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Multiubiquitin Chain Binding Subunit MCB1 (RPN10) of the 26S Proteasome Is Essential for Developmental Progression in *Physcomitrella patens*

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The 26S proteasome, a multisubunit complex, is the primary protease of the ubiquitin-mediated proteolytic system in eukaryotes. We have recently characterized MCB1 (RPN10), a subunit of the 26S complex that has affinity for multiubiquitin chains in vitro and as a result may function as a receptor for ubiquitinated substrates. To define the role of MCB1 further, we analyzed its function in *Physcomitrella patens* by generating *MCB1* gene disruptions using homologous recombination. PpMCB1, which is 50 to 75% similar to orthologs from other eukaryotes, is present in the 26S proteasome complex and has a similar affinity for multiubiquitin chains, using a conserved hydrophobic domain within the C-terminal half of the polypeptide. Unlike yeast Δmcb1 strains, which grow normally, *P. patens* Δmcb1 strains are viable but are under developmental arrest, generating abnormal caulonema that are unable to form buds and gametophores. Treatment with auxin and cytokinin restored bud formation and subsequent partial development of gametophores. Complementation of a Δmcb1 strain with mutated versions of *PpMCB1* revealed that the multiubiquitin chain binding site is not essential for the wild-type phenotype. These results show that MCB1 has an important function in the 26S proteasome of higher order eukaryotes in addition to its ability to bind multiubiquitin chains, and they provide further support for a role of the ubiquitin/26S proteasome proteolytic pathway in plant developmental processes triggered by hormones.

INTRODUCTION

Selective protein degradation is essential for many aspects of plant cell homeostasis, growth, and development (Callis, 1995; Vierstra, 1996). It is important not only for removing aberrant polypeptides but also for dismantling existing enzyme and regulatory protein networks as cells switch from one metabolic/developmental state to another. One major route in eukaryotes employs the 76–amino acid protein ubiquitin as a reusable proteolytic signal (Coux et al., 1996; Vierstra, 1996; Hershko and Ciechanover, 1998). In this pathway, chains of ubiquitin are covalently attached to proteins committed for degradation. The resulting ubiquitin–protein conjugates are then broken down by the 26S proteasome, a multisubunit proteolytic complex that degrades the target protein but releases the ubiquitin molecules intact. The 26S proteasome is composed of two subcomplexes, a 20S cylindrical core particle that contains the protease active sites and a 19S regulatory particle that confers substrate specificity (Löwe et al., 1995; Groll et al., 1997; Glickman et al., 1998a). In animals and yeast, the ubiquitin/26S proteasome pathway plays a role in a number of cellular processes, primarily by controlling the degradation of one or more short-lived enzymes/regulators. Examples include cell cycle progression, morphogenesis, neuronal function, transcriptional regulation, DNA silencing and repair, the stress response, signal transduction, and programmed cell death (Coux et al., 1996; Hershko and Ciechanover, 1998).

Although the biochemical and molecular aspects of the ubiquitin/26S proteasome pathway in higher plants have been characterized in considerable detail, an understanding of the pathway's role in plant growth and development is still rudimentary (Vierstra, 1996). Numerous enzymes required for ubiquitin conjugation and most of the subunits of the 26S proteasome have been identified, with an emphasis on using Arabidopsis as a model (Vierstra, 1996; Parmentier et al., 1997; Fu et al., 1998a, 1999). Nonetheless, we still do not know the identity of most pathway targets or the phenotypic consequences of their breakdown. To date, phytochrome

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A and the A- and B-type mitotic cyclins are the only known natural targets in plants (Clough and Vierstra, 1997; Genschik et al., 1998).

One of the main obstacles has been the lack of informative ubiquitin/26S proteasome pathway mutants. Identification of such mutants has been hindered by the lack of predictable phenotypes and by the fact that most pathway components are encoded by multiple genes in plants (Vierstra, 1996). However, several pathway mutants have been described. The first was created by ectopic expression of a ubiquitin variant bearing a lysine to arginine substitution at position 48 (Bachmair et al., 1990). This variant interferes dominantly with the ubiquitin pathway by blocking the polymerization of multiubiquitin chains internally linked through residue 48 (Chau et al., 1989). When expressed in tobacco, Arg-48 ubiquitin disrupted vascular tissue development, induced leaf curling, and initiated necrosis, suggesting a role in programmed cell death (Bachmair et al., 1990; Becker et al., 1993).

Shirley and Goodman (1993) identified fortuitously an Arabidopsis mutant missing a gene encoding the 26S proteasome subunit α6 (designated PAF1 [Fu et al., 1998a]). Although a comparable mutation is lethal in yeast, Arabidopsis *Δ*paf1 is phenotypically normal, consistent with subsequent studies showing that a *PAF1* paralog exits (Fu et al., 1998a). Bachmair et al. (1993) identified a possible Arabidopsis mutant in the N-end rule pathway, a subpathway within the ubiquitin/26S proteasome system that selects targets for breakdown based on the nature of their N-terminal residue (Varshavsky, 1997). Although this mutant has a compromised N-end rule pathway, it appears phenotypically normal (Potuschak et al., 1998).

More recently, Arabidopsis mutants defective in the auxin and jasmonate response pathways (*transport inhibitor response 1* [*tir1*] and *coronatine-insensitive-1* [*coi1*] [Ruegger et al., 1998; Xie et al., 1998]) and related Arabidopsis and Antirrhinum floral development mutants (*unusual floral organs* [*ufo*] and *fimbriata* [*fim*] [Ingram et al., 1995]) have been described. All four genes encode proteins related to the F-box family proposed to function in enzyme complexes that selectively recruit proteins for ubiquitination. It has been proposed that both TIR1 and COI1 are involved in the ubiquitination (and presumably degradation) of short-lived proteins necessary for auxin and jasmonate signaling.

In recent years, an alternative to the forward genetic isolation of mutants has been the use of reverse genetic approaches to impair the function of known genes. Unfortunately, two common methods (antisense and cosuppression) have failed to generate useful ubiquitin/26S proteasome pathway mutants in Arabidopsis, presumably due to the presence of paralogs and/or isologs with overlapping functions (van Nocker et al., 1996a; P. Bates, S. van Nocker, and R.D. Vierstra, unpublished data). Given the lack of efficient homologous recombination, disruption of individual genes in higher plants has been limited, for the most part, to random mutagenesis with Agrobacterium T-DNA and transposable elements (Krysan et al., 1996; Long and Coupland, 1998). However, Schaefer and Zyrd (1997) recently overcame this barrier in the haploid moss *Physcomitrella patens* with the development of an efficient method for targeted gene disruption. They showed that this seedless terrestrial plant will integrate introduced DNA at high frequency by homologous recombination, thus providing the first reliable method for generating genetic “knockouts” in a plant. The power of this approach has been demonstrated by the creation and analysis of *P. patens* knockouts in the *FTSZ* tubulin and *PPDES6* Δ6-acyl desaturase genes, which revealed the function of the encoded proteins in chloroplast division and fatty acid biosynthesis, respectively (Girke et al., 1998; Strepp et al., 1998).

We have begun to exploit *P. patens* for the reverse genetic analysis of the ubiquitin/26S proteasome pathway. Here, we report the creation of a disruption in the *MCB1* gene (also designated *RPN10* [Finley et al., 1998]), encoding a component of the 19S regulatory complex of the 26S proteasome. PpMCB1 orthologs from other species (Arabidopsis MPB1, Drosophila p54, yeast MCB1, and human S5a) have affinity for multiubiquitin chains in vitro, suggesting that they function within the 26S complex as receptors for multiubiquitinated substrates (Deveraux et al., 1994; van Nocker et al., 1996b, 1996c; Haracska and Udvardy, 1997; Fu et al., 1998b). Like its yeast counterpart (van Nocker et al., 1996c), we found that moss MCB1 is not essential, providing further evidence that additional ubiquitin receptors besides MCB1 must associate with the complex. However, unlike yeast *Δ*mcb1 mutants, *P. patens Δ*mcb1 strains arrest developmentally, being impaired in caulonema differentiation and unable to make gametophores. This developmental block was partially relieved by application of auxin and cytokinin, suggesting that PpMCB1 is required for a hormonal signaling pathway. The multiubiquitin binding site is not essential for this developmental transition, indicating that MCB1 has another function(s) in the ubiquitin/26S proteasome proteolytic pathway in addition to ubiquitin recognition. Based on this example, *P. patens* should be an excellent reverse genetic model for uncovering the functions of many factors within this complex proteolytic pathway.

RESULTS

Cloning and Analysis of *P. patens MCB1*

PpMCB1 was chosen as a suitable target for generating our first *P. patens* knockout in the ubiquitin/26S proteasome pathway. Our choice was based on preliminary immunoblot and genomic DNA gel blot analyses that showed that the moss contains a protein related to higher plant MCB1 that, like its Arabidopsis ortholog (van Nocker et al., 1996a), is encoded by a single gene (data not shown). Using Arabidopsis *MCB1* as a probe, we isolated nine *PpMCB1* cDNAs from a λ ZAP cDNA library prepared from polyadenylated *P. patens* RNA. All nine clones represented overlapping cDNAs derived from the same locus based on similar restriction maps and subsequent DNA sequencing. From alignments with orthologs from other species, the longest cDNA appeared to contain the entire coding region of 414 amino acids (Figure 1). Derived amino acid sequence identity and similarity between PpMCB1 and its plant, yeast, and animal orthologs are 65 and 75% (Arabidopsis), 65 and 73% (ice plant; Genbank accession number AF069324), 47 and 58% (yeast), 44 and 53% (human), and 41 and 50% (Drosophila). Significant conservation was evident for the N-terminal half, a domain near the C terminus, and an interior domain containing a hydrophobic seven–amino acid motif, LALALRV (Figures 1 and 2A). Previous studies of MCB1 proteins from several species have shown that this hydrophobic motif is essential for multiubiquitin chain recognition (Fu et al., 1998b; Young et al., 1998). Like its plant and metazoan counterparts, PpMCB1 contains a 146-residue C-terminal extension not present in the yeast ortholog. Coimmunoprecipitation experiments subsequently revealed that PpMCB1 is part of the moss 26S proteasome complex (see below).

When expressed in *Escherichia coli*, the *PpMCB1* cDNA directed the synthesis of a soluble protein with an apparent molecular mass of 44 kD, in close agreement with its predicted mass (Figure 2B). As can be seen in Figure 2C, the *E. coli*–expressed PpMCB1 protein, like its animal, yeast, and plant homologs (van Nocker et al., 1996b, 1996c; Haracska and Udvardy, 1997; Fu et al., 1998b), readily bound Lys-48–linked multiubiquitin chains after SDS-PAGE and electrophoretic transfer of the recombinant protein onto nitrocellulose membranes. Similarly, PpMCB1 preferred interacting with multiubiquitin chains over free ubiquitin. When probed with a mixture of free ubiquitin and multiubiquitin chains of

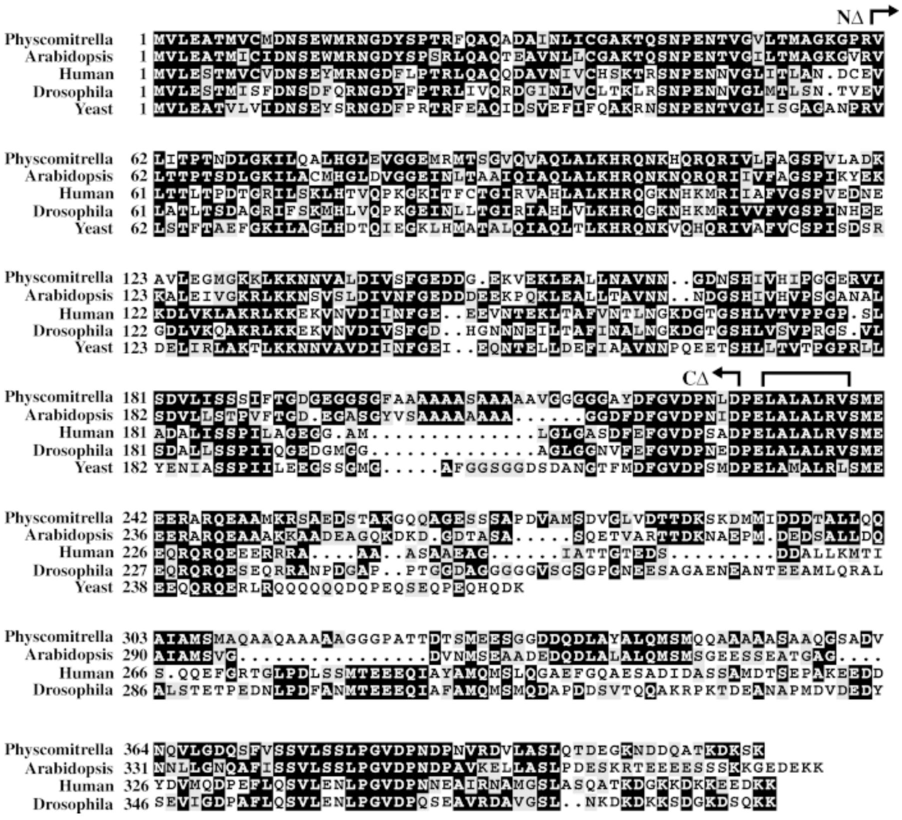

Figure 1. Amino Acid Sequence Comparison of *P. patens* MCB1 with Orthologs from Other Eukaryotes.

Orthologs include Arabidopsis MCB1 (MBP1 [van Nocker et al., 1996a]), human S5a (Ferrell et al., 1996), Drosophila μ54p (Haracska and Udvardy, 1995), and yeast MCB1 (RPN10 [van Nocker et al., 1996b]). Residues that are identical and similar among the sequences are displayed in black and gray boxes, respectively. Dots indicate gaps in the amino acid sequence. The figure was generated using the computer program BoxShade 2.7 (vax0.biomed.uni-koeln.de). The hydrophobic patch required for interaction of MCB1 proteins with multiubiquitin chains is identified by the bracket (Fu et al., 1998b). The beginning of the NΔ and the end of the CΔ coding regions are shown by arrows (see Figure 2).

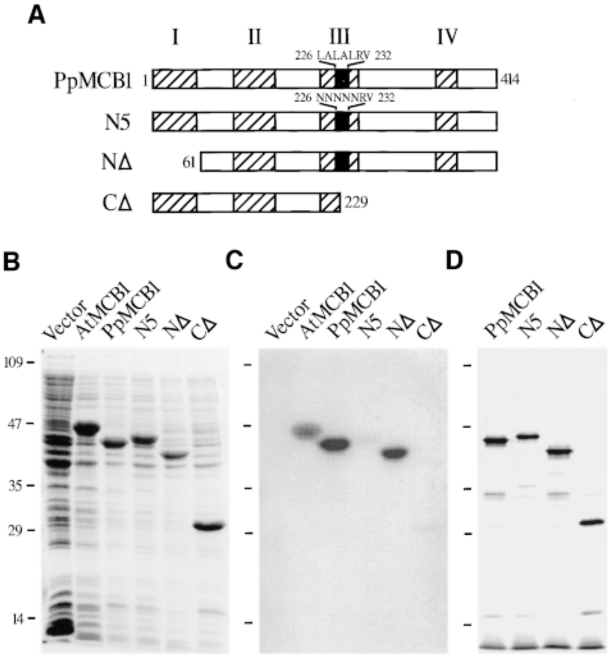

Figure 2. Affinity of *P. patens* MCB1 for Multiubiquitin Chains.

(A) Schematic diagram of wild-type and mutated versions of PpMCB1. The hatched boxes locate four regions of high amino acid sequence similarity among the MCB1 proteins from various eukaryotes as identified by Fu et al. (1998b). The black box identifies the hydrophobic domain required for multiubiquitin chain binding; alteration of this sequence in the N5 mutation is shown above the box.

(B) to **(D)** Expression, in vitro multiubiquitin chain binding activity, and antigenicity of wild-type and mutant verions of PpMCB1. **(B)** Coomassie Brillant Blue R 250–stained gel of *E. coli*–expressed proteins. **(C)** Autoradiogram of a duplicate gel shown in **(B)** in which the proteins were electrophoretically transferred onto nitrocellulose and probed with a heterogeneous mix of Lys-48–linked multiubiquitin chains labeled with iodine-125. **(D)** Immunoblot of a duplicate gel shown in **(B)** in which the proteins were probed with Arabidopsis anti-MCB1 antibodies. AtMCB1, wild-type MCB1 protein from Arabidopsis similarly expressed in *E. coli.* Molecular mass markers for **(B)** to **(D)** are given at left in kilodaltons.

varying lengths, only those chains containing three or more ubiquitin molecules interacted well with the immobilized PpMCB1 protein (data not shown).

Previous studies with Arabidopsis, yeast, and human MCB1 identified a hydrophobic patch (LAL/MALRV/L) that is essential for multiubiquitin chain binding (Fu et al., 1998b; Young et al., 1998). To confirm that this conserved patch is also required for ubiquitin recognition by PpMCB1, a set of three mutations was created, expressed in *E. coli*, and tested for chain binding (Figures 2A and 2B). The N5 mutation converted the hydrophobic LALAL sequence to a more hydrophilic stretch of five asparagine residues, and the CΔ mutation deleted the C-terminal 185 residues, including the LALALRV domain. Similar alterations in the yeast and Arabidopsis orthologs eliminated multiubiquitin chain binding (Fu et al., 1998b). The NΔ mutation had the N-terminal 61 amino acids removed. Although comparable mutants of yeast and Arabidopsis MCB1 can still bind multiubiquitin chains, this N-terminal domain is necessary in yeast for degradation of a subset of ubiquitin/26S proteasome pathway targets and for resistance to amino acid analogs (Fu et al., 1998b). When we tested the *P. patens* MCB1 mutants for their affinity for free multiubiquitin chains, only the NΔ deletion retained activity (Figure 2C). Neither the N5 nor CΔ mutants showed significant binding, implicating the C-terminal region, and more specifically the LALALRV sequence, in chain recognition.

Targeted Disruption of *PpMCB1*

Using the method of Schaefer and Zyrd (1997), we created a disruption of *P. patens MCB1* by homologous recombination. The knockout construction contained 67 bp of 5′ untranslated region (UTR) and 672 bp of 5′-*PpMCB1* coding sequence, the hygromycin phosphotransferase (*HPH*) gene, the expression of which was driven by the cauliflower mosaic virus (CaMV) 35S promoter, followed by 562 bp of 3′-*PpMCB1* coding sequence and 28 bp of 3′ UTR (Figure 3A). A linear DNA fragment was amplified from this sequence by polymerase chain reaction (PCR) with primers 1-F and 1-R (see Figure 3A) and used to transform *P. patens*. From 1.2×10^6 protoplasts derived from haploid protonema, 55 hygromycin-resistant colonies appeared. PCR analysis of genomic DNA using primers outside the knockout DNA fragment (primers 11-F and 11-R; Figure 3A) in conjunction with primers within the *HPH* gene revealed two colonies bearing an insertion in the *PpMCB1* locus (data not shown).

The genomic organization of one of the targeted hygromycin-resistant colonies provided further evidence that the *PpMCB1* locus was disrupted (Figure 3B). As compared with that from a wild-type strain, DNA gel blot analysis of the *PpMCB1* locus from the *Δ*mcb1 strain had a distinct, more complex fragment pattern for all restriction endonucleases used. DNA sequence analysis of the junctions between the *PpMCB1* gene and the knockout cassette revealed that the insertion of the *HPH* gene into this strain occurred by homologous recombination at the 3′ end and by illegitimate recombination at the 5′ end. (This *Δ*mcb1 strain was used subsequently for the complementation studies, amino acid analog sensitivity, and 26S proteasome isolation [see below].) As expected, this disruption eliminated expression of the PpMCB1 protein. Whereas PpMCB1 could be easily detected in wild-type *P. patens* by immunoblot analysis with Arabidopsis anti-MCB1 antibodies, none could be detected in the *Δ*mcb1 strain (Figure 3C).

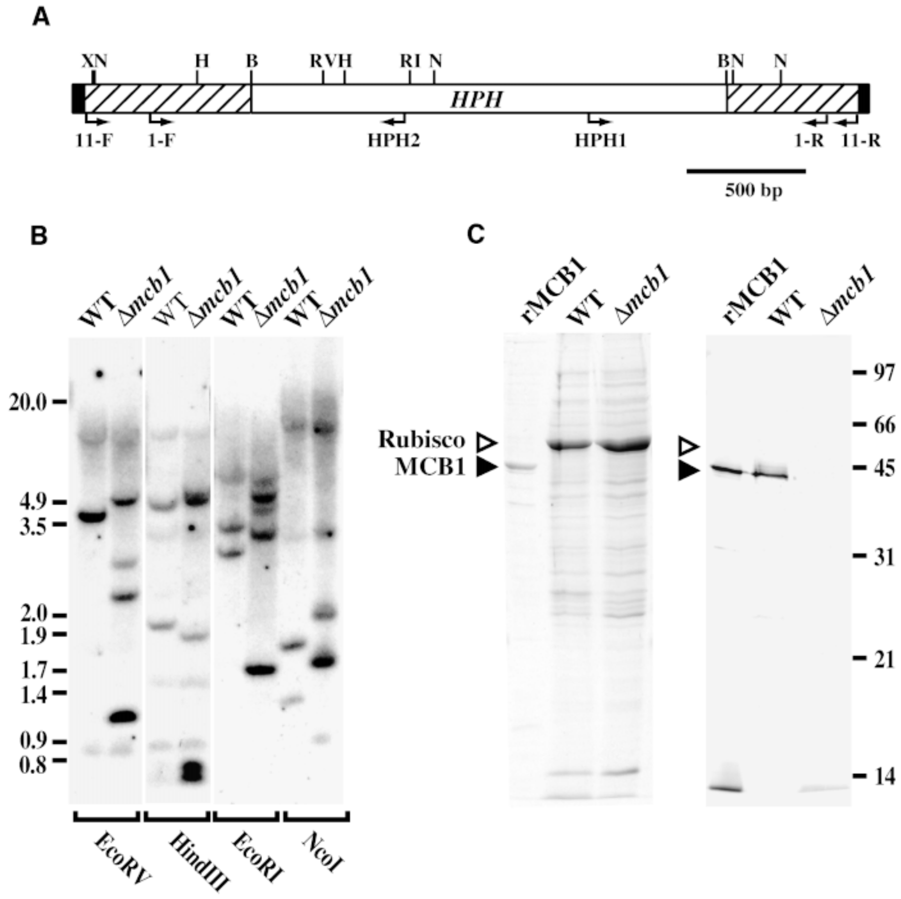

Figure 3. Molecular Analysis of a *P. patens Δ*mcb1 Mutant Generated by Targeted Disruption.

(A) Schematic diagram of the vector used to disrupt the *PpMCB1* gene by homologous recombination. White, hatched, and black boxes denote the hygromycin phosphotransferase (*HPH*) gene, the *PpMCB1* gene fragments, and pBluescript sequences, respectively. Location of the PCR primers used to amplify the knockout construction before transformation, to confirm insertion of the *HPH* gene into the *PpMCB1* genomic locus, and to generate the radiolabeled probes for DNA gel blot analysis are indicated below. F and R denote forward and reverse primers, respectively. Restriction endonuclease cut sites used for DNA gel blot analysis and/or various DNA reconstructions are shown above. B, BamHI; H, HindIII; N, NcoI; RI, EcoRI; RV, EcoRV; X, XbaI.

(B) DNA gel blot analysis of genomic DNA from wild-type (WT) *P. patens* and a *Δ*mcb1 strain. Genomic DNA was digested with EcoRV, HindIII, EcoRI, and NcoI and probed with a 1.3-kb *PpMCB1* cDNA fragment amplified by PCR. Molecular mass markers are given at left in kilobase pairs.

(C) Immunoblot detection of PpMCB1 proteins. The left gel is a Coomassie Blue–stained gel of total soluble protein isolated from the wild type (WT) and the *Δ*mcb1 strain. Full-length PpMCB1 protein expressed in *E. coli* is shown on the left (MCB1). Gel on the right is an immunoblot of a duplicate gel with Arabidopsis anti-MCB1 antibodies. Migrations of PpMCB1 and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are identified by arrowheads. Molecular mass markers are given at right in kilodaltons. rMCB1, recombinant *P. patens* MCB1 expressed in *E. coli*.

Disruption of *PpMCB1* Impairs *P. patens* Development

Following spore germination, the life cycle of *P. patens* begins with the formation of a filamentous haploid protonemal colony (reviewed in Cove, 1992; Cove and Knight, 1993; Reski, 1998). Chloronema cells, which develop first, have abundant large chloroplasts and perpendicular septa separating the individual cells in the filament. A second cell type—caulonema—arises from chloronema by anticlinal cell divisions. Caulonema have oblique septa, are very elongate, and contain fewer and smaller chloroplasts. Buds eventually develop as side branches on the caulonemal filaments; the buds then differentiate into large leafy gametophores bearing antheridia and archegonia in which the male and female

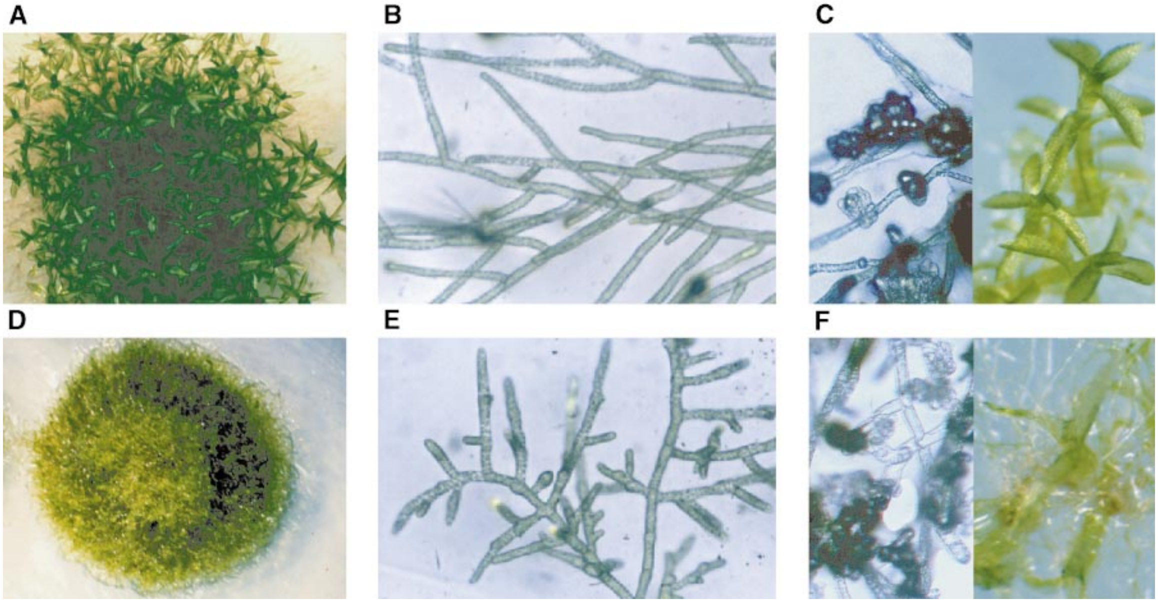

Figure 4. Disruption of *PpMCB1* Inhibits Developmental Progression.

Wild-type *P. patens* (**[A]** to **[C]**) and a Δmcb1 strain (**[D]** to **[F]**) were grown for 14 days in 16-hr-light/8-hr-dark photoperiods on minimal NO_3 media without or with 50 nM of the auxin IAA and 50 nM of the cytokinin IPA.

(A) and **(D)** Colonies of wild-type and Δmcb1 strains.

(B) and **(E)** Enlargements showing the protonemal cells.

(C) and **(F)** Colonies of wild-type and Δmcb1 strains treated with IAA and IPA, showing the formation of buds (left panels) and gametophore-like structures (right panels) from the Δmcb1 protonema.

gametes are generated, respectively. Fertilization creates a diploid zygote that produces haploid spores by meiosis, thus completing the life cycle.

Wild-type *P. patens* proceeds normally through its life cycle of protonemal growth, bud formation, and gametophore development when grown on either NH_4- or NO_3-containing minimal media (Figures 4A and 4B). By contrast, we observed that the Δmcb1 strains arrested at the early stages of caulonema development (Figures 4D and 4E). The chloronema cells that formed from the mutant colonies were slightly shorter but otherwise normal in appearance. Cells with oblique septa and resembling caulonema cells originated from the chloronema; however, these cells were severely stunted and had swollen tips as compared with the highly elongate morphology that is typical of wild-type *P. patens* caulonema cells. The Δmcb1 strains failed to progress developmentally beyond this point. Consequently, whereas wild-type *P. patens* generated an expanding protonemal colony decorated with numerous buds and gametophores, the Δmcb1 strains remained as a solid expanding clump of filaments without buds or gametophores even after months of growth (Figure 4D).

For wild-type *P. patens*, formation of buds and subsequent gametophores is hormonally induced and can be accelerated by the addition of auxin and cytokinin to the medium (Ashton et al., 1979). Here, we observed a similar promotive effect when the wild-type strain was grown on the auxin indoleacetic acid (IAA) and the cytokinin isopentenyladenine (IPA) (Figure 4C). For the Δmcb1 strains, this treatment restored the formation of caulonema cells and stimulated the development of buds and gametophores. When exposed to as little as 50 nM IAA and IPA, buds arose from the Δmcb1 cultures and eventually progressed to form gametophores (Figure 4F). However, these gametophore structures did not expand normally and did not develop beyond the four- to six-leaf stage (Figure 4F). In addition, these restored gametophores were substantially shorter and had thinner leaves containing less chlorophyll. (It was not determined if antheridia or archegonia were formed.) A combination of both hormones was most effective. IAA alone, at concentrations up to 5 μM, induced only caulonema differentiation, whereas concentrations of IPA up to 5 μM induced bud formation but little gametophore development in the Δmcb1 strains (data not shown).

Yeast Δmcb1 strains are unable to degrade a subset of ubiquitin/26S proteasome pathway targets (van Nocker et al., 1996b). As a consequence, the *P. patens* Δmcb1-conferred phenotype could have arisen by a stabilization of one or more proteins (repressors?) whose removal is required for protonema to proceed beyond early caulonema devel-

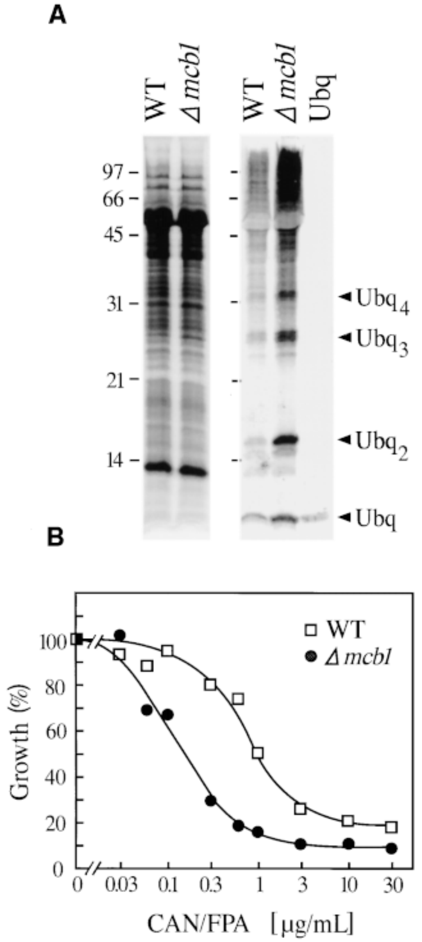

Figure 5. Disruption of *PpMCB1* Elevates the Levels of Ubiquitin and Ubiquitin Conjugates and Increases the Sensitivity to Amino Acid Analogs.

(A) Immunoblot analysis of total protein extracted from wild-type (WT) and Δmcb1 protonema. Gel at left shows equal amounts of protein subjected to SDS-PAGE and Coomassie Blue staining. Gel at right shows equal amounts of protein subjected to SDS-PAGE and immunoblot analysis with plant anti-ubiquitin antibodies. Ubq, purified ubiquitin. Arrowheads identify free ubiquitin and multiubiquitin chains of various lengths. Molecular mass markers are given at left in kilodaltons.

(B) Growth of wild-type WT (□) and Δmcb1 (●) strains on various concentrations of the amino acid analogs CAN and FPA. After 18 days of growth on minimal NO_3 medium plus analogs, colony area was measured and expressed as a percentage of the area of the same strain grown on analog-free medium. CAN and FPA were used at a constant ratio of 1:3. Concentrations indicated reflect those for CAN.

opment. Analysis of ubiquitin conjugates in the Δmcb1 strain was consistent with this possibility. As compared with the wild type, an equivalent amount of total protein from Δmcb1 protonema had three- to fourfold higher levels of the ubiquitin monomer, free multiubiquitin chains, and high molecular mass ubiquitin conjugates that represent multiubiquitin chains attached to protein targets awaiting degradation (Figure 5A). Presumably, the increase in higher mass conjugates reflected a stabilization of ubiquitinated targets that were inefficiently degraded by 26S proteasomes lacking PpMCB1.

A reduced capacity to degrade protein was also inferred by growth of the Δmcb1 strain on amino acid analogs. Translational incorporation of these analogs generates aberrant proteins that require the ubiquitin/26S proteasome pathway for removal (Bachmair et al., 1990; Seufert and Jentsch, 1990; van Nocker et al., 1996b). Consequently, cells impaired in ubiquitin-dependent proteolysis are often hypersensitive to such analogs because the resulting abnormal proteins accumulate to toxic levels. In fact, we have shown previously that the growth of yeast Δmcb1 strains is hypersensitive to canavanine (CAN) and *p*-fluoro-L-phenylalanine (FPA), analogs of arginine and phenylalanine, respectively (van Nocker et al., 1996b; Fu et al., 1998b). When *P. patens* was grown on minimal media (minus NH_4) containing various concentrations of CAN and FPA, we found that the Δmcb1 strain also was hypersensitive, showing reduced protonema growth and increased chlorosis as compared with the wild type (Figure 5B and data not shown). Even though high analog concentrations ($>$1 and 3 μg mL^{-1} CAN and FPA, respectively) also retarded the growth of wild-type protonema, these colonies remained alive and green in contrast to the pale yellow Δmcb1 colonies that eventually died after 3 to 4 weeks of growth on similarly high analog levels (data not shown).

Complementation of Δmcb1 with Wild-Type and Mutant Versions of *PpMCB1*

To help confirm that the Δmcb1-conferred phenotype was caused solely by disruption of the *PpMCB1* gene and to identify domains in the MCB1 protein important for its activity, one of the Δmcb1 strains was transformed with the wild-type *PpMCB1* gene or the mutants *N5*, *CΔ*, and *NΔ* (see Figure 2A). The complementation plasmid contained the neomycin phosphotransferase II (*NPTII*) gene, which confers kanamycin resistance, and the *PpMCB1* coding region expressed under the control of the CaMV 35S promoter. This plasmid was cotransformed with a related plasmid containing 3.6 kb of *P. patens* genomic DNA (Schaefer and Zryd, 1997). Schaefer and Zryd (1997) showed previously that recombination between these plasmids creates a chimeric vector flanked by the same *P. patens* genomic DNA. This flanking DNA often promotes integration of the different constructions into the same chromosomal site, thus

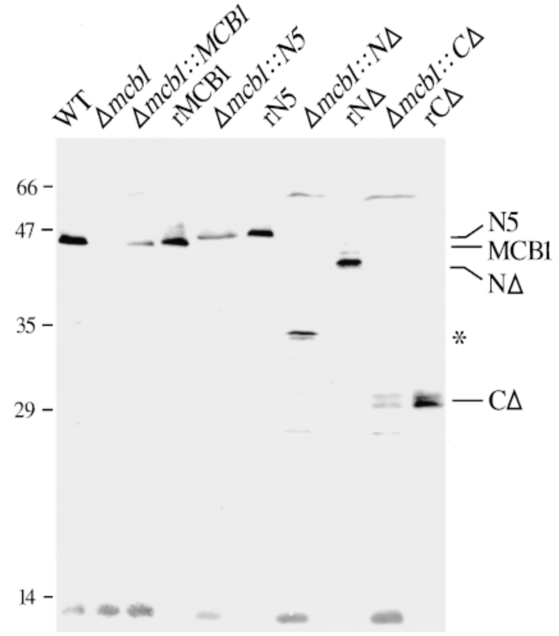

Figure 6. Expression of Full-Length and Mutated Versions of PpMCB1 in *P. patens* Δmcb1.

Coding regions for *PpMCB1* and the mutants *N5*, *NΔ*, and *CΔ* (see Figure 2 for descriptions), expressed under the control of the CaMV 35S promoter, were transformed into a Δmcb1 strain. Protenema from the transformed colonies were homogenized, and equal amounts of total protein were subjected to SDS-PAGE and immunoblot analysis with Arabidopsis anti-MCB1 antibodies. WT, wild-type *P. patens.* rMCB1, rN5, rNΔ, and rCΔ represent recombinant versions of each protein expressed in *E. coli* (see Figure 2); their migration positions are indicated to the right. The asterisk denotes the migration position of an abundant truncated product of NΔ that accumulated in the Δmcb1::*NΔ* strain. Molecular mass markers are given at left in kilodaltons.

minimizing the effect of genomic position on gene expression in different transformants.

As can be seen in Figure 6, introduction of the expression vectors created transgenic *P. patens* lines expressing immunologically detectable levels of each of the four proteins. For the Δmcb1 strains harboring the *PpMCB1*, *N5*, and *CΔ* vectors, the ectopically expressed proteins were easily detected and had apparent molecular masses indistinguishable from the *E. coli*–expressed versions. However, for the Δmcb1 strains harboring *NΔ*, only trace amounts of the full-length NΔ protein were evident (data not shown). Instead, a more abundant protein of 34 kD accumulated, which likely represented an incomplete translation product from the *NΔ* mRNA or a stable proteolytic cleavage fragment of the NΔ polypeptide (Figure 6). In support of the latter possibility, a similarly sized product was also evident when the *NΔ* gene was expressed in *E. coli* (see Figure 2D). As compared with those in wild-type plants, the immunologically detectable levels of the PpMCB1 proteins ectopically expressed in the Δmcb1 strain were substantially less (Figure 6). One factor contributing to these low accumulation levels was the use of the CaMV 35S promoter, which expresses weakly in *P. patens* (P.-A. Girod, unpublished data). For CΔ and NΔ, another contributing factor could have been poor cross-reaction of the deletions with the Arabidopsis anti-MCB1 antibodies. This appears unlikely because the *E. coli*–expressed versions of both CΔ and NΔ were easily detected by the immunoblot assay used here (Figure 2D).

Despite the low protein levels, the expression of wild-type PpMCB1 restored gametophorogenesis to the Δmcb1 strain (Figure 7). Whereas the Δmcb1 strain alone or one transformed with an empty *NPTII* vector failed to form buds and gametophores when grown on minimal media (minus hormones and NH_4) (Figures 7A and 7D, and data not shown), protonema of the Δmcb1::*MCB1* strain developed normal caulonema that eventually differentiated to form buds and gametophores (Figure 7B). There was a slight delay in bud and gametophore initiation, but this effect could have been caused by the lower protein levels in the Δmcb1::*MCB1* strain as compared with that of the wild type. A near normal phenotype also was rescued in the Δmcb1::*N5*, Δmcb1::*NΔ*, and Δmcb1::*CΔ* strains. Although delayed, the development of caulonema and the formation of buds and gametophores was evident (Figures 7C, 7E, and 7F and data not shown). The Δmcb1::*NΔ* strain showed the greatest delay in developmental progression from chloronema. Those gametophores that did arise were abnormal, being shorter and containing leaves that were curled and not strictly opposed (as compared with the wild type or Δmcb1::*MCB1* strains). In fact, when cultured on minimal medium containing NH_4, the Δmcb1::*NΔ* strain exhibited the same phenotype as did the Δmcb1 strain, indicating that complementation by the *NΔ* gene was weak.

Association of PpMCB1 with the 26S Proteasome

Previous studies showed that the yeast and animal MCB1 proteins are found in a free form as well as tightly associated with the 19S regulatory complex of the 26S proteasome (Haracska and Udvardy, 1995; van Nocker et al., 1996b, 1996c). To confirm that PpMCB1, likewise, is a subunit of the *P. patens* 26S proteasome, we attempted to coimmunoprecipitate PpMCB1 and the 20S proteasome from crude protonemal extracts. Anti-MCB1 antibodies were raised against the Arabidopsis protein (van Nocker et al., 1996b). The anti-20S proteasome antibodies were generated against the entire *P. patens* complex and can detect most, if not all, of the 7 α and 7 β subunits of the purified 20S complex when separated by two-dimensional PAGE (P.-A. Girod, unpublished data). However, after one-dimensional SDS-PAGE, three 20S subunits of 20, 24, and 27 kD were most evident from the analysis of crude extracts (Figure 8).

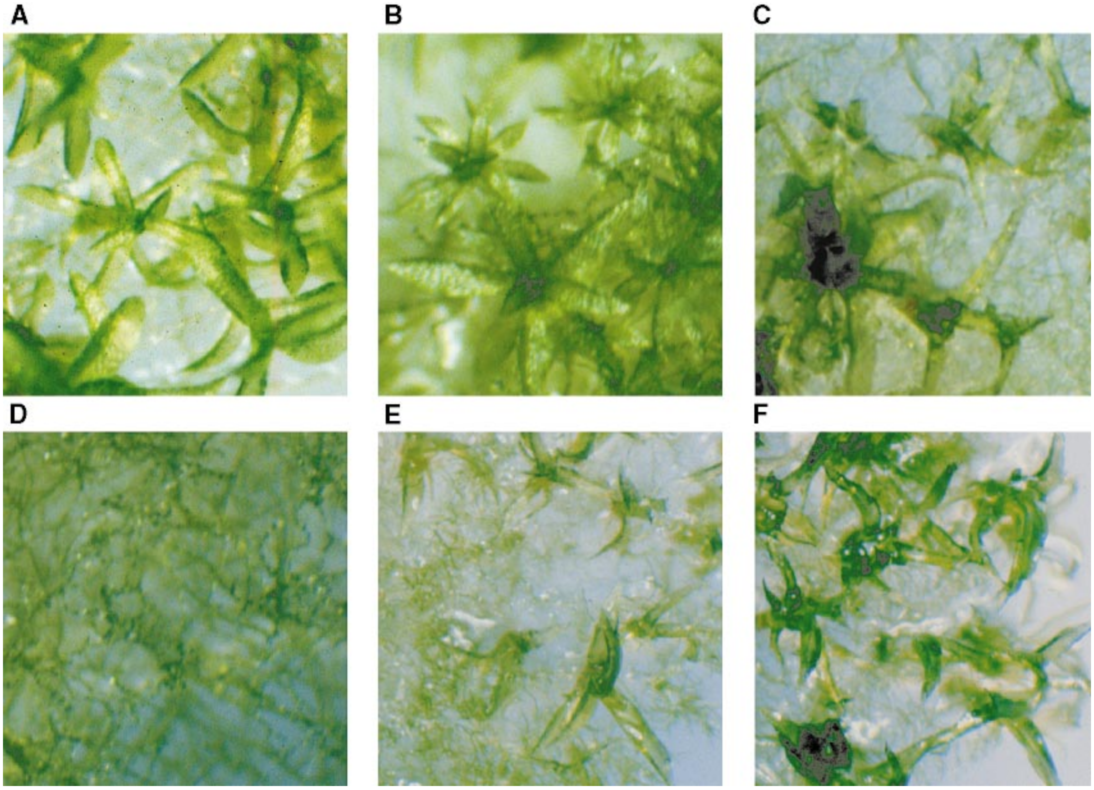

Figure 7. Complementation of a *P. patens* *Δ*mcb1 Strain with Full-Length and Mutated Versions of *PpMCB1*.

Coding regions of *PpMCB1* or the mutants *N5*, *NΔ*, and *CΔ* (see Figure 2 for descriptions), expressed under the control of the CaMV 35S promoter, were transformed into a *Δ*mcb1 strain. Protein expression levels for each of the complemented strains are shown in Figure 6. The colonies were grown on minimal NO_3 medium for 3 weeks and assessed for gametophore development. *P. patens* strains are as follows.
(A) Wild type.
(B) *Δ*mcb1::*MCB1*.
(C) *Δ*mcb1::*N5*.
(D) *Δ*mcb1.
(E) *Δ*mcb1::*NΔ*.
(F) *Δ*mcb1::*CΔ*.

Results from the coimmunoprecipitation experiments indicate that *P. patens* MCB1 is associated with the 20S proteasome in the 26S complex. As can be seen in Figure 8, Arabidopsis anti-MCB1 antibodies immunoprecipitated not only PpMCB1 (detected as a 44-kD species just below the IgG heavy chain) but also the same 20-, 24-, and 27-kD subunits of the 20S proteasome easily detected in crude extracts. These 20S subunits were not evident in the anti-MCB1 antibody immunoprecipitates from the *Δ*mcb1 extracts. PpMCB1 remained associated with the 20S subunits with or without the addition of ATP to the extraction and coimmunoprecipitation buffers. Conversely, the anti-20S proteasome antibodies immunoprecipitated the 20S proteasome subunits from both the wild type and the *Δ*mcb1 strains (Figure 6). No PpMCB1 could be detected in the immunoprecipitates from the *Δ*mcb1 strain (as expected), but a trace amount of PpMCB1 could be seen in immunoprecipitates from the wild type as a result of its association with the 20S complex. It should be emphasized that the antibodies used to detect PpMCB1 were prepared against the Arabidopsis counterpart. As a consequence, they were much less sensitive in detecting its corresponding antigen than were the *P. patens* anti-20S proteasome antibodies.

DISCUSSION

By exploiting the ability to disrupt *P. patens* genes with high efficiency (Schaefer and Zryd, 1997), we report here our first attempt to resolve the function of the ubiquitin/26S proteasome pathway in plants by using reverse genetics. The gene

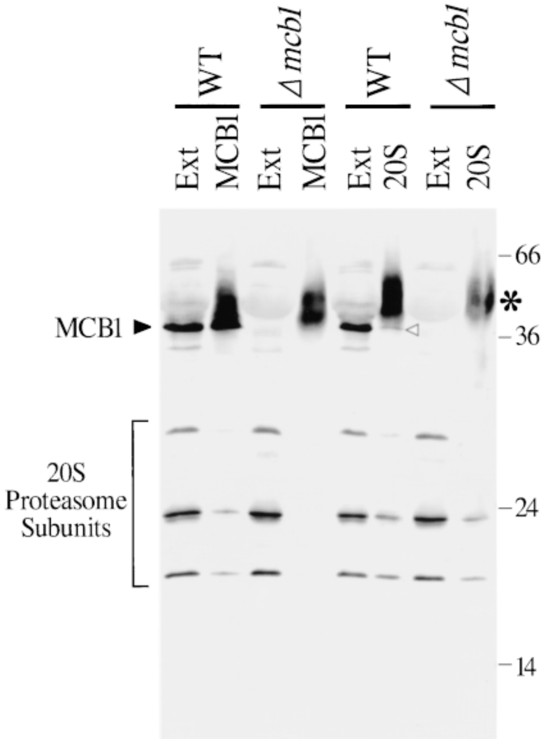

Figure 8. Association of PpMCB1 with the *P. patens* 26S Proteasome.

Crude extracts (Ext) were prepared from either wild-type (WT) or Δmcb1 protonema and immunoprecipitated with Arabidopsis anti-MCB1 antibodies (MCB1) or antibodies prepared against the *P. patens* 20S proteasome (20S), the catalytic subcomplex of the 26S proteasome. The immunoprecipitates were then subjected to immunoblot analysis with a mixture of anti-20S proteasome antibodies and anti-MCB1 antibodies. The SDS-PAGE migration positions of PpMCB1 are indicated by the open and closed arrowheads. Three of the *P. patens* 20S proteasome subunits that are strongly recognized by the anti-20S antibodies are indicated by the bracket. The asterisk identifies the migration position of the immunoglobulin heavy chains used in the immunoprecipitations and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase that was stained nonspecifically. Molecular mass markers are given at right in kilodaltons.

targeted was *PpMCB1* that encodes a subunit of the 26S proteasome with in vitro affinity for multiubiquitin chains. Based on its binding activity and presence within the 26S proteasome, we proposed previously that MCB1 functions as a receptor for multiubiquitin chains, thus helping the proteolytic complex identify and capture ubiquitinated proteins that are then degraded by the 20S protease (van Nocker et al., 1996b, 1996c; Fu et al., 1998b). However, the fact that both yeast (van Nocker et al., 1996c) and *P. patens* (this report) are viable without this protein implies that the 26S proteasome must possess other receptors that recognize ubiquitinated proteins in addition to MCB1. In contrast to a limited role in yeast, we discovered here that MCB1 has an expanded role in *P. patens*, being necessary for normal developmental progression. Surprisingly, this role does not require the multiubiquitin chain recognition site, further demonstrating that MCB1 has an important function within the 26S proteasome beyond its ability to bind multiubiquitin chains.

In yeast, MCB1 is not essential. Although yeast Δmcb1 strains grow and develop normally on rich media, they are more sensitive to amino acid analogs and unable to degrade a subset of ubiquitin pathway targets (van Nocker et al., 1996b; Fu et al., 1998b). These targets include those catabolized by the ubiquitin–fusion degradation pathway, a subpathway that recognizes proteins translationally fused to the C terminus of ubiquitin (Johnson et al., 1995). In contrast to yeast, we show here that MCB1 has a more critical function in the moss. Like yeast Δmcb1 strains, *P. patens* Δmcb1 strains have elevated levels of ubiquitin conjugates and are hypersensitive to growth on amino acid analogs. However, even under normal growth conditions, *P. patens* missing PpMCB1 is developmentally impaired; the Δmcb1 strains are unable to form phenotypically normal caulonema and are blocked in bud and gametophore differentiation. This effect further highlights the importance of the ubiquitin/26S proteasome pathway in plant growth and development and suggests that MCB1, in particular, has a more complex role in plants as compared with yeast (van Nocker et al., 1996c; Fu et al., 1998b).

Like its higher plant, yeast, and metazoan counterparts, PpMCB1 binds multiubiquitin chains through a hydrophobic patch in its C-terminal half. Nonetheless, in agreement with complementation studies of yeast Δmcb1 strains (van Nocker et al., 1996c; Fu et al., 1998b), we find that the ability of PpMCB1 to bind chains is not essential for its phenotypic function in *P. patens*. In yeast, counterparts to the N5 and CΔ mutants restored the resistance to amino acid analogs and rescued the ubiquitin–fusion degradation pathway in the Δmcb1 strains, even though they could not bind multiubiquitin chains in vitro (Fu et al., 1998b). In a similar fashion, the *P. patens* N5 and CΔ mutants, which also fail to bind chains in vitro, at least partially rescued the phenotypic defects in the Δmcb1 moss.

Consequently, it is possible that the binding activity observed in vitro for MCB1 proteins is not relevant to its in vivo function(s) or that the MCB1 proteins possess another more critical role(s) within the 26S proteasome in addition to their ability to recognize multiubiquitin chains. At present, no definitive evidence exists for or against the former possibility. However, MCB1 remains the only 26S proteasome subunit with a demonstrated affinity for ubiquitin chains (Deveraux et al., 1994; van Nocker et al., 1996b), and the in vitro binding specificity of the isolated 26S proteasome for multiubiquitinated proteins mimics that of MCB1 (Beale et al., 1998). With respect to the latter possibility, recent data indicate that the 19S regulatory particle of the 26S proteasome is actually composed of two subparticles that may require MCB1 for tight association (Glickman et al., 1998b). Thus, it is possible that the 26S proteasome in the Δmcb1 strains lacks sufficient stability to degrade ubiquitinated (and possibly nonubiquitinated) substrates effectively. It is remotely conceivable that MCB1 has additional functions outside of the 26S complex. For example, Anand et al. (1997) showed that the free form of mammalian MCB1 (designated S5a) can interact with and affect the activity of Id1, a protein that appears to help positively regulate muscle differentiation by inhibiting the dimerization and transcriptional activity of the basic helix-loop-helix protein MyoD. The possibility that binding of Id1 to MCB1 initiates the degradation of Id1 by the 26S proteasome or affects Id1 activity without proteolysis remains to be investigated.

Interestingly, low levels of all three of the mutated PpMCB1 proteins (N5, NΔ, and CΔ) restored normal caulonema differentiation to the Δmcb1 strain. However, these levels were insufficient to support the development of completely normal gametophores. Taken together with the hormone rescue data, these observations point toward an increased need for MCB1 during the latter stages of gametophorogenesis. In yeast, expression of the NΔ protein accentuates the sensitivity of the Δmcb1 strain cells to amino acid analogs, indicating that the truncated protein is somewhat cytotoxic to yeast in the absence of the wild-type protein (Fu et al., 1998b). We expected a similar toxicity when the comparable *P. patens* NΔ mutant was expressed in the Δmcb1 moss. Instead, we found that the Δmcb1::*NΔ* strain reverted to a more normal phenotype. One possible explanation is that the truncated 34-kD proteins that did accumulate in the Δmcb1::*NΔ* strains may be much less cytotoxic than is the entire NΔ polypeptide.

Based on the possible function(s) of MCB1 in the 26S proteasome, the phenotypic defects in the *P. patens* Δmcb1 strains are likely caused by the stabilization of one or more short-lived proteins whose removal is required for normal developmental progression beyond chloronema. In a more global way, the Δmcb1 defect could stabilize specific targets by slowing the rate of overall protein turnover by the 26S proteasome. In support of this possibility are the observations that the levels of multiubiquitin chains and multiubiquitinated proteins are elevated in the Δmcb1 mutants and that the Δmcb1 mutants are more sensitive to growth on amino acid analogs, a condition that should increase the need for ubiquitin/26S proteasome–dependent proteolysis (Figure 5). Alternatively, the failure to remove proteins adequately could generate a stress response. This stress, in turn, could indirectly block differentiation. The ubiquitin/26S proteasome pathway is required for the stress response in many eukaryotes, and stressful conditions, such as growth on amino acid analogs, upregulates the synthesis of ubiquitin and other components of the pathway in yeast, animals, and plants (Finley et al., 1987; Burke et al., 1988; Feussner et al., 1997; Mathew et al., 1998; McCafferty and Talbot, 1998). The observation that the overall levels of free ubiquitin and ubiquitin chains are elevated in the Δmcb1 strains is congruent with this stress effect (Figure 5A).

A more intriguing possibility is that loss of MCB1 preferentially stabilizes only a specific group of regulatory proteins. Consistent with this possibility are the observations that only a subset of ubiquitin/26S proteasome targets is stabilized in the yeast Δmcb1 background (van Nocker et al., 1996c) and that the chloronema from the moss Δmcb1 strains are viable and continue to divide despite the expected need of the ubiquitin/26S proteasome pathway for removing a variety of mitotic checkpoint and other regulatory proteins (King et al., 1997; Genschik et al., 1998). Both observations suggest that the routing of ubiquitinated proteins to the 26S proteasome through MCB1 is limited. We show here that this block in *P. patens* development can be partially bypassed by treatment of the Δmcb1 strain with cytokinin and auxin. Treatment with low levels of both hormones triggered caulonema development, bud formation, and partial rescue of gametophores. It is possible that low levels of these hormones increase the overall degradation rate and/or attenuate the general stress of the Δmcb1 strain. A more likely scenario is that the hormones bypass a specific mechanism that normally represses developmental progress but fails to be deactivated in the Δmcb1 mutant. This bypass could involve stimulating the turnover of critical protein(s) by another mechanism or overriding their action even when present in abnormally high levels. Whatever the nature of the defect caused by the loss of MCB1, it is clearly not lethal because these Δmcb1 strains show no loss of viability.

The first indications that the ubiquitin/26S proteasome pathway plays an important role in plant development came from the morphogenic defects induced in tobacco after expression of a dominant-negative ubiquitin variant that blocks the assembly of multiubiquitin chains (Bachmair et al., 1990; Becker et al., 1993). The more recent identification of UFO/FIM, TIR1, and COI1 as possible F-box proteins has provided specific evidence that flower development and the auxin and jasmonate hormone signaling pathways may require components of the ubiquitin pathway responsible for conjugating ubiquitin to protein targets (Ingram et al., 1995; Ruegger et al., 1998; Xie et al., 1998). We extend these observations to the development of *P. patens* and to another hormone—cytokinin—and implicate the 26S proteasome as well in plant development/hormone signaling. In fact the Δmcb1 strains described here are strikingly similar to the previously described *caulonema*$^-$ (*cal*$^-$) mutant, which is blocked in caulonema development as a result of an insensitivity to auxins and cytokinins (Ashton et al., 1979). The nature of the *cal*$^-$ defect is not known.

Taken together, the evidence suggests that removal of specific short-lived proteins may be essential for many hormonally triggered processes in plant. These unstable proteins could act as transcriptional repressors or function to inhibit the activity or transport of hormones and other activators.

With respect to auxin-mediated responses, candidates include the small auxin-upregulated proteins that have very short half-lives and are expressed under auxin control (Abel et al., 1994). For flower development, indirect evidence suggests that the homeotic protein APETALA3 is turned over rapidly (Jacks et al., 1994). For cytokinins and jasmonate, potential candidates are not known.

Certainly, the ability to target the disruption of specific genes as well as methods that allow for successful complementation make *P. patens* a useful model in which to study plant growth and development. With respect to the ubiquitin/26S proteasome pathway, also important was the finding that many components identified in Arabidopsis have closely related moss orthologs. In addition to MCB1, these include ubiquitin, 20S proteasome subunits, and proteins involved in ubiquitin conjugation (ubiquitin-conjugating enzymes and ubiquitin–protein ligases) (data not shown). Thus, studies with *P. patens* should provide useful information for all higher plants. The more complex phenotype elicited by disruption of *PpMCB1* in *P. patens* as compared with yeast also highlights the more expanded role of the ubiquitin/26S proteasome pathway in multicellular eukaryotes and thus the need for parallel studies in more complex organisms.

METHODS

Plant Material and Growth

Physcomitrella patens B.S.G. was grown axenically on solid minimal NH_4- or NO_3-containing media (NH_4 medium without ammonium tartrate) (Ashton and Cove, 1977) in a growth chamber under controlled conditions (25°C). Light was provided from above by fluorescent Grolux lights (Sylvania Corp., Danvers, MA) under a regime of 16 hr of light and 8 hr of darkness. Plants were subcultured every 7 days. Filter-sterilized canavanine (CAN) and 5-fluoro-L-phenylalanine (FPA) (Sigma) were added to the autoclaved media. Effects of amino acid analogs on protonemal growth were determined by measuring the colony area after 18 days of growth and expressing this area as a percentage of that obtained for colonies grown on analog-free media.

Isolation of *P. patens MCB1* cDNA

A λ ZAP cDNA library (titer of 1.3×10^8), prepared from polyadenylated RNA isolated from 5-day-old protonema (P.-A. Girod, unpublished data), was screened at low stringency (washing in 3 × SSC at 65°C [20 × SSC = 3 M NaCl and 0.3 M sodium citrate]) by using the ^{32}P-labeled *Arabidopsis thaliana MCB1* cDNA as the probe (van Nocker et al., 1996b). Immobilization of λ DNA onto Zeta-Probe membranes (Bio-Rad, Richmond, CA), probe hybridization, and in vivo excision of pBluescript SK+ plasmids (Stratagene, La Jolla, CA) containing *P. patens MCB1* cDNAs were performed according the manufacturer's protocols. The complete nucleic acid sequence of the clone containing the longest 5′ untranslated region (UTR) was determined by automated DNA sequencing. The sequence for the *PpMCB1* cDNA was deposited in the GenBank database under accession number AF076610.

PpMCB1 Plasmid Constructions

The *PpMCB1* knockout construction (5′-*PpMCB1*::*HPH*::3′-*PpMCB1*) was created by replacement of a 12-bp BamHI fragment (nucleotides 738 to 750) in the *PpMCB1* cDNA with a 2.0-kb BamHI fragment containing an expression cassette for the selectable *HPH* gene (Schaefer and Zryd, 1997). The *PpMCB1* cDNA was first digested with XbaI to remove a 3′ fragment that contained an additional BamHI site. To generate the 2.8-kb DNA fragment used for disruption, the *PpMCB1* knockout construct was used as a template in polymerase chain reactions (PCRs) with primers 1F (5′-GGGAGTTCAAGTTGCTCAGC-3′) and 1R (5′-CCAGAACCTGGTTCACATCG-3′), which annealed at positions 333 to 352 and 1149 to 1168, respectively (see Figure 3A). For complementation of the *P. patens* *Δ*mcb1 strain, the β-glucuronidase (*GUS*) coding sequence in p*NPTII*::35S–*GUS* was replaced by the coding sequence for *PpMCB1*, *N5*, *NΔ*, or *CΔ*, designed as described below. p*NPTII*::35S–*GUS* was assembled in pBluescript SK+ by subcloning sequentially the cauliflower mosaic virus (CaMV) 35S promoter and *GUS* coding sequence from the CaMV 35S promoter/*GUS*/*NOS* (nopaline synthase) terminator gene that is present in pBI121 (Jefferson et al., 1987) and the *NPTII* coding region and *NOS* 3′ transcription termination signal from the *NOS* promoter/*NPTII*/*NOS* terminator gene present in pZM104A (Naio and Lam, 1995).

To synthesize the various PpMCB1 proteins in *Escherichia coli*, the corresponding DNA fragments were cloned into pET30c (Novagen, Madison, WI) and expressed using the manufacturer's recommendations. Mutants were generated by PCR strategies using the *PpMCB1* cDNA as the template. Wild-type *PpMCB1* was amplified using 5′-CGGCCATATGGTGCTCGAGGCGACCATGGTC-3′ (primer 1) and 5′-CCAACTTGAATTCCATACTCATTTGCTCTTATCC-3′ (primer 2) as the 5′ and 3′ primers, respectively. The underlined nucleotides in the 5′ and 3′ primers indicate NdeI and EcoRI sites, respectively, engineered to facilitate subsequent cloning of the PCR products. *CΔ* was generated with primer 1 and 5′-GGGAATTCTC***TCA***ATCCAGATTTGGATCCACACCAAAG-3′ designed to introduce a stop codon at codon 230 (shown in italics). *NΔ* was generated with primer 2 and 5′-CCAT***ATG***GTTCTTATCACTCCAACCAACGAC-3′ designed to introduce a new start codon at codon 61 (shown in italics). The mutant *N5* was created by QuickChange site-directed mutagenesis (Stratagene) using pET30c harboring wild-type *PpMCB1* as the template.

Protoplast Isolation, Transformation, and Growth

P. patens protoplasts were isolated and regenerated as described previously (Schaefer et al., 1991). Protoplasts were resuspended at 1.2×10^6 protoplasts/mL in MMM solution (8.8% mannitol, 15 mM $MgCl_2$, and 0.1% 2-[N-morpholino]ethane-sulfonic acid, pH 5.6). For transformation with the various *PpMCB1*constructs, the PCR reaction products were first treated with DpnI, precipitated with phenol/chloroform, and resuspended in water at a concentration of 0.5 μg μL^{-1}. For the complementation experiments, transformation was performed with plasmid DNA resuspended in water at a concentration of 0.5 μg μL^{-1}.

For transformation, 300 μL of a protoplast suspension was added to either 15 μL of the PCR products or 15 μL of a plasmid solution containing *PpMCB1* (or its derivatives) and 15 μL of pUC18 containing the 3.6-kb genomic *P. patens* fragment 108 (Schaefer and Zryd, 1997). After gentle mixing, 300 μL of polyethylene glycol (40% polyethylene glycol, 0.1 M $CaNO_3$, 0.38 M mannitol, and 10 mM Tris-HCl, pH 8.0) was added, and the suspension was incubated with occasional mixing at 42°C for 5 min and for an additional 10 min at room temperature. The protoplast suspension was slowly diluted to a final volume of 10 mL with liquid NH_4 media (Ashton and Cove, 1977) supplemented with 6.8% mannitol (NH_4M) and incubated for 15 hr under indirect light. Thereafter, protoplasts were collected by centrifugation (3 min at 120*g*), resuspended in melted NH_4M media containing 0.8% LMT agarose (FMC, Rockland, ME), and overlayed onto solid NH_4M media covered with cellophane. After 4 days of culture in direct light, the cellophane overlays were transferred to fresh NH_4 media, and after 6 additional days, the cellophane overlays were transferred to NH_4 media supplemented with 50 mg L^{-1} hygromycin, 50 mg L^{-1} G418 (Gibco), or 20 mg L^{-1} kanamycin sulfate. Stable antibiotic-resistant clones were further selected by a second round of growth on NH_4 media followed by growth on NH_4 media containing antibiotic to select for stable clones. For induction of gametophores, plants were cultured on NO_3 media.

Analysis of Transgenic Plants

P. patens DNA was isolated with the DNeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The integration of the *HPH* cassette into the *PpMCB1* locus was analyzed by PCR. Pairs of primers specific for the 5′ junction were 11F (5′-GGCAAGATGGTGCTCGAGGCGACCATGGA-3′) and HPH2 (5′-CCGATGCAAAGTGCCGATAAACATAA-3′) and for the 3′ junction were 11R (5′-TGCTCTTATCCTTTGTTGCTTGATCATCGAT-3′) and HPH1 (5′-AGAAGTACTCGCCGATAGTGGAAACC-3′) (see Figure 3). For DNA gel blot analyses, genomic *P. patens* DNA was isolated using the method of Rogers and Bendich (1988). A 1.3-kb fragment, encompassing the entire coding region, was amplified from the *PpMCB1* cDNA by PCR with the 11F and 11R primer pair (Figure 3A) and used to probe 2 μg of *P. patens* genomic DNA digested with various restriction endonucleases and blotted onto Immobilon P membranes (Millipore, Bedford, MA).

Immunological Analysis

Extracts were prepared from *P. patens* protonema by grinding frozen tissue in extraction buffer (10 mM Na_2EDTA, 50 mM NaH_2PO_4, 10 mM 2-mercaptoethanol, 0.1% sarcosine, and 0.1% Triton X-100, pH 7.0) and clarifying the crude extract at 10,000*g*. Equal amounts of protein (as determined by Bio-Rad protein assay) were subjected to SDS-PAGE and electroblotted onto Immobilon P or nitrocellulose membranes (HAHY; Millipore) for detecting PpMCB1 or ubiquitin, respectively. Immunoblot analyses were performed with rabbit antiserum against Arabidopsis MCB1, plant ubiquitin (van Nocker et al., 1996b), or purified *P. patens* 20S proteasomes (P.-A. Girod, unpublished data) and used in conjunction with alkaline phosphatase–labeled goat anti–rabbit immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

For the coimmunoprecipitation assays, 1 g of 5-day-old protonema was homogenized in a mortar and pestle at room temperature in 330 μL of 200 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 2 mM Na_2EDTA, 4 mM phenylmethylsulfonyl fluoride, and 40% glycerol with or without 1 mM ATP. Clarified extracts (100 μL) were incubated at 4°C with the anti-20S proteasome antiserum (1:100 dilution) or the anti-MCB1 antiserum (1:500 dilution) for 1 hr, followed by a 12-hr incubation with 10 μL of protein A–Sepharose (Sigma). Beads were collected by centrifugation and washed in the extraction buffer (with or without ATP). Protein was subjected to SDS-PAGE, blotted onto Immobilon P membranes, and incubated with the anti-MCB1 antiserum followed by anti-20S proteasome antiserum (see above).

Assay for Multiubiquitin Chain Binding

pET30c-expression plasmids harboring the various *PpMCB1* constructions were introduced into *E. coli* strain BL21 (DE3) and expressed according to manufacturer's protocols (Novagen). Total protein from cell lysates was fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes (HAHY; Millipore). Membranes were incubated with ^{125}I-labeled multiubiquitin chains as described previously (Fu et al., 1998b). Radiolabeled multiubiquitin chains linked via residue Lys-48 were prepared according to the method of van Nocker and Vierstra (1993).

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