoided. Genetic manipulation of chloroplasts is important because it will render plants that grow more efficiently through more proficient use of the energy produced by the chloroplast. This will in turn provide more food to feed the 800 million hungry people and the 185 million malnourished children in the world today (Herd, 1998).

Studies such as the one described here provide a fundamental beginning for the delineation of a general mechanism of chloroplast division by stimulating other basic research questions about chloroplast biogenesis. New experiments need to be designed to more conclusively define the function of the protein, coded for by this nuclear gene in rice, during chloroplast division. Ultimately, a thorough understanding of chloroplasts and their process of division will facilitate the use of genetically modified chloroplasts in agriculturally beneficial capacities in the future.

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