Chloroplast Development at Low Temperatures Requires a Homolog of DIM1, a Yeast Gene Encoding the 18S rRNA Dimethylase

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Poikilothermic organisms require mechanisms that allow survival at chilling temperatures (2 to 15°C). We have isolated chilling-sensitive mutants of Arabidopsis, a plant that is very chilling resistant, and are characterizing them to understand the genes involved in chilling resistance. The T-DNA–tagged mutant paleface1 (pfc1) grows normally at 22°C but at 5°C exhibits a pattern of chilling-induced chlorosis consistent with a disruption of chloroplast development. Genomic DNA flanking the T-DNA was cloned and used to isolate wild-type genomic and cDNA clones. The PFC1 transcript is present at a low level in wild-type plants and was not detected in pfc1 plants. Wild-type Arabidopsis expressing antisense constructs of PFC1 grew normally at 22°C but showed chilling-induced chlorosis, confirming that the gene is essential for low-temperature development of chloroplasts. The deduced amino acid sequence of PFC1 has identity with rRNA methylases found in bacteria and yeast that modify specific adenosines of pre-rRNA transcripts. The pfc1 mutant does not have these modifications in the small subunit rRNA of the plastid.

INTRODUCTION

Chilling resistance is an adaptation that plants in temperate climates require to survive and grow in suboptimal, non-freezing temperatures. Arabidopsis germinates in the autumn, overwinters in a vegetative state, and then flowers during the lengthening days of spring. It grows despite the chilling temperatures it experiences seasonally and diurnally in its native temperate climate (Rédei, 1969). Temperature adaptation is a complex trait, as is evident by the broad variability in chilling resistance among plant species and the varying sensitivity of some plants to chilling during their life cycle. Chilling tolerance has been shown to be inherited as a dominant or codominant trait in the progeny of crosses between chilling-tolerant and chilling-sensitive individuals. In such a cross with orchids, subsequent filial generations show a progressive breakdown of tolerance, thus demonstrating that chilling tolerance is a polygenic trait reflecting the sum of many individual adaptive mechanisms (Patterson and Reid, 1990). To understand better the genes and processes specifically required for chilling resistance but not for growth at warm temperatures, we initiated a screen to isolate and characterize chilling-sensitive mutants of Arabidopsis (Tokuhisa et al., 1997). One of the advantages of a mutational approach is the ability to isolate and define components of a complex response through the characterization of individual mutants. The growth of our mutants was similar to that of their wild-type progenitors at 22°C; however, these mutants were damaged when grown at 5°C. They showed an array of symptoms, including chlorosis, necrosis, growth cessation, reduced growth, or high anthocyanin content. Such mutants appear with a high frequency—3% in chemically mutated and 0.3% in T-DNA–tagged populations of Arabidopsis. We have focused our research efforts on mutants with the most dramatic phenotypes isolated from the T-DNA–tagged populations. The mutations in these populations should predominantly consist of null alleles and therefore identify genes in this screen that are not required at normal growth temperatures but are required at chilling temperatures.

Chlorophyll-deficient tissue is a frequent symptom in plants of tropical origin, such as sugarcane (Faris, 1927), when they experience low temperatures (2 to 15°C). These symptoms also arise with a high frequency in mutated populations of plants, such as maize (Bálint and Herczegh, 1981), barley (von Wettstein et al., 1971; Smillie, 1979), and Arabidopsis (Schneider et al., 1995). One of the mutants that we have isolated, the T-DNA–tagged mutant paleface1 (pfc1), shows chilling-induced chlorosis and reduced growth at 5°C. Genetic analyses indicated that the pfc1 phenotype is caused by the recessive mutation of a nuclear-encoded gene, most likely by the insertion of a T-DNA (Tokuhisa et al., 1997). We have exploited the fact that tagged mutants simplify the cloning of mutated genes and report here on the
identity of the pfc1 allele. This tagged mutation identifies a
gene that encodes the 16S rRNA methylase. The gene has a
specific role in normal chloroplast development at low tem-
peratures, but the gene and the methylation activity are not
required at 22°C.

RESULTS

Patterns of Chilling-Induced Chlorosis in the
pfc1 Mutant

Under growth conditions of 22°C, the pfc1 mutant produces
green tissue that is indistinguishable from wild-type tissue at
all stages of the life cycle. Figure 1 (top) shows wild-type
and pfc1 Arabidopsis grown at 22°C to a late vegetative
stage of the life cycle. After transfer to 5°C, wild-type plants
continued to produce green tissue, whereas all of the stem
and leaf tissue that developed in pfc1 individuals at the chill-
ing temperature lacked chlorophyll (Figure 1, bottom). This
new tissue was visible after 10 days of chilling, and all sub-
sequent growth was chlorotic. The chlorotic pfc1 bolt stems
and leaves showed typical morphology, with the exception
of more angular and slightly smaller leaves. This chlorosis
was not detected in pfc1 plants grown at 22°C and sub-
jected to drought, fungal attack (powdery mildew or Pyth-
ium), or wounding by thrips (Thysanoptera).

The pfc1 mutant was subjected to a prolonged chilling fol-
lowed by rewarming to determine whether the chlorosis was
irreversible and to identify developmental stages affected by
the mutation. The pfc1 plants were grown at 22°C for 2
weeks, transferred to 5°C for 5 months, and then transferred
back to 22°C for up to 3 weeks. Figure 2A illustrates the typ-
ical appearance of pfc1 individuals at the beginning of
the rewarming. The cotyledons and first leaves (outermost de-
tached leaves), which are the leaves that developed at 22°C,
remained green even after 5 months at 5°C. Leaves with tis-
sues that matured at both 22 and 5°C (detached leaves closest
to the center) had chimeric pigmentation, green tips, and
pale leaf bases. All other leaves and the apical meristem
that developed entirely at 5°C were entirely chlorotic; they
lacked chlorophyll but contained carotenoids that gave a
yellow hue to the tissue. After 10 days of rewarming at 22°C
(Figure 2B), green tissue began to develop in the apical mer-
istem and at the leaf bases of immature chlorotic leaves.

The carotenoid pigmentation in the mature chlorotic leaves
decreased and changed the leaf color from yellow to white.
After 21 days (Figure 2C), all of the chlorotic tissues that de-
veloped during the initial 22°C growth, the coty-
ledons and first leaves, had senesced, as had leaves that
were entirely chlorotic. This warming treatment demon-
strates that the chlorotic phenotype was limited to tissue
developing at the reduced temperature and that the chloro-
phyll level in a tissue maturing at a particular growth temper-
ature was not dramatically altered by other temperatures
experienced after maturation.

The pfc1 Mutation Affects Chloroplast Development

This distinct pattern of symptom expression in chilled and
rewarmed pfc1 mutants was investigated further at the phy-
siological level. The observation that pfc1 plants con-
tinued to grow at 5°C despite having a large proportion of het-
erotrophic tissue indicates that photosynthesis in the green
tissue was sufficient for growth. To confirm this, the photo-
synthetic capabilities of wild-type and pfc1 plants were as-
sessed by chlorophyll fluorescence analysis after 20 days at
22°C and after an additional 30 days at 5°C (Table 1). At
22°C, the quantum yield of photosystem II (PSII), measured
as the maximum variable fluorescence/maximum fluores-
cence (Fv/Fm) in dark-adapted leaves, and the quantum yield
of PSII under steady state photosynthesis (φPSII) were similar
in wild-type and pfc1 leaves. After 30 days, green pfc1
leaves had Fv/Fm and φPSII values that were 71 and 59%, re-
spectively, of the values of the green pfc1 leaves grown at
22°C. As expected, the fluorescence measurements of chlo-
rotic pfc1 leaves that had developed entirely at 5°C indi-
cated little or no photosynthetic activity.
These results confirmed that green pfc1 leaves have sufficient photosynthetic activity to allow survival of the mutant for at least 5 months at 5°C. The principal effect of the pfc1 mutation on photosynthesis is through the inhibited development of chloroplasts under chilling conditions. There is a much lesser effect on mature, green chloroplasts. Therefore, these physiological data indicate that the wild-type PFC1 gene is essential for chloroplast biogenesis at low temperatures.

A Gene Spans the T-DNA Insertion Site

Previously, we demonstrated the 3:1 segregation ratio in an F2 population of the wild-type and pfc1 phenotypes, indicating that the mutation is a recessive trait inherited in a Mendelian fashion (Tokuhisa et al., 1997). In this same F2 population, the segregation of kanamycin resistance, a marker for the T-DNA insert, indicated a single segregating T-DNA in the pfc1 genome. Each of the F2 plants that exhibited chilling-induced chlorosis produced a pool of F3 progeny that was 100% kanamycin resistant. This cosegregation of the T-DNA inserts and the chilling-induced chlorosis demonstrated that there is a high probability that the T-DNA is inserted at the pfc1 locus.

The plasmid rescue technique (Yanofsky et al., 1990) was used to clone the genomic DNA flanking the T-DNA insert. This strategy exploits the fact that the T-DNA insert contains copies of the bacterial plasmid pBR322 (Feldmann et al., 1989). DNA fragments containing genomic plant DNA/T-DNA junctions and pBR322 sequences can be recovered as functional plasmids. The pfc1 genomic DNA was incubated with BamHI, EcoRI, PstI, or SalI restriction enzymes and allowed to ligate at a low concentration to promote self-ligation. The ligation products were electroporated into Escherichia coli. The transformed bacteria were screened by antibiotic selection. Three plasmids were isolated from the SalI-digested DNA that conferred ampicillin resistance and had a fragment after EcoRI and SalI digestion that had sequence consistent with 70 bp of the left border of the T-DNA insert adjacent to a 1.0-kb fragment of putative plant DNA.

This 1.1-kb EcoRI-SalI fragment was subcloned to generate the plasmid pPR1.E/S (Figure 3A). The sequence of this fragment was used in a BLAST search of the GenBank database (Altschul et al., 1990) and shown to have identity with a gene encoding a tRNAV$_{Glu}$ (Figure 4, bracketed nucleotides).

Table 1. Chlorophyll Fluorescence Measurement of PSII and Quantum Yield in Leaves of Arabidopsis Wild-Type and pfc1 Plants

<table>
<thead>
<tr>
<th>Leaf Sample (Color)</th>
<th>Days at 5°C</th>
<th>F$_v$/F$_m$$^b$</th>
<th>(\Phi_{PSII}) at Steady State$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (green)</td>
<td>0</td>
<td>0.763 (0.004)$^d$</td>
<td>0.599 (0.011)$^c$</td>
</tr>
<tr>
<td>pfc1 (green)</td>
<td>0</td>
<td>0.751 (0.006)$^d$</td>
<td>0.593 (0.014)$^c$</td>
</tr>
<tr>
<td>Mature leaf</td>
<td>30</td>
<td>0.742 (0.022)$^d$</td>
<td>0.613 (0.011)$^c$</td>
</tr>
<tr>
<td>Wild type (green)</td>
<td>30</td>
<td>0.530 (0.029)$^d$</td>
<td>0.352 (0.019)$^c$</td>
</tr>
<tr>
<td>pfc1 (green)</td>
<td>30</td>
<td>0.755 (0.001)$^d$</td>
<td>0.564 (0.028)$^c$</td>
</tr>
<tr>
<td>pfc1 (chlorotic)</td>
<td>30</td>
<td>0.391 (0.011)$^d$</td>
<td>0.161 (0.011)$^c$</td>
</tr>
</tbody>
</table>

$^a$ Plants were grown for 20 days at 22°C and 30 days at 5°C.

$^b$ Standard errors are indicated in parentheses.

$^c$ $n = 9$.

$^d$ $n = 3$.

$^e$ $n = 6$.

Figure 2. The Appearance of the pfc1 Mutant during Rewarming.

(A) The pfc1 mutant was grown for 2 weeks at 22°C and then transferred to 5°C for 5 months.

(B) The pfc1 mutant was grown for 2 weeks at 22°C, transferred to 5°C for 5 months, and then transferred to 22°C for 10 days.

(C) The pfc1 mutant was grown for 2 weeks at 22°C, transferred to 5°C for 5 months, and then transferred to 22°C for 21 days.

The detached leaves have the approximate orientation that they had on the plant, with the older leaves being placed farther from the center.
from positions 12 to 95). The T-DNA insert was unlikely to disrupt the expression of this gene. Genes encoding tRNAs contain internal promoters for transcription by RNA polymerase III, and the 5' end of the gene was 137 bp away from the T-DNA insertion (Figure 4, vertical arrow).

To characterize this genomic region further, a clone (lp6.4.8) that hybridized with the 1.1-kb fragment was isolated from a wild-type genomic DNA library prepared from the Columbia ecotype of Arabidopsis. Subclones of lp6.4.8, p648B3 and p648S2, that hybridized with the 1.1-kb fragment were isolated after BamHI and SalI digestion, respectively.

Sequence analysis of a 0.49-kb HindIII-BamHI fragment of p648B3 showed open reading frames in both orientations, which were compared with the GenBank database at the amino acid level. One frame had a very significant match (P = 0.00017) with an S-adenosyl methionine (SAM) binding motif associated with various methyltransferase-encoded genes. This fragment of p648B3 containing the SAM binding motif was used as a hybridization probe to screen two Arabidopsis cDNA libraries. One clone was isolated, subcloned (Figure 3B, p20.1.1), and sequenced. The cDNA was incomplete; it lacked an ATG 5' of the SAM binding motif and contained an additional G residue (Figure 4, position 607) when compared with the genomic sequence. To obtain a more complete cDNA, we screened the λPRL-2 library, using it as a template in a polymerase chain reaction (PCR) with a vector-specific primer (T7 promoter) and a CDNA-specific primer (2aRMT-B; Figure 4). The sequence of the larger of two products obtained (pmp42.9) had an additional 243 bp at the 5' end relative to the initial cDNA p20.1.1. However, there was no initiator codon, and a different frameshift (Figure 4, position 398) disrupted the longest open reading frame.

To isolate the 5' end of the cDNA, poly(A) RNA from Arabidopsis was used as a template for first-strand cDNA synthesis by using an oligo(dT) primer and reverse transcriptase. The cDNA synthesis products were used as a template for a nested PCR. The first PCR used a primer complementary to the putative initiator codon identified in the genomic sequence (HH-HE; Figure 4) and another 3' of the sequence encoding the SAM binding motif (2aRMT-B). This reaction product was used for a second PCR with primers 1RMT510 and 2bRMT-B. A single band of 450 bp was isolated and cloned (p5'-RMT). The sequence of the p5'-RMT insert was identical to the genomic DNA sequence, except for the absence of two introns (Figure 4; boxed nucleotides). To generate a plasmid containing the entire reading frame, the XhoI-BamHI fragment of p5'-RMT was ligated to a BamHI-PstI fragment from p20.1.1. This plasmid was designated pORM (Figure 3B).

Figure 3. The Genomic Organization of the Wild-Type and Mutant Alleles of PFC1.
(A) The genome structure of the pfc1 locus showing the location of the plasmid rescue clone pPR1.E/S and the T-DNA insertion with the orientation of left border (LB), a restriction map with selected restriction enzymes, the genomic clones, the exon/intron structure of PFC1, and the location of the tRNA-encoding gene. B, BamH; D, DraIl; E, EcoRI; H, HindIII; P, PstI; and S, SalI.
(B) Three cDNA clones, p20.1.1, pmp42.9, and p5'-RMT, and pORM, the reconstructed open reading frame of PFC1. The location of a base addition (+G) or deletion (−G) is indicated. Additional restriction enzymes are abbreviated as follows: StuI; XhoI.

Figure 4. Nucleotide and Derived Amino Acid Sequence at the 5' End of the PFC1 Locus.
The tRNA gene is identified by brackets. Restriction enzyme sites and primer names are indicated. The placement of horizontal arrows designates the primer sequence for the sense strand (above) or antisense (below) sequence. The boxed nucleotides identify introns. The vertical arrow marks the T-DNA insertion site.
Sequence comparisons of the cDNA and the genomic DNA revealed a gene with eight introns (Figure 3A). The transcription start site has not been determined, but only 102 bp separate the 5’ end of the putative gene encoding the rRNA and the start of the 1032-nucleotide reading frame (Figure 4).

The deduced polypeptide of PFC1 consists of 343 amino acids. Amino acid sequence comparisons with the GenBank and dbEST databases identified homologous genes from such diverse organisms as bacteria, yeast, nematodes, Synchocystis, and humans. These genes encode rRNA methylases comprising a class of enzymes that methylate the base moiety of specific adenosines in rRNA transcripts. The genes that have been shown to encode rRNA methylase activity included ksgA from the Gram-negative bacterium E. coli, ermD from the Gram-positive bacterium Bacillus licheniformis, and DIM1 from Saccharomyces cerevisiae. By homology with these genes, other genes have been identified, including one from the Caenorhabditis elegans sequencing project and a human fibroblast cDNA. An overall comparison of five of these sequences with gaps introduced to optimize alignment showed identities in the range of 26 to 30% (Figure 5). A five-way alignment, as shown in Figure 6, revealed three regions at an identity of 63% or greater. They include the SAM binding site between residues 99 and 112 of the Arabidopsis sequence. Two other domains whose functions remain unknown span residues 183 to 197 and 252 to 268 and are common to both eukaryote and prokaryote forms of the enzyme.

Two regions of the deduced Arabidopsis polypeptide showed little or no identity with the other methylases. The C-terminal 25% of the polypeptide is highly variable among the five sequences. The 49-residue peptide at the N terminus is conspicuously unique to Arabidopsis. It contains a high proportion of serines and threonines (25%) and is very low in acidic residues (4%). These characteristics are associated with transit peptide motifs that target preproteins to the chloroplast (Von Heijne et al., 1989).

The cDNA was used to confirm that the mutant pfc1 allele was interrupted by a DNA insert. Gel blots of genomic DNA extracted from wild-type and pfc1 Arabidopsis plants and digested with BamHI were probed with the radiolabeled XhoI-PstI fragment of poRM (Figure 7). The PFC1 gene contains one internal BamHI restriction site creating two radiolabeled bands of 6.0 and 3.7 kb. The 3’ end of the gene (3.7 kb) is of similar mobility in both the wild type and mutant, but the 5’ end in the pfc1 genomic DNA (Figure 7, second lane) shows a band in excess of 8.5 kb (the largest marker on the gel in Figure 7), which is consistent with the addition of at least one T-DNA insert.

**Antisense Expression of the PFC1 Gene in Wild-Type Arabidopsis Generates Chilling-Induced Chlorosis**

Because of our difficulties in identifying and cloning a full-length cDNA, we used a partial cDNA to establish by an antisense approach that the cloned gene was the locus responsible for the phenotype. Despite a frameshift and a premature termination of the 5’ end in the cDNA, we anticipated that the derivative antisense construct would suppress PFC1 transcript levels and produce a plant with chilling-induced chlorosis. The construct was prepared from the ligation of the cDNA sequence 5’ of the BamHI site in pmp42.9 with the cDNA sequence 3’ of the BamHI site in the original clone p20.1.1 (Figure 3B). The sequence was ligated in an antisense orientation relative to the 35S transcript promoter of cauliflower mosaic virus and the nopaline synthase transcript terminator in the binary vector pHIN420. Transformation of wild-type Arabidopsis plants with this construct was mediated by Agrobacterium. Putative Arabidopsis transformants were identified initially by the acquired kanamycin resistance conferred by a gene within the T-DNA encoding neomycin phosphotransferase II. The progeny of these plants were assayed by PCR for the presence of the antisense gene construct and the absence of the pfc1 allele and were screened for chilling-induced chlorosis. As shown in Figure 8, chilling treatments that resulted in the chlorotic phenotype of pfc1 plants (Figure 8A, right) also caused identical phenotypes in transformant lines PT5-51 (Figure 8B, right), PT5-57 (Figure 8C, left), and PT5-71 (Figure 8C, right). Under these same growth conditions, transformant PT5-33 (Figure 8B, left) showed a phenotype similar to wild-type Arabidopsis plants (Figure 8A, left). Transformant lines PT5-33, PT5-51, PT5-57, and PT5-71 tested positive for the antisense construct. These lines tested negative for the mutant (T-DNA–disrupted) pfc1 allele.

These transformant lines were tested for levels of PFC1 transcript. As shown in Figure 9, a gel blot of 40 μg of total RNA extracted from wild-type, antisense transformant PT5-71 and pfc1 leaves of plants grown at 22°C was probed with a radiolabeled antisense pfc1 RNA prepared from poRM. A band at 1.4 kb was apparent with wild-type Arabidopsis but undetectable with the antisense-expressing plant PT5-71 and the pfc1 mutant. The PFC1 transcript was also undetectable in the transformants PT5-51 and PT5-57 (data not shown).
shown). The absence of pfc1 transcript in both of the plants with chilling-induced chlorosis, the original pfc1 mutant and the PT5-71 antisense plant, provides further evidence for the critical role of the PFC1 gene in chloroplast development at chilling temperatures. By contrast, some other Arabidopsis transformants containing the antisense construct but lacking chilling-induced chlorosis such as PT5-33 (Figure 8B, left) had PFC1 transcript levels ranging from 4 to 97% of wild-type levels (data not shown).

The pfc1 Mutant Lacks Adenosine Modification in the Small Subunit rRNA of the Plastid

The methylation activities encoded by the DIM1 and ksgA genes modify two specific adenosines in the highly conserved 3'-terminal region of the small subunit of rRNA. A primer extension method can detect modified nucleotides of rRNA. Reverse transcriptase will extend a DNA oligonucleotide primer that hybridizes with RNA templates but will terminate prematurely if it encounters an adenosine dimethylated at the N-6 position of the base (Hagenbüchle et al., 1978; Sigmund et al., 1980). Therefore, a primer extension protocol can distinguish the methylation status of adenosines in the rRNA of wild-type E. coli and the ksgA2 mutant (data not shown). A primer complementary to the 3'-end of the small subunit of plastid rRNA was generated that terminated seven bases before the adenosines, which in E. coli are the methylation sites. As shown in Figure 10, sequencing gel analysis visualized a six-base extension as the principal product derived from the primer and the template by using total RNA extracted from wild-type plants. This corresponds

Figure 6. Conserved Domains in the rRNA Methylases.

The deduced amino acid sequences from five genes were aligned with the GAP and Pileup Programs (Genetics Computer Group, version 8) at default settings. The genes include an open reading frame from C. elegans, DIM1 from S. cerevisiae, PFC1 from Arabidopsis, ksgA from E. coli, and ermD from B. lichenformis. Gaps introduced to optimize alignment are represented by dots. Regions containing at least four of five matches of identity (black) and similarity (gray) are identified (Boxshade Program; http://ulrec3.unil.ch/software/BOX_form.html). Similar residues are grouped as follows: (V, L, I, M), (S, T), (Q, N, E, D), (K, R), (G, A), and (F, W, Y). The numbers are the residue number relative to the N terminus of the deduced polypeptide. The GenBank accession number for the PFC1 cDNA sequence is AF051326.
to the anticipated termination before the two adenosines. In contrast, the 27-base extension observed using pfc1 mutant RNA extends through the adenosines and terminates before a uracil. The corresponding uracil in E. coli rRNA is methylated in the base moiety; thus, it is possible that the same modification in the plastid rRNA is the cause of the termination demonstrated for pfc1 rRNA in Figure 10. These results indicate that pfc1 lacks methylation of the 3’ adenosines in the small subunit of plastid rRNA.

**DISCUSSION**

The **PFC1 Gene Is Required for Chilling Resistance in Arabidopsis**

The phenotype of the pfc1 mutation coincides with chloroplast maturation at chilling temperatures. This pattern was most evident in the temperature shift experiments in which leaves developed at both temperatures. Leaves that were initiated at 22°C and matured at 5°C showed green tips and chlorotic leaf bases. Conversely, leaves that were initiated at 5°C and matured at 22°C showed the reverse pattern. These patterns are consistent with leaf maturation in dicots, which begins at the tip and extends to the base (Pyke et al., 1991). The possibility that the mutation inhibited plastid replication at low temperatures is unlikely. The chlorotic apical meristems of pfc1 plants left for 5 months at 5°C retained the ability upon return to 22°C to produce green leaves and stems (Figure 2C). In addition, the mutation did not directly affect the maintenance of chloroplasts because green tissues at 5°C remained photosynthetically active at levels sufficient to support the plant for at least 5 months (Figure 2A and Table 1). One effect of the pfc1 mutation on green tissue occurred when the plants that had been chilled for five months were returned to 22°C and the old green tissues senesced more rapidly in pfc1 individuals than in wild-type plants.

**The 16S rRNA Methyrase of Arabidopsis**

We have shown that the deduced amino acid sequence of PFC1 has 26 to 30% identity (Figure 5) with rRNA methylases from different organisms. Given the evolutionary distance between these organisms, this level of identity indicates that these genes encode polypeptides with closely related functions. Two other lines of evidence support this
conclusion. The yeast DIM1 gene has sufficient identity at the amino acid level (27%) with the bacterial ksgA gene to complement a methylase-deficient strain (ksgA<sup>2</sup>) of E. coli (Lafontaine et al., 1995). Three domains within the deduced polypeptide have 63% identity among the five organisms. The N-terminal domain is a SAM binding site, which has the consensus sequence VLEIGXGXG (where X represents any amino acid) found in protein, RNA, and DNA methyltransferases (Ingrosso et al., 1989; Kagan and Clarke, 1994). The two other domains with high identity include one in the middle of the polypeptide, NXPYXISXP, and another toward the C terminus, FXPPPKVDSAVVR (Figure 6).

The methylation activities encoded by the DIM1 and ksgA genes modify the N-6 position of the base moiety of adenosine with two methylations. This is in contrast with the more frequent and less conserved methylation of the ribose moiety of the nucleotide (2'-O-methylation) observed in vertebrate ribosomal RNA but absent in bacteria (Maden, 1996). The modified bases occur at two specific adenosines in the 3' terminus of the small subunit of rRNA at a late stage in pre-rRNA processing. The sequence of this region of the rRNA is highly conserved in prokaryotes and eukaryotes (Maden, 1990). The ability of reverse transcriptase to extend a primer specific to the 3' end of the small subunit rRNA of plastids by using an RNA template from pfc1 mutants but not from wild-type plants indicates that the pfc1 mutation disrupts a gene that encodes rRNA methylation activity.

The 49-mer peptide at the N-terminal end of the Arabidopsis sequence is unique among the five sequences. The high serine/threonine content and the low frequency of acidic amino acid residues in this sequence are characteristic of transit peptides that target polypeptides to the chloroplast (Von Heijne et al., 1989). A short region beginning at residue 41 of this peptide (Figure 6), RRRTVKVSCG, closely resembles the arginine-rich consensus motif (Val/Ile)-X-(Ala/Cys)Ala (where X represents any amino acid) derived from 32 chloroplast-targeted polypeptides in the vicinity of the known peptide cleavage site of the transit peptides (Gavel and Von Heijne, 1990). The peptide from Arabidopsis contains three arginines at positions −9, −8, and −7 relative to the glycine, followed by a valine at position −3 and a cysteine residue at position −1 within a region predicted to have a β sheet conformation (Chou and Fasman, 1974; Devereux et al., 1984). The only difference with the consensus is the glycine at position +1, which is found in one of the 32 transit peptides used for the consensus determination.

The cleavage of this transit peptide would generate a mature protein with a theoretical molecular weight of 32,800 and a pI of 8.0. The primer extension results, using a primer complementary to the plastid rRNA together with the sequence analysis predictions and the chlorotic phenotype of pfc1 plants, indicate that PFC1 is localized to the plastid.

Although not a principal focus of this investigation, two features of the PFC1 gene structure are worth noting. A double ATG occurs at the 5' end of the open reading frame of PFC1. Four genes encoding ribosomal proteins targeted to the chloroplast have been shown to have two 5' ATGs separated by three to 24 nucleotides (Giese and Subramanian, 1990). Translational initiation of the mRNA encoding the plastid L12 ribosomal protein was shown to be enhanced by tandem AUGs separated by 15 nucleotides. Of the translation initiation events, 70 to 80% occurred at the first site and

Figure 9. Transcript Levels of PFC1 in Wild-Type, pfc1, and PT5-71 Plants.
Total RNA was extracted from wild-type Arabidopsis (WT), antisense transgenic PT5-71, and pfc1 plants. Forty micrograms of RNA was subjected to gel blot analysis using a radiolabeled antisense transcript derived from the poRM insert as a probe. Molecular length markers are shown at left in kilobases.

Figure 10. Primer Extension Products Using a Primer Complementary to the Small Subunit rRNA from Plastids and Total RNA from Wild-Type and pfc1 Plants.
An oligonucleotide primer complementary to the 3' end of the small subunit rRNA of plastids was extended by reverse transcriptase using RNA from wild-type (WT) and pfc1 plants as templates. The products were fractionated on a 10% sequencing gel. The length of extension is indicated at left as the number of bases added by reverse transcriptase to the end of the primer.
to 30% at the downstream site. The slightly different polypeptide lengths of the two preproteins would be irrelevant because cleavage of the transit peptide sequences would create the same-sized mature protein.

The second notable feature is the close head-to-head proximity of the genes encoding the tRNA and the rRNA methylase. This 102-bp separation between the gene encoding the tRNA and the initiation codon of PFC1 may indicate roles for the tRNA gene as a template and internal promoter for tRNA<sub>val</sub> synthesis and as a promoter element for the RNA polymerase II transcription of the rRNA methylase. Such configurations have been identified in a trypanosome in which three small RNA genes are each spaced 95 to 97 bp from a tRNA gene in a divergent orientation. Mutational analysis of the A- and B-boxes of these tRNA genes demonstrated that they served as intragenic promoters for tRNA transcription and as extragenic regulatory elements for the small RNAs transcribed by RNA polymerase III (Nakaar et al., 1994).

The Function of an rRNA Methylase in Chilling Resistance

Although rRNA methylases have been characterized for some time, their biological function is unknown. rRNA methylases were first characterized in the context of bacterial antibiotic resistance. The methylase activity alters the affinity between the antibiotics and specific domains on the rRNA (Helser et al., 1971). The rRNA methylase encoded by ksgA confers kasugamycin sensitivity (Formenoy et al., 1994). Aside from the antibiotic response, bacteria lacking rRNA methylation are only slightly less competent than are the wild-type bacteria for translational initiation and fidelity of translation (Van Gemen and Van Knippenberg, 1990).

In contrast to the minor role of ksgA in bacteria, DIM1 is required for viability in yeast. DIM1 from yeast was identified by complementation of the antibiotic response in an E. coli strain mutated for ksgA (Lafontaine et al., 1994). The methylase modifies the same rRNA domain as in bacteria at a late stage of pre-rRNA processing in yeast. A conditional mutation of DIM1 demonstrated that the absence of the methylase in yeast eliminates the methylation as expected, but interestingly, the mutation inhibits pre-rRNA processing of the 18S precursor at a step before adenosine dimethylation. Processing of the precursors of the 5.8S and 25S rRNAs is unaffected and by default leaves an aberrant 22S rRNA that is not processed to the mature small subunit (18S) form. This disruption blocks ribosome biogenesis and is the probable cause for the observed lethal phenotype (Lafontaine et al., 1995).

Although the pfc1 mutant defines a function for PFC1 in chilling-resistant development of chloroplast, and we have shown a biochemical lesion in RNA methylation, the initial chilling-sensitive defect created by the mutation remains unknown. In principle, the pfc1 mutation could affect translation of chloroplast-encoded proteins. The absence of four methyl groups could create a chilling-sensitive conformation of the ribosome and disrupt translation, as observed in the bacterial ksgA<sup>−</sup> mutant. However, the symptoms of the pfc1 mutant are not consistent with a direct effect on translation.

In the tissues that matured before the chilling treatment, photosynthesis is maintained (Table 1), albeit at a reduced level. The high turnover of the D1 reaction center protein of PSII (Russell et al., 1995) requires that photosynthetically active tissues continually synthesize D1 protein to maintain photosynthesis. Because D1 protein is derived from a chloroplast-encoded gene, it follows that protein synthesis is active in these fully developed chloroplasts. In the chlorotic tissues, the plastids are developed sufficiently to allow import and function of the nuclear-encoded enzymes required for the synthesis of carotenoids, which produce the yellow pigmentation and the synthesis of fatty acids (Figures 1 and 2A). The mutation does not appear to affect cytosolic protein synthesis as shoot development and leaf expansion continue, despite the dramatic inhibition of chloroplast development in these tissues.

Another possibility for the biochemical defect created by the pfc1 mutation is a chilling-sensitive step in pre-rRNA processing that inhibits ribosome biogenesis. In yeast, Dim1p has been proposed to be an integral component of a protein–RNA complex required for early stages of rRNA processing in addition to its activity as the 18S rRNA dimethylase (Lafontaine et al., 1995). By analogy, the absence of PFC1 would eliminate the adenosine methylation and independently destabilize a 16S rRNA–protein complex in a temperature-dependent manner. Alternatively, the absence of methylation may directly destabilize pre-rRNA processing or a subsequent step of ribosome assembly. These possibilities will be addressed by an analysis of 16S rRNA processing and plastid ribosome assembly in the pfc1 mutant.

Several features of chloroplast biogenesis and ribosome turnover may minimize the impact of the inhibited biogenesis of plastid ribosomes. The plastid is a semiautonomous organelle, with many of the critical early components for plastid biogenesis derived from the nucleus and cytosol independent of plastid gene expression (Link, 1996). Therefore, functional plastids may require minimal translational activity within the plastid. In contrast to the hundreds of chloroplasts present in a leaf mesophyll cell, the plastids in apical meristem cells are maintained at a low number as undifferentiated proplastids (Mullet, 1988). It is likely that the chilled apical meristem can maintain the potential to produce chloroplasts upon rewarming because the limited proliferation and minimal differentiation of the proplastids require little or no plastid translational activity. The barley mutant albostrians has mature leaf sectors containing ribosome-free plastids, indicating that proplastids can survive in the absence of plastid translation (Dorne et al., 1982; Hess et al., 1993). These factors could allow the proplastid population of the mutant meristems to survive 5 months of chilling and differentiate into chloroplasts upon rewarming (Figure 2).
The survival of the green leaves of the pfc1 mutant for at least 5 months of chilling, despite the inhibition of plastid ribosome biogenesis, probably reflects the overabundance and slow turnover of plastid ribosomes. The greatest requirement for plastid protein synthesis is during chloroplast development (Mullet, 1988). Mature chloroplasts require only a portion of the translational capacity for maintenance of photosynthesis. In Lemna, the plastid ribosomes have a longer half-life than do cytosolic ribosomes, with a turnover rate of 15 versus 5 days at 25°C growth temperatures (Trewavas, 1970). At 5°C, the turnover rate of proplastid ribosomes is likely to be slower. During the chilling regime, these tissues would have sufficient translational activity in the plastids to maintain photosynthesis, as shown in Table 1, despite a significant loss of ribosomes.

Chilling-induced chlorosis is a frequent symptom of chilling injury. Mutants have been identified in maize and barley that show chilling-induced chlorosis. Various biochemical lesions are attributed to this chlorosis, and some include deficiencies in chloroplast ribosomes. The plastids of the maize mutant M-11 appear normal at 27°C, show no chlorophyll accumulation, and are markedly deficient in ribosomes when they develop at 17°C (Miller et al., 1969). Similar observations of reduced densities of plastid ribosomes were made of the extremely chlorotic tissues of the virescent maize mutant v16 grown below 25°C (Hopkins and Elffman, 1984). Barkan (1993) has shown that a component of pre-16S rRNA processing is disrupted at low temperatures in another virescent mutant of maize, hcf7.

Conclusions

We have used a mutational approach to identify the 16S rRNA methylase in higher plants and have shown that it is required for the development of chloroplasts at low temperature. These results indicate that one general function of the 16S rRNA methylase is to compensate for a chilling-sensitive step in ribosome biogenesis. We have demonstrated that T-DNA-tagged mutants can be used to elucidate the components of chilling-resistant development in a poikilothermic organism. The T-DNA tag allowed for the facile identification of PFC1 function. The pfc1 mutant permitted us to characterize a single aspect of the chilling resistance response. Together, the molecular identification and the chlorotic phenotype provide a foundation to investigate the complete pathway for this mechanism of chilling resistance and to determine the initial chilling-sensitive defect.

METHODS

Plant Growth Conditions

Arabidopsis thaliana plants were grown at 22 and 5°C with light intensities of 100 to 150 μE m⁻² sec⁻¹ and a photoperiod of 10 hr of light and 14 hr of darkness. The growth medium was either Metromix 350 (Scotts-Sierra, Maryville, OH) supplemented with macronutrients and micronutrients or a local soil-less potting. Plants transferred from 22 to 5°C were covered completely with plastic wrap for 1 or 2 days and partially exposed to the ambient atmosphere for another 1 or 2 days before the plastic was removed entirely.

Chlorophyll Fluorescence Measurements

The quantum yield of the photosystem II (PSII) partial reaction was measured in detached leaves as a ratio of the variable fluorescence to maximum fluorescence emission (Fv/Fm) induced by a short pulse of photosynthetically saturating light applied to dark-adapted leaves. The quantum yield of steady state photosynthetic electron transport (ΦPSII) was measured subsequently with the same leaves used for the Fv/Fm measurements, after steady state photosynthesis was established by illuminating them for 5 min with 100 μE m⁻² sec⁻¹ of actinic light. All measurements were done with a PAM fluorometer (Waltz, Effelbrich, Germany).

Genomic Plant DNA Isolation

Genomic DNA was isolated from Arabidopsis by the method of Rogers and Bendich (1985). In brief, leaf tissue was frozen and pulverized in liquid nitrogen. A volume equivalent (milliliters) of 2× CTAB grinding buffer (2% cetyltrimethylammonium bromide [w/v], 100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, and 1% PVP [molecular weight 40,000]) at 65°C was added. The mixture was heated for 5 min at 65°C. The samples were cooled on ice. An equal volume of chloroform–isoamyl alcohol (24:1) was added. The samples were shaken to mix the two phases and partitioned by centrifugation for 1 min at 20,000g. The top phase was retained. A 0.1 volume of 10% CTAB solution (10% CTAB [w/v] and 0.7 M NaCl) and 1 volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris, pH 8.0, and 10 mM EDTA, pH 8.0) were added, and the mixture was incubated for at least 8 hr at 5°C. The precipitate was recovered by centrifugation at 20,000g for 10 min. The pellet was resuspended in high-salt TE (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, and 1 M NaCl) for 10 min at 65°C. Two volumes of ethanol were added. The precipitate was recovered by centrifugation for 5 min at 20,000g and washed with 80% ethanol. This final pellet was resuspended in 0.1× TE (1 mM Tris, pH 8.0, and 0.1 mM EDTA, pH 8.0).

Isolation of a pfc1 Genomic DNA/T-DNA Junction

The paleface1 (pfc1) genomic DNA (2 μg) was digested to completion with SalI. Aliquots of the restricted DNA (250 ng) were incubated at 0.5 ng/μL with 4 units of T4 DNA ligase. The ligated DNA was mixed with 10 μg of rRNA and concentrated by ethanol precipitation. Escherichia coli DH10B (mcrA−, mcrB−, mcrC−, and mrr−; Gibco BRL, Gaithersburg, MD) was transformed with the ligation mixture by electroporation. Three ampicillin-resistant colonies were identified containing plasmids that, when digested with EcoRI and SalI, generated two fragments. The 1.1-kb fragment was subcloned into pUC19 and designated pPR1.E/5.
Genomic and cDNA Clone Isolation

The 1.1-kb fragment of pPR1.E/S was radiolabeled by random priming and used to screen a genomic library of the Arabidopsis ecotype Columbia (a gift from R. Davis, Stanford University, Stanford, CA). One phage was isolated (λ648) containing a BamHI-derived fragment that hybridized with the pPR1.E/S insert. The fragment was subcloned and designated p648B3. A HindIII-BamHI subclone of p648B3 (0.49 kb) was radiolabeled and used to screen the Arabidopsis cDNA libraries λE/S (a gift from R. Davis; Elledge et al., 1991), λMT (a gift from M. Thomasow, Michigan State University, East Lansing; Hajela et al., 1990), and a λPR-2 (provided by the Arabidopsis Biological Resource Center, Ohio State University, Columbus; Newman et al., 1994). Three positive hybridizing plaques were identified in the λMT library. One phage was successfully converted to a plasmid, as described by the suppliers (Stratagene, La Jolla, CA). The entire insert was isolated by partial digest with EcoRI and cloned into pUC19, thereby generating p20.1.1. This incomplete cDNA was used as a probe to isolate a genomic subclone from λ648 that contained the entire pFCC1 gene (p648BS2).

The λPR-2 library was screened by polymerase chain reaction (PCR) by using primers for the T7 promoter and the 5' end of the original cDNA clone 2aRMT-B (5'-CAAATGTTGCGTACC-3'). The reaction products were used for a second PCR with the T7 primer and a primer 2bRMT-B (5'-CAAATGTTGCGTACC-3') adjacent to the first primer. The larger of the two products (505 bp) was subcloned as pmp42.9 and sequenced. This fragment had 418 bp of PFC1 5' sequence and a frameshift. To isolate the full-length open reading frame of PFC1, first-strand cDNA synthesis was generated from poly(A)+ RNA of wild-type Arabidopsis with Moloney murine leukemia virus reverse transcriptase (Epicentre, Madison, WI). Poly(A)+ RNA was isolated (PolyATtract mRNA isolation system; Promega, Madison, WI). The cDNA strand was used for PCR with primers HH-HE (5'-GGAATTCTGAATGGAT-3') and 2aRMT-B. The reaction products were used for a second PCR with a primer ending with an Xhol site (RMT510) and a primer downstream of a BamHI site (2bRMT-B). A single product of 430 bp was generated, digested with Xhol and BamHI, and cloned into pBluescript SK+ (Stratagene) as p5'-RMT. The 3' end of the original clone p20.1.1 was excised at the internal BamHI site and a PstI site in the multiple cloning sites of the 3' end and inserted in p5'-RMT opened at the BamHI and PstI sites and designated poRM.

DNA and RNA Analyses

Genomic DNA was digested with BamHI and separated by electrophoresis on a 0.8% agarose gel. The DNA was transferred to a Zeta-PROBE nylon membrane (Bio-Rad) by alkaline blotting, according to the vendor instructions. Radiolabeled DNA fragments were made by random primer extension by using the Klenow fragment of DNA polymerase I with [32P]dCTP. Hybridization and washes were performed at 65°C using the standard protocol (Bio-Rad). Total RNA was extracted from Arabidopsis leaves according to the phenol-SDS method (Ausubel et al., 1987). The RNA was analyzed by electrophoresis in a 1.8 M formaldehyde and 1.3% agarose gel and transferred to a Zeta probe membrane by capillary blotting with 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate). Radiolabeled RNA was made by in vitro transcription using a riboprobe (Promega) with [32P]-ATP. Quantitation of RNA gel blots was performed using a Molecular Imager system GS-525 (Molecular Analyst Software version 2.1; Bio-Rad).

DNA Sequence Analysis

DNA sequences were obtained by cycle sequencing (PRISM, version 2.1.1; Applied Biosystems, Inc., Foster City, CA). Sequence compilations and analyses were conducted using the programs available in the Genetics Computer Group (Madison, WI) package versions 8 and 9 (Devereux et al., 1984), using default settings for parameters, unless indicated. The genes that were analyzed included ksgA from E. coli (GenBank accession number M11054; Van Buul and Van Knippenberg, 1985), ermD from Bacillus licheniformis, (GenBank accession number M29382; Gryczan et al., 1984), DIM1 from Saccharomyces cerevisiae (GenBank accession number L26480; Lafontaine et al., 1994), an open reading frame from the Caenorhabditis elegans sequencing project (GenBank accession number Z47075; Wilson et al., 1994), and a human fibroblast cDNA (GenBank accession number W30719). World Wide Web sites accessed include the yeast genome database (http://genome-www.stanford.edu/Saccharomyces), the Synechocystis genome database (http://www.kazusa.or.jp/cyano/cyano.html), the TIGR Arabidopsis expressed sequence tag database (http://www.tigr.org), and BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

Gene Constructs and Plant Transformation

A near-full-length cDNA clone (mp44.2) was constructed for the antisense gene construct. The plasmid pmp42.9 containing the 5' cDNA fragment isolated by PCR screening of the λPR-2 library was linearized at the BamHI site. A 3' cDNA fragment was isolated by BamHI digestion of the original cDNA insert in p20.1.1 and ligated with the linearized pmp42.9. A clone containing the inserted fragment in the correct orientation was identified by restriction analysis (mp44.2). The 3' noncoding region plus 55 bp of the coding region were removed by digestion of mp44.2 with Stul and Smal followed by intramolecular ligation. The remaining cDNA sequence was excised by digestion with KpnI followed by partial digestion with BamHI. The largest fragment (980 bp) was isolated and ligated into the KpnI and BamHI sites of the binary vector pBIN420 (Psychalla et al., 1997) and designated pBIN420-RMT-1. These sites are between the cauliflower mosaic virus 35S promoter and the nopaline synthase 3' terminator and place the PFC1 construct in the antisense orientation for plant gene expression.

The wild-type Arabidopsis plants of the Columbia and Wassilewskija ecotypes used for transformation were 4 months old. The binary vector pBIN420-RMT-1 was transformed into the Agrobacterium tumefaciens GV3101 by the freeze-thaw method (Holsters et al., 1978). Plant transformation was accomplished by using the in-the-plant vacuum infiltration method (Bechtold et al., 1993) with one modification. The plants were infiltrated by inverting the pots and submerging the aerial portions in the Agrobacterium suspension. Seeds produced by the infiltrated plants were germinated on plates containing Murashige and Skoog basal salts (4.3 g/L; Gibco BRL), 1% (w/v) sucrose, 0.8% (w/v) Bacto-agar, 200 mg/L carbenicillin, and 100 mg/L kanamycin and adjusted to pH 5.8 with KOH. Putative transformants were identified by the acquired kanamycin resistance conferred by a gene within the T-DNA encoding neomycin phosphotransferase II.

Primer Extensions with rRNA Templates

A primer extension protocol (Ausubel et al., 1987) was used for RNA analysis with modifications as described below. Oligonucleotide
primers were end-labeled with \(^{32}\)P-ATP by using T4 polynucleotide kinase. The primer 5'-TAAGGAGGTGATCCAA-3' was the reverse complement of the 3' end of the small subunit rRNA of \(E\). coli. The primer 5'-AAGAGGATCCAGC-3' was designed for the same region of plastid rRNA from Arabidopsis (rRNA sequence provided by S. Tabata and S. Sato, Kazusa DNA Research Institute, Kisarazu, Japan). For primer RNA annealing, 2.5 \(\mu\)g of total RNA from \(E\). coli or Arabidopsis was incubated with 2 pmol of primer for 40 min at 65°C and cooled over 10 min to 25°C. Moloney murine leukemia virus reverse transcriptase was used rather than the avian myeloblastosis virus reverse transcriptase. The RNase \(I\) solution contained 10-fold less single-stranded DNA. The reaction products were fractionated on a 10% acrylamide sequencing gel and visualized using a Molecular Imager (model GS525; Bio-Rad).

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Chloroplast Development at Low Temperatures Requires a Homolog of DIM1, a Yeast Gene Encoding the 18S rRNA Dimethylase

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