Plastid Movement Impaired 2, a New Gene Involved in Normal Blue-Light-Induced Chloroplast Movements in Arabidopsis

Darron R. Luesse, Stacy L. DeBlasio, and Roger P. Hangarter*
Department of Biology, Indiana University, Bloomington, Indiana 47405

Chloroplasts move in a light-dependent manner that can modulate the photosynthetic potential of plant cells. Identification of genes required for light-induced plastid movement is beginning to define the molecular machinery that controls these movements. In this work, we describe plastid movement impaired 2 (pmi2), a mutant in Arabidopsis (Arabidopsis thaliana) that displays attenuated chloroplast movements under intermediate and high light intensities while maintaining a normal movement response under low light intensities. In wild-type plants, fluence rates below 20 μmol m⁻² s⁻¹ of blue light lead to chloroplast accumulation on the periclinal cell walls, whereas light intensities over 20 μmol m⁻² s⁻¹ caused chloroplasts to move toward the anticlinal cell walls (avoidance response). However, at light intensities below 75 μmol m⁻² s⁻¹, chloroplasts in pmi2 mutants move to the periclinal walls; 100 μmol m⁻² s⁻¹ of blue light is required for chloroplasts in pmi2 to move to the anticlinal cell walls, indicating a shift in the light threshold for the avoidance response in the mutant. The pmi2 mutation has been mapped to a gene that encodes a protein of unknown function with a large coiled-coil domain in the N terminus and a putative P loop. PMI2 shares sequence and structural similarity with PMI15, another unknown protein in Arabidopsis that, when mutated, causes a defect in chloroplast avoidance under high-light intensities.

Light-directed chloroplast movements have been observed throughout the plant kingdom (Zurzycki, 1961; Lechowski, 1974; Haupt and Scheuerlein, 1990; Gorton and Vogelmann, 1996; Trojan and Gabrys, 1996). Under low-intensity light, chloroplasts migrate to periclinal cell walls in what has been termed the low-light or accumulation response. In contrast, high-intensity light induces movement to anticlinal cell walls in what is referred to as the high-light or avoidance response. Accumulation along the periclinal cell walls under low-light conditions is believed to maximize light capture for photosynthesis, whereas the avoidance response to high light protects chloroplasts from photodamage by positioning chloroplasts in areas where light intensities are lowest (Zurzycki, 1961; Lechowski, 1974; Gorton and Vogelmann, 1996; Park et al., 1996; Trojan and Gabrys, 1996). Indeed, upon transfer to high light, Arabidopsis (Arabidopsis thaliana) mutants that lack light-induced chloroplast movements displayed signs of photodamage more rapidly than wild type (Kasahara et al., 2002).

In Arabidopsis and other angiosperms studied, light-induced chloroplast movement is initiated by blue light via members of the phototropin family of photoreceptors, whereas in algae, moss, and ferns, red light can also initiate movement (Kagawa and Wada, 1994, 1996, Kagawa and Wada, 2000; Jarillo et al., 2001; Kagawa et al., 2001, 2003; Sakai et al., 2001). Previous studies have shown that phototropin1 (phot1) and phototropin2 (phot2) function redundantly under low-intensity blue light to mediate the accumulation response, whereas phot2 appears to drive the avoidance response to high-intensity blue light. Specifically, mutations in phot1 show a slight attenuation in accumulation under low-intensity blue light, but behave normally under high-intensity blue light (Kagawa and Wada, 2000). Mutants in phot2 show chloroplast accumulation on the periclinal cell walls in response to all blue-light intensities tested, even when exposed to high-light conditions that would initiate an avoidance response in wild-type and phot1 plants (Kagawa et al., 2001; Sakai et al., 2001). Plants with mutations in both phototropins lack light-induced chloroplast movement under all tested light conditions (Sakai et al., 2001).

In Lemna triscula, the actin-depolymerizing agent cytochalasin B eliminated chloroplast movement, whereas microtubule-depolymerizing drugs had no impact on movement (Tlalka and Gabryś, 1993). In Arabidopsis mesophyll cells, actin is associated with the outer chloroplast envelope and forms a network of thick and thin filaments, which, when disrupted by lantrunculin B, resulted in abnormal chloroplast aggregation (Kandasamy and Meagher, 1999). Chloroplasts in the chloroplast unusual positioning 1 (chup1)
Chloroplast Movement Mutant

RESULTS

Chloroplast Movements in pmii2 Leaves

The pmii2 mutant was isolated in a screen designed to identify chloroplast movement mutants by measuring changes in red-light transmittance through leaves (Walczak and Gabryś, 1980; DeBlasio et al., 2005). In Arabidopsis, red light does not initiate chloroplast movement, but it is absorbed efficiently by chlorophyll. As chloroplasts relocate in leaf cells, the amount of red light transmitted through the leaf changes, leading to decreases in light transmittance as chloroplasts accumulate along the periclinal cell walls and increases in transmittance when chloroplasts gather on anticlinal walls (Trojan and Gabryś, 1996; Kagawa and Wada, 2000).

Wild-type leaves exposed to sequential 60-min treatments of low (0.2 μmol m⁻² s⁻¹), intermediate (20 μmol m⁻² s⁻¹), and high (60 μmol m⁻² s⁻¹) blue light responded with distinct chloroplast movement responses, as indicated by changes in red-light transmittance (Fig. 1A). Exposure to 0.2 μmol m⁻² s⁻¹ resulted in chloroplast accumulation along periclinal cell walls and a corresponding drop in red-light transmittance. Subsequent treatments of 20 and 60 μmol m⁻² s⁻¹ led to an avoidance response as seen by increased red-light transmittance as the chloroplasts moved to anticlinal sides of cells. Leaves from pmii2 displayed a normal accumulation response under 0.2 μmol m⁻² s⁻¹, but failed to achieve an avoidance response under higher light intensities and instead showed accumulation on the periclinal walls (Fig. 1A). There were, however, slight transient increases in red-light transmittance immediately following initiation of the high-blue-light treatments, indicating that pmii2 can perceive this signal but is unable to develop normal chloroplast movements to anticlinal walls.

To better understand chloroplast movements in pmii2, detailed fluence-response relationships were examined. Upon exposure to continuous low fluence rates of blue light (5 μmol m⁻² s⁻¹ and below), both wild type (Fig. 1, B and C) and pmii2 (Fig. 1, B and D) produced accumulation responses of similar magnitude and kinetics. In wild-type leaves, 10 μmol m⁻² s⁻¹ of blue light induced a biphasic response characterized by an initial increase in transmittance for approximately 10 min, followed by a decrease to about 0.5% below the transmittance of dark-acclimated leaves. In contrast, pmii2 did not display a biphasic response under 10 μmol m⁻² s⁻¹, but instead the chloroplasts moved to a low-light position with the decrease in transmittance reaching about 1.5% below the initial dark level.

The pmii2 phenotype is most pronounced under high fluence rates of blue light. In wild type, 100 μmol m⁻² s⁻¹ of blue light caused robust avoidance movement of chloroplasts to the anticlinal cell walls, which increased red-light transmittance by about 3% (Fig. 1, B and C). At that intensity of blue light, the response in pmii2 leaves was strongly attenuated, reaching a change in light transmittance slightly above the initial dark value (Fig. 1, B and D). At light intensities between 20 and 100 μmol m⁻² s⁻¹, which induce a robust avoidance response in wild type, pmii2 showed biphasic transmittance changes similar to those displayed by wild type under 10 μmol m⁻² s⁻¹ of blue light. As the intensity of blue light increased above about 20 μmol m⁻² s⁻¹, the magnitude of the accumulation phase decreased and the longevity of the avoidance phase of the biphasic response increased in pmii2. These data show that, although pmii2 can perceive high-light treatments, it is defective in some aspect of chloroplast movement following perception of intermediate and high-light intensities because it fails to develop a normal movement response at all fluence rates above 10 μmol m⁻² s⁻¹ (Fig. 1B).

To confirm that the observed changes in red-light transmittance reflected changes in chloroplast movement in pmii2, chloroplast movements were recorded by time-lapse microscopy of dark-acclimated leaves during exposure to sequential treatments of low- and high-intensity white light (Supplemental Movie 1). Under low-intensity white light, chloroplasts of both wild type and pmii2 were found to accumulate along the periclinal walls of palisade cells. Subsequent irradiation with high-intensity white light initiated movement to the anticlinal walls. However, the high-light-induced movements in pmii2 showed a decrease in the rate of movement as well as differences in chloroplast positioning.

Analysis of cross sections of leaves that had been irradiated for 60 min with low (5 μmol m⁻² s⁻¹) or high (60 μmol m⁻² s⁻¹) blue light allowed visualization of the final chloroplast position in all cell layers. After dark treatment, both wild type and pmii2 had about 57% of their chloroplasts located on the periclinal cell walls and 43% on the anticlinal walls (Fig. 2A). After...
exposure to low-fluence-rate blue light, 70% of chloroplasts in both wild-type and pmi2 cells were located along periclinal cell walls. However, under high fluence rates, only 40% of wild-type chloroplasts remained along periclinal cell walls, whereas pmi2 had 53% residing there. Although the pmi2 defect is seen throughout the leaf, the defect is most pronounced in the palisade layer, which shows a 17% difference in chloroplast location between wild type and pmi2 compared to a 10% difference in both lower mesophyll layers (Fig. 2B). Consistent with the light transmittance results, pmi2 chloroplasts have roughly the same distribution after dark and high-light treatments.

Chloroplast movement requires photoreception by the phototropin family of photoreceptors and use of the actin cytoskeleton for movement (Tlalka and Gabrys, 1993; Sakai et al., 2001). The pmi2 mutant was examined for secondary phenotypes that could result from defects in light signaling or the actin cytoskeleton. Phototropism, gravitropism, cell size, and chloroplast size were analyzed in pmi2 and no abnormalities were found (data not shown). To determine whether mitochondrial movement is altered in pmi2, live wild-type and pmi2 leaf segments were stained with MitoTracker Green and examined by time-lapse microscopy (Presley et al., 2003; Bari et al., 2004). Although quantitative measurements of movement were not made, mitochondrial movements appeared to be similarly robust in both wild type and pmi2 (Supplemental Movie 2). Furthermore, under our normal growth conditions, pmi2 plants were visually indistinguishable from wild type and showed no apparent developmental phenotypes.

Lines expressing a green fluorescent protein (GFP) reporter fused to the actin-binding domain of mouse talin (GFP:mTalin) were crossed with pmi2 to allow the

Figure 1. Chloroplast movements in the pmi2 mutant. A, Chloroplast movements in wild-type and two pmi2 mutants in response to sequential treatments of increasing fluence rates of blue light. Red-light transmittance was measured for 90 min in dark-acclimated leaves to establish a baseline, followed by sequential, 60-min treatments of 0.2, 20, and 60 μmol m⁻² s⁻¹ blue light. Blue-light intensity changes occurred at 90, 150, and 210 min (indicated by arrows) for low-, intermediate-, and high-light treatments. Red-light transmittance was recorded every 5 min. For wild type, pmi2-1, and pmi2-2, n = 17, 7, and 7, respectively. B, The fluence-rate response of wild type and pmi2 after 90-min exposures to single fluence rates of blue light. Data points represent the final time points from C and D. C and D, Time course of red-light transmittance changes through leaves in response to different fluence rates of blue light. Red-light transmittance was measured for 60 min in dark-acclimated leaves to establish a baseline before the blue-light treatments were initiated. Red-light transmittance was recorded every 3 min. Data at 5 μmol m⁻² s⁻¹ were similar to data at 1 μmol m⁻² s⁻¹ and were omitted to reduce crowding on the graph. The data are the average ± se from leaves of five to 15 plants per light treatment.
actin cytoskeleton of living pm2 cells to be examined (Kost et al., 1998). As previously reported, Arabidopsis mesophyll cells have thick and thin filaments of actin, as well as shells of polymerized actin surrounding wild-type and pm2 chloroplasts (Kost et al., 1998; Kandasamy and Meagher, 1999). No noticeable differences in the arrangement of the actin network in wild-type (Fig. 3, A and B) and pm2 (Fig. 3, C and D) leaf cells were observed by confocal microscopy. It should be noted, however, that although GFP:mTalin is often used to study actin-based processes in living cells, in our experiments it attenuated light-induced chloroplast movements to roughly one-half the magnitude of that seen in wild-type leaves (Fig. 3E). The cause of this inhibition is unknown, but raises the possibility that the GFP:mTalin may alter the actin cytoskeleton sufficiently to obscure subtle differences in mutant plants.

Identification of PM2

Genetic polymorphisms and recombination frequencies were analyzed in F2 plants obtained from a cross between pm2 Columbia (Col) and Landsberg erecta (Ler) to map pm2 to a 134-kb region on chromosome I, which was covered by one complete and two partial bacterial artificial chromosomes (BACs). The largest of these, F4N21, spanning 97 kb, was partially digested and transformed into Escherichia coli. A contiguous group of clones was used to transform pm2-1 plants. The mutant phenotype was repeatedly rescued by two clones, analysis of which identified a 13-kb interval that contained one partial and four complete open reading frames. Sequencing of this region in pm2 revealed a cytosine to Tyr mutation resulting in a premature stop codon at amino acid 323 of At1g66840 (Fig. 4A). A Salk insertion line (SALK_088187 designated pm2-2), with a T-DNA insert in the second exon of At1g66840 (Alonso et al., 2003), also displayed the pm2 phenotype (Fig. 1A) and failed to complement pm2-1 (data not shown). Taken together, these results indicated that PM2 is At1g66840.

Analysis of the predicted amino acid sequence of PM2 with publicly available programs revealed the
presence of several possible functional domains (Fig. 4B). The main structural feature of PMI2 is a long stretch of coiled-coil regions spanning the N-terminal two-thirds of the protein. The program MultiCoil predicts the presence of three coiled-coil regions spanning amino acids 53 to 87, 119 to 169, and 403 to 437 (Wolf et al., 1997). The Marcoil program predicts five distinct coiled coils from amino acids 58 to 82, 115 to 237, 285 to 393, 407 to 421, and 424 to 428 (Delorenzi and Speed, 2002). Marcoil also identified some lower-scoring coiled-coil motifs between these high-scoring stretches. It is possible that the N-terminal two-thirds of the protein functions as a long coiled coil with one or more low-complexity uncoiled segments in the interior.

A search of the PROSITE database (Gattiker et al., 2002) indicated a putative ATP-/GTP-binding motif A (P loop) from amino acids 560 to 567 (Saraste et al., 1990) and a putative nuclear-localization signal starting at amino acid 584. Further analysis with PredictNLS identified an additional potential nuclear-localization signal in PMI2 in addition to the one identified by PROSITE (Cokol et al., 2000). The first is located at the C terminus of the protein, spanning amino acids 584 to 603. The other, located in the coiled-coil region and extending from amino acids 81 to 88, is also predicted by PredictNLS to bind DNA.

Localization of PMI2

A GFP:PMI2 fusion containing the putative P loop and the C-terminal nuclear-localization domain was created by inserting the carboxy terminus of PMI2 (Glu-509 to Gln-607) into the vector pEGAD (creating construct pP2CTPGD). Wild-type and pmi2-1 plants expressing pP2CTPGD and the control pEGAD showed strong GFP fluorescence in the cytoplasm, but no noticeable localization to the nucleus or any organelle was observed for pP2CTPGD (data not shown). Furthermore, analysis of chloroplast movement in pmi2 plants transformed with pP2CTPGD showed that this construct is not sufficient to rescue the pmi2 mutant phenotype (Fig. 5). Leaves from wild-type plants transformed with pEGAD or pP2CTPGD and visibly expressing GFP showed normal chloroplast movements upon exposure to 60 μmol m⁻² s⁻¹ blue light (Fig. 5C). However, leaves from wild-type plants transformed with pP2CTPGD, but with no visible GFP, displayed only about 0.8% change in red-light transmittance. Although RNA and protein levels in these plants were not examined, activity of the GFP, pP2CTPGD, and native PMI2 were most likely limited by gene silencing, providing further evidence that the mutation found in At1g66840 is responsible for the pmi2 phenotype.

PMI2 Expression

To determine where PMI2 is transcribed, reverse transcription (RT)-PCR was performed on RNA collected from different parts of wild-type and pmi2 plants (Fig. 6). Results indicate that PMI2 is expressed in rosette and cauline leaves, stems, flowers, and roots of wild-type plants as well as in leaves of pmi2-1 and pmi2-2. The presence of PMI2 RNA in pmi2 mutant leaves indicates that transcription is not inhibited by the mutations in these lines. The presence of PMI2 RNA in nonphotosynthetic tissue suggests that PMI2 may have functions in addition to the regulation of chloroplast movement. RT-PCR using primers downstream of the T-DNA insert in pmi2-2 resulted in cDNA amplification, indicating that transcription in this mutant continues through the insert (data not shown).

Analysis of publicly available microarray experiments confirms PMI2 expression throughout the plant (https://www.genevestigator.ethz.ch). Those data also indicate that wild-type seedlings show a 4-fold increase in PMI2 expression over dark-grown controls after a 4-h treatment with white or blue light, and treatments with far-red light lead to a 7-fold increase (Zimmermann et al., 2004). Also, decreased environmental O₂ in
PMI2-Related Genes

Analysis of the Arabidopsis genome revealed that At5g38150 (GenBank accession no. NM_123175) has 55% identity and 65% similarity to PMI2. Structurally, At5g38150 is also predicted to contain a long coiled-coil domain in the N terminus, but lacks the consensus sequences for a P-loop or nuclear localization. To determine whether At5g38150 functions in chloroplast movement, lines with T-DNA insertions in the predicted At5g38150 open reading frame were obtained from the SALK collection and tested for light-induced chloroplast movements. Leaves from SALK_047862 plants displayed attenuated chloroplast movement in response to a high blue-light treatment (Fig. 7). The change in red-light transmittance was 2%, compared to 3% in wild type, after 60 min of 60 μmol m⁻² s⁻¹ of blue light. Due to the pmi phenotype, we have designated this T-DNA mutant as pmi15.

Examination of pmi2 pmi15 double mutants showed movement abnormalities under all intensities of blue light tested (Fig. 7). Under 0.2 μmol m⁻² s⁻¹ blue light, the double mutant displayed an enhanced accumulation response showing a 1.8% change in red-light transmittance compared to the 1.2% seen in the single mutants or wild type. At 20 μmol m⁻² s⁻¹ of blue light, the double mutant showed a greater change in light transmittance associated with chloroplast accumulation than seen in either single mutant, whereas under high blue light (60 μmol m⁻² s⁻¹), the double mutant displayed chloroplast movement similar to pmi2. These results indicate that both PMI2 and PMI15 affect chloroplast avoidance under high-intensity blue light, although the effect of PMI2 is more severe. Furthermore, these genes appear to work redundantly under low-light conditions to regulate the magnitude of chloroplast accumulation and may represent a small family of genes involved in the chloroplast avoidance response.

Analysis of available genomes has revealed a protein in rice (Oryza sativa; BAD82493, GenBank accession no. NP_947041) that is predicted to encode a 1,051-amino acid protein that shares 31% identity and 45% similarity with PMI2 in its carboxy terminus. Like PMI2, it has a long coiled-coil segment (amino acids 480–980) followed by a region with no known motifs. The N-terminal region of BAD82493 does not appear to share significant homology with other known proteins. The consensus sequences for nuclear localization and the putative P loop found in PMI2 are not present in BAD82493; however, PredictNLS indicates three other putative nuclear-localization signals located at positions 271, 326, and 340. Alignments of PMI2, PMI15, and BAD82493 revealed the presence of two highly conserved regions between amino acids 166 to 191 and 16 to 17, 7, 11, and 8 plants, respectively.

DISCUSSION

PMI2 Structure and Function

We have identified PMI2 and PMI15 as genes that are required for proper light-induced chloroplast movements. Under low blue light, pmi2 mutants displayed normal chloroplast movement, whereas under high blue light, pmi2 had markedly attenuated chloroplast movements. Whereas wild-type leaves displayed an avoidance response when exposed to light intensities >20 μmol m⁻² s⁻¹, pmi2 leaves required at least 75 to 100 μmol m⁻² s⁻¹ to produce avoidance movement (Fig. 1). Analysis of leaf cross sections confirmed that the blue-light-induced changes in red-light transmittance measured in pmi2 were a result of fewer chloroplasts moving from the periclinial to the anticlinal cell walls in all cell layers of leaves (Fig. 2).

The two alleles of pmi2 we have identified displayed slightly different phenotypes, with pmi2-2 (T-DNA) showing a less severe reduction in movement under intermediate and high-light conditions than pmi2-1 (ethyl methanesulfonate nonsense; Fig. 1A). The cause

Figure 7. Chloroplast movements in pmi15 and pmi2 pmi15. Chloroplast movements in wild type, pmi2, pmi15, and pmi2 pmi15 double mutants measured as described in Figure 1A. For wild type, pmi2-1, pmi15, and pmi2 pmi15, n = 17, 7, 11, and 8 plants, respectively.
of this is unknown. It is possible that pmii-2 contains a full-length protein, including an insert, which reduces its functionality but does not eliminate it. Conversely, it is possible that the early stop codon in pmii-1 produces a truncated protein that can have a dominant negative effect on chloroplast movement. However, this is less likely because pmii-1 appears to be fully recessive. Antibodies to PMI2 will be required to determine whether there is a stable protein product in either allele. In any case, the phenotypes of both pmii-1 and pmii-2 support the requirement of PMI2 for normal light-induced chloroplast movement under intermediate and high-light intensities.

The phenotype of pmii-2 is distinct from that of other known chloroplast movement mutants. For example, the pmi1 and chup1 mutants display no light-induced chloroplast movement (Oikawa et al., 2003; DeBlasio et al., 2005), the phot1 mutant has a slightly attenuated low-light response (Kagawa and Wada, 2000), phot2 has an accumulation response under any fluence rate of light (Kagawa et al., 2001; Sakai et al., 2001), and the jac1 mutant lacks the low-light accumulation response while retaining the high-light avoidance response (Suetsugu et al., 2005). To some degree, the phenotype of pmii-2 resembles that of phot2 because it shows an accumulation response under high light, although the phenotype is not as severe as in the pmii-2 mutant (Fig. 7). The pmii-2 pmii-15 double mutant had a reduced chloroplast movement response similar to pmii-2 single mutants. Although the double mutant had a slightly greater attenuation under intermediate light conditions, it is apparent that PMI2 is the major player for chloroplast movement.

Structural comparison of PMI2 and PMI15 may provide some insight into the potential functional domains in PMI2. Analysis of the predicted structure of PMI2 has provided few clues to its possible function. Publicly available motif databases suggest three possible functional domains within PMI2: a long coiled-coil domain, two putative nuclear-localization signals, and a putative P loop. BLAST analysis of the Arabidopsis genome with PMI2 revealed one homologous gene, PMI15, for which the predicted amino acid sequences have 55% identity and 65% similarity. Analysis of chloroplast movement in T-DNA mutant pmii15 indicates that it has an attenuated response under high blue light, although the phenotype is not as severe as in the pmii-2 mutant (Fig. 7). The pmii-2 pmii-15 double mutant had a reduced chloroplast movement response very similar to pmii-2 single mutants. Although the double mutant had a slightly greater attenuation under intermediate light conditions, it is apparent that PMI2 is the major player for chloroplast movement.

Figure 8. Alignment of PMI2 (top) and PMI15 (bottom). Black shading indicates identical amino acid sequence in both proteins. Gray shading indicates similar amino acid composition. Dashes indicate gaps inserted to facilitate alignment.

Luesse et al.
that the carboxy-terminal putative nuclear-localization domain is not localized in the cytoplasm (Fig. 5). These results suggest that expressing this construct indicated that it was localized to the nucleus. Our analysis of plants expressing PMI2 using a GFP:PMI2-C-terminal fusion protein that fused to the GFP did not indicate nuclear localization, and therefore, it may not be localized to the nucleus.

Furthermore, no identifiable nuclear-localization signals are present in PMI15, suggesting that PMI2 acts in the phot2 portion of the pathway. The similarity between the phenotypes of pmi2 and phot2 certainly suggests this. It is also possible that PMI2 is not involved in the signaling cascade, but rather has a structural role providing support for phot2 or other components of the pathway. For example, given the function of other coiled-coil proteins, PMI2 could function to orient or hold phot2 to the plasma membrane where it is known to locate (Sakamoto and Briggs, 2002).

In conclusion, we have identified two new genes that are required for a normal chloroplast avoidance response to high fluence rates of blue light. Plants with mutations in the pmi2 gene have normal chloroplast movement under low light intensities, but display aberrant chloroplast orientation under both intermediate and high blue light. PMI2 encodes a protein of unknown function composed mostly of a long coiled-coil motif. A mutation in PMI15, a gene related to PMI2 in Arabidopsis, also affects chloroplast avoidance under high light intensities. Although mutations
in phot2 eliminate the avoidance response, we are not aware of other mutants that specifically attenuate the high-light chloroplast movement. These two proteins add to the growing list of proteins of unknown biochemical function that are associated with light-induced chloroplast movements (Oikawa et al., 2003; DeBlasio et al., 2005; Suetsugu et al., 2005). Because several of the proteins required for chloroplast motility, including phot1, phot2, CHUP1, JAK1, and PMII, appear to be unique to plants, the mechanism of chloroplast motility may be different from other organelles.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Arabidopsis (Arabidopsis thaliana) ecotype Col-01 was used as the wild type in this study. The pmii-1 mutant is in the Col background and was isolated from a population mutagenized with ethyl methanesulfonate (Sigma-Aldrich). The pmii-2 seeds used for these studies were backcrossed three times. The pmii-2 T-DNA insertion line was isolated from the SALK collection (http://signal.salk.edu). Phototropin mutants (Col ecotype) were obtained from Emmanuel Liscum (University of Missouri, Columbia; phot1-5) and Takatoshi Kagawa (University of Tsukuba, Japan; phot2-3). GFP::Talin-expressing lines (Ler ecotype) were provided by Zenbiosh Yang. Seeds were sown in damp Scott’s plug mix (Scotts-Sierra) and incubated at 4°C for 72 h. Plants were germinated and grown at 23°C in a growth room with a 12-h light/dark cycle. Changes in chloroplast movement were determined by blue-light treatments. High-light chloroplast movement. These two proteins appear to be unique to plants, the mechanism of chloroplast motility may be different from other organelles.

**Mutant Isolation and Light Transmittance Measurements**

Details of the procedure for identifying chloroplast movement mutants have been previously described (DeBlasio et al., 2005). For characterization of chloroplast movements in mutant and wild-type plants, leaves were excised from plants after 11 h of darkness near the end of their subjective 12-h night period. The excised leaves were placed in a dark humid chamber with their petioles held in water-filled microfuge tubes for 15 min to 6 h. To prepare for measurement, a leaf blade was sandwiched between two glass microscope slides (VWR International) with its petiole protruding from the slides. The petiole was wrapped with wet paper towel to ensure hydration. The assembly was then arranged on a stage so the leaf covered a 5-mm diameter red Plexiglas window (Rohm and Haas no. 2423; Dayton Plastics). The sensor from a LI-COR 1800 spectroradiometer (LI-COR) was placed directly below the red Plexiglas window and a red light-emitting diode (660 nm; Radio Shack) was mounted directly above the leaf to provide 20 to 30 mW cm⁻² red light for measurements of leaf transmittance. Blue light to induce movement was generated by passing light from a halogen fiber-optic light microscope illuminator (Cole Palmer) through a blue interference filter 450 ± 25 nm (03FB304; Melles Griot). The light from the fiber-optic assembly illuminated the leaf at an angle of 60° relative to the leaf surface. Changes in blue-light intensity were achieved using neutral density filters. Red-light transmittance through leaves was measured with a LI-COR 1800 spectroradiometer by integrating the quantum flux between 650 and 670 nm for each time point. For each leaf, the change in percent of red-light transmittance was calculated as (I_r/I_o) × 100/I_o, where I_r and I_o are the incident and transmitted red-light fluence rate, respectively, and I_o is the average red-light transmittance value measured prior to the blue-light treatments. Results are presented as the average change in percent of red-light transmittance ± SE.

**Mapping and Identification of PMII**

Crosses were made between pmii-2 (Col ecotype) and the Ler ecotype. Analysis of polymorphisms in 94 F2 plants homozygous for pmii-2 placed the mutation in a 135-kb region between markers at 35,308 bp on BAC T12I7 and 35,116 bp on BAC T4024.

Plasmids containing BAC F4N21 (Arabidopsis Biological Resource Center [ABRC]) were partially digested with Sau3AI (New England Biolabs) for 30 min at 37°C to obtain 15- to 25-kb fragments. These fragments were ligated (T4 DNA ligase; New England Biolabs) at 4°C overnight to binary vector pCLD04541 (ABRC) that had been digested with BamHI (New England Biolabs). Plasmid DNA was introduced into Escherichia coli (DH5α) using a Gigapack III XL cosmid packaging kit (Stratagene). E. coli colonies were screened with PCR markers located every 10 kb along the length of BAC F4N21. Colonies containing 10- to 30-kb fragments were identified that formed a contiguous sequence across the entire BAC. These were mated into Agrobacterium tumefaciens (GV3101) using E. coli helper strain pRK2013 and used to transform pmii-2 plants by floral dipping (Clough and Bent, 1998). Transformants were isolated as described in DeBlasio et al. (2005).

**Microscopy**

Cross sections of leaves were made to measure chloroplast positions in leaf cells. Leaves that had been exposed to darkness, low-intensity (1 μmol m⁻² s⁻¹), or high-intensity (60 μmol m⁻² s⁻¹) blue light were cut into small pieces (1 × 0.5 mm), dehydrated in acetone, fixed in a solution of 4% formaldehyde and 5% gluteraldehyde (Electron Microscopy Sciences), and embedded in soft Spurr’s resin (Electron Microscopy Sciences). The embedded leaf pieces were sectioned using an automated ultramicrotome and a glass knife. Chloroplasts and cell walls were stained using bromphenol blue (Sigma-Aldrich). Micrographs obtained from the sections were analyzed by counting chloroplasts positioned along the top, bottom, left, and right cell wall. The data from each micrograph were considered one data point.

To analyze chloroplast movement in palisade mesophyll cells with time-lapse microscopy, 1-mm pieces of live, dark-acclimated leaves, excluding the midvein, were cut with a razor and mounted on a slide in water. Images of the adaxial surface of palisade mesophyll cells were captured every 30 s with MetaMorph software (Universal Imaging) and a Hamamatsu ORCA-ER CCD camera mounted on a Nikon E800 microscope. While imaging, the leaf sections were given 1-h sequential irradiations of low and high light from below using the built-in bright-field lamp. To measure cell and chloroplast area, leaf pieces were prepared as described above. The diameter of palisade mesophyll cells and the chloroplasts within were determined using ImageJ (http://rsb.info.nih.gov/ij/).

Mitochondria were observed in live tissue by vacuum infiltrating 1-mm pieces of freshly cut leaves with the green fluorescent dye MitoTracker Green (Invitrogen). Images of cells with stained mitochondria were captured every second to detect mitochndria movements with the same equipment used for visualization of chloroplast movement.

To view PMII localization, a gene construct was made by fusing the carboxy terminus of PMII with GFP. A 300-bp C-terminal region of PMII was amplified with primers (forward primer 5′-GGATCCTAGGAGAGAGGAAGCATTAGGAGATG-3′ and reverse primer 5′-GGATCCTAATTCGACTGAGGAATGAAATCTG-3′) containing BamHI restriction sites. The amplified fragment was ligated into the vector pGEM-T (Invitrogen). The insert was then excised from pGEM-T by digestion with BamHI and ligated into vector pEAGG (ABRC) that had been cut with BamHI to create plasmid p2PCTPGD. The transformation vector pEAGG contains a polynucleoty cloning region downstream of GFP. Using the floral-dip method, p2PCTPGD was transformed into wild-type and pmii-2 plants. Leaves were prepared for microscopy as described above. GFP fluorescence from leaves expressing GFP::Talin, p2PCTPGD, and pEGAD was recorded using a spinning disc confocal microscope (Perkin-Elmer). The images shown are projected views from between 10 and 40 optical sections taken every 0.5 to 0.1 nm.

**Tropisms**

The phototropic response of pmii-2 was determined by germinating seeds on vertical square petri dishes containing 0.5 × Murashige and Skoog medium (Sigma-Aldrich). After 60 h of etiolated growth, seedlings were exposed to 6 h of 1 μmol m⁻² s⁻¹ unilateral blue light. Gravitropism was tested by a 90° reorientation of 60-h-old etiolated seedlings grown on vertical petri dishes containing 0.5 × Murashige and Skoog and 1% Suc. Curvature was measured using ImageJ (http://rsb.info.nih.gov/ij/).

**Confirmation of T-DNA Inserts from the SALK Database**

The position of the T-DNA insert in pmii-2 (SALK_088187) was confirmed through PCR amplification with primer Lba-1 (located on the T-DNA insert:

1336


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5′-TGTTACCGTATGTTGCCATCG-3′) and primers flanking the predicted insert (5′-AAACATACATATCCAATCTCC-3′ and 5′-CTTAATCCGCAATCAACATCT-3′). The T- DNA insert in AT5g38150 in plants homozygous for SALK_047862 was confirmed by PCR using primers Lba-1 and primers flanking the predicted insertion site (5′-GTTGGAATGTTGATCTATGCC-3′ and 5′-TCAACATTTCTGACAACATATAAC-3′).

RT-PCR

Isolation of total RNA was performed using the RNeasy plant mini kit (Qagen). Rosette leaf and root pieces were collected from 6-week-old plants. Cauline leaves, stem segments, and flowers were collected formed 10-week-old plants. RT-PCR was performed on a 0.5-μg sample of RNA from each sample using the Titan One Tube RT-PCR system (Roche Diagnostics). Primers using the Titan One Tube RT-PCR system (Roche Diagnostics). Primers (5′-GAATTTAGATGGAACTGTATCAG-3′ and 5′-CTTACGAGACTCA-GACA-3′) were designed to flank the intron in PM12 to differentiate RNA amplification from amplification of contaminating DNA. For a loading control, amplification of the Arabidopsis ubiquitin-conjugating enzyme (At5g25760) was used (5′-TCAACATTTCTGACAACATATAAC-3′ and 5′-TCAACATTTCTGACAACATATAAC-3′), designed around an intron.

Sequence Alignments

Sequence alignments of PM12, At6g35180, and BAD82493 were produced by the publicly available program TOCOFFEE. Outputs were produced by BOXSHADE (Swiss EMBl node server, Swiss Institute of Bioinformatics; http://www.ch.embnet.org/index.html).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_105355 (pmi1), NM_123175 (pmi15), and NP_947041 (BAD82493).

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LITERATURE CITED


