



The protein turnover of Arabidopsis BPM1 is involved in regulation of flowering time and abiotic stress response

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Key message Protein degradation is essential in plant growth and development. The stability of Cullin3 substrate adaptor protein BPM1 is regulated by multiple environmental cues pointing on manifold control of targeted protein degradation.

Abstract A small family of six MATH-BTB genes (*BPM1-6*) is described in *Arabidopsis thaliana*. BPM proteins are part of the Cullin E3 ubiquitin ligase complexes and are known to bind at least three families of transcription factors: ERF/AP2 class I, homeobox-leucine zipper and R2R3 MYB. By targeting these transcription factors for ubiquitination and subsequent proteasomal degradation, BPMs play an important role in plant flowering, seed development and abiotic stress response. In this study, we generated *BPM1*-overexpressing plants that showed an early flowering phenotype, resistance to abscisic acid and tolerance to osmotic stress. We analyzed *BPM1*-GFP protein stability and found that the protein has a high turnover rate and is degraded by the proteasome 26S in a Cullin-dependent manner. Finally, we found that *BPM1* protein stability is environmentally conditioned. Darkness and salt stress triggered *BPM1* degradation, whereas elevated temperature enhanced *BPM1* stability and accumulation *in planta*.

Keywords MATH-BTB · Early flowering · Water deprivation · ABA · Abiotic stress · Elevated temperature

Introduction

The addition of ubiquitin is a common posttranslational modification that controls the stability, function or subcellular localization of many proteins and has important regulatory roles in different cellular and physiological processes (Chen and Hellmann 2013). Cullin-based ubiquitin ligases are modular ubiquitin ligases that control selective protein turnover in eukaryotic cells (Petroski and Deshaies 2005; Hua and Vierstra 2011). They contain three major

elements—a Cullin scaffold, a RING finger protein that recruits an ubiquitin-charged E2 enzyme, and a substrate adaptor that places substrates in proximity to the E2 enzyme to facilitate ubiquitin transfer. In *Arabidopsis* there are six Cullin-like proteins CUL1, CUL2, CUL3A, CUL3B, CUL4 and ANAPHASE PROMOTING COMPLEX2 (Choi et al. 2014). Central to formation of an active Cullin-based ubiquitin ligase complex (except for ANAPHASE PROMOTING COMPLEX2) is neddylation, the modification of a single conserved lysine residue in the Cullin subunit with the NEDD8 protein. Neddylation promotes the structural reorganization of the C-terminal RING binding domain of the Cullin, promoting the processivity of ubiquitin transfer (Duda et al. 2011). MATH-BTB proteins, commonly found in plants and animals, are shown to function as substrate-specific adaptors of Cullin3 (CUL3)-based E3 complexes that facilitate the transfer of ubiquitin moieties to substrates (Weber et al. 2005; Pintard et al. 2003).

In *Arabidopsis thaliana* a small family of six MATH-BTB genes (*BPM1-6*) is described which codes for at least 16 different protein isoforms due to alternative splicing (www.uniprot.org; Fig. S1). *Arabidopsis* BPM proteins

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bind ERF/AP2 family of transcription factors (TFs), a class I homeobox-leucine zipper TFs, as well as R2R3 MYB family of TFs and protein phosphatases type 2C. By targeting these proteins for ubiquitination and subsequent proteasomal degradation, BPMs play important roles in plant flowering, seed development and abiotic stress responses (Weber and Hellmann 2009; Lechner et al. 2011; Chen et al. 2013, 2015; Morimoto et al. 2017; Julian et al. 2019). Besides being important regulators of transcription, BPM proteins were shown to be essential in mediating CUL3 binding to DNA (Chen et al. 2015) and might alter target TFs activity by intervening in their interaction with other cellular components.

There are several T-DNA insertion mutant lines described for each BPM gene (<https://www.arabidopsis.org>). Many of them are unavailable or described to have expression pattern similar to wild type and, to date, no null mutant was described for any *BPM* gene. To downregulate the expression of MATH-BTB members, different research groups have expressed 35S-driven artificial microRNAs in *Arabidopsis*, and three types of mutants with downregulated BPMs were recovered: plants expressing 20–50% of wild type *BPM* levels (*6xami-bpm*; Chen et al. 2013), plants with downregulation of *BPM 1, 4, 5* and *6* (*amiR-bpm*; Lechner et al. 2011) and plants with downregulation of *BPM1, 2, 3, 4* and *5* (*amiBPM*; Morimoto et al. 2017). Each mutant line with downregulated BPMs shows developmental alterations. Moreover, the phenotypes of these mutant lines differ slightly from one another, suggesting specific roles of individual *BPM* genes.

Relative expression levels and tissue-specific expression patterns of all *BPM* genes have been analyzed previously (Weber and Hellmann 2009; Lechner et al. 2011). In PBPM:GUS transgenic lines, *BPM1* expression is highest in buds, flowers, root and leaf vasculature, while semi-quantitative gene expression analysis shows induced *BPM1* expression upon salt, osmotic and drought stress (Weber and Hellmann 2009). To examine *BPM1* protein and to determine *BPM1*-specific roles, we produced *A. thaliana* transgenic plants overexpressing GFP-tagged *BPM1* under control of the 35S promoter. Due to assumed functional redundancy of BPM proteins (Weber and Hellmann 2009), we analyzed the impact of overexpressed *BPM1*-GFP on expression of native *BPM* genes. Additionally, we analyzed the expression patterns of endogenous *BPM* genes under stress conditions. We described the developmental features of *BPM1*-GFP transgenic lines and examined *BPM1*-GFP subcellular localization and stability in conditions of abiotic stress, during daily rhythm changes and after exposure to elevated temperature. Taken together, our results indicated a role of *BPM1* protein stability and turnover in regulation of flowering time and abiotic stress response.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (accession Col-0) plants were grown on soil in 16 h light/8 h dark cycles at 21 °C and a light intensity of approximately 120 to 130 $\mu\text{mol}/\text{m}^2 \text{ s}$ with 50% relative humidity. For germination, seeds were surface sterilized with 1% Izosan G (100% sodium dichloroisocyanurate dihydrate, Pliva, Croatia) and 0.01% Mucosal for 10 min, cold treated at 4 °C for 2–3 days, and then planted on Murashige and Skoog (MS) medium with 0.8% agar and 2% sucrose (germination plates). Plates were incubated in 16 h light/8 h dark cycles at 24 °C.

Stress treatments

To examine the role of *BPM1* in stress response, *BPM1*-overexpressing lines (L003 and L104) and wild type were subjected to different stress conditions. For germination assay, surface sterilized seeds were plated on Murashige and Skoog (MS) medium supplemented with 0.5 g MES (MS-MES), 0.8% agar and 2% sucrose, and with 25, 50 and 100 mM NaCl; 100, 200 and 300 mM mannitol, or 0.5 and 1 μM abscisic acid (ABA). ABA treatments were on MS-MES plates without sucrose. Plates were kept for 3 days at 4 °C and germination rate (percentage of seeds with radicle emergence) was measured 2 days after transfer to plant growth chamber with constant light at 24 °C. For each treatment at least three independent experiments ($n > 100$) were performed. To analyze the effect of ABA, mannitol and NaCl on *BPMs* expression, seeds were germinated on MS-MES plates and 12-day-old seedlings were transferred to liquid MS-MES medium supplemented with 50 μM ABA, 300 mM mannitol or 150 mM NaCl. For ABA treatments, the medium did not contain sucrose. For control, seedlings were incubated in a mock solution (liquid MS-MES medium with or without sucrose). Solution was vacuum infiltrated into seedlings, and RNA was extracted after 3 h of incubation at 24 °C. To test the effect of elevated temperature on *BPMs* expression, seeds were germinated on MS plates and 12-day-old seedlings were incubated at 24 °C or 37 °C for 3 h, followed by RNA extraction. To monitor *BPM1* protein stability and intracellular localization in stress conditions, seeds were germinated on MS-MES plates and 12-day-old seedlings were incubated for 6 h in liquid MS-MES medium without sucrose and supplemented with 50 μM ABA, 150 mM NaCl or 300 mM mannitol. For control, seedlings were incubated in a liquid MS-MES medium without supplements. To analyze the effect of light on *BPM1* protein

stability and intracellular localization 12-day-old seedlings were cultivated in 16 h day/8 h night regime with the dark period beginning at 11 p.m. and ending at 7 a.m. Seedlings were harvested every 4 h. To monitor temperature-dependent BPM1 stability, seeds were germinated on MS plates and 12-day-old seedlings were, after 8-h dark period, incubated in the dark at 24 °C or 37 °C for 6 h. For all stability assays, BPM1-GFP signal was monitored by fluorescence microscopy, followed by whole protein extraction, western blotting and immunodetection.

Plasmid construction and plant transformation

Binary vector pB7FWG2-BPM1 was constructed as described by Leljak Levanic et al. (2012), electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90), and then introduced to wild type *A. thaliana* Col-0 via floral-dip method (Clough and Bent 1998). Transgenic plants were selected on germination plates containing 30 mg/L glufosinate-ammonium. Selected lines were selfed and T3 or T4 transgenic progeny of lines L003 and L104 was used in most experiments.

Fluorescence microscopy

Confocal images were acquired using a Zeiss LSM780 confocal microscope system (Carl Zeiss) or Leica TCS SP2 AOBs. Laser settings for GFP were as follows: excitation 488 nm/emission: 495–534 nm.

Images were processed in IMAGEJ (<http://rsb.info.nih.gov/ij/>).

Quantitative reverse transcription PCR (RT-qPCR)

For gene expression profiling under physiological conditions, 12-day-old seedlings of wild type and BPM1 overexpression lines were grown in standard conditions and sampled at the same time for RNA extraction. For gene expression profiling under stress conditions, 12-day-old seedlings of wild type and BPM1 overexpression lines were sampled prior and after 3-h-long treatment with 50 μ M ABA, 300 mM mannitol, 150 mM NaCl, as well as prior and after 3-h-long incubation at 37 °C. For expression profiling during daily rhythm, 12-day-old seedlings of wild type and BPM1 overexpression lines were grown in standard 16 h day/8 h night regime and sampled at 12 p.m. (control), 5 p.m. and 6 a.m.

RNeasy plant mini kit (Qiagen) was used for extraction of 500 ng total RNA which was transcribed using high capacity cDNA reverse transcription kit (ThermoFisher Scientific). For RT-qPCR, cDNA was diluted two times, mixed with GoTaq qPCR Master mix (Promega) and gene-specific primers were added (total volume of 15 μ L). All primers used in

RT-qPCR are listed in Table S1. PCR was performed on a Lightcycler LC480 apparatus (Roche) or Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. The mean value of three replicates was normalized to expression of *RHIP1* (AT4G26410) or *RHIP1* and *TIP4.1* (AT4G34270) genes as internal controls and results were analyzed with delta delta Ct method (Livak and Schmittgen 2001). For gene expression analysis of *BPM1-GFP* transgene in BPM1-overexpressing plants, the amount of transgene was measured using GFP-specific primers (listed in Table S1 under 'GFP') and calibrated to expression of endogenous *BPM1.1* and *BPM1.3* in wild type plants.

Protein Gel-Blot analysis

Whole proteins were extracted from tissues frozen in liquid nitrogen and ground in the mixer mill. One volume of extraction buffer (50 mM Tris-HCl—pH 6.8, 3% SDS, 10% glycerol, 0.1% bromophenol blue and 2.5 mM DTT) was added and mixed until material thawed. Samples were heated to 95 °C for 5 min. The extracts were cleared by centrifugation at 14,000 \times g for 15 min at 25 °C. Concentration of proteins was determined by using amido black (Popov et al. 1975). Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous Tris-Gly buffer system using polyacrylamide mini gels (4% stacking gel and 12% resolving gel) with the buffer system of Laemmli. For immunoblotting 20 μ g of proteins per sample was loaded. The proteins were electroblotted at 200 mA for 120 min onto Immobilon-P polyvinylidene difluoride membrane. The transfer buffer was 20 mM Tris-HCl, 150 mM glycine, and 10% methanol. Precision plus protein dual color standard (BioRad) was used. The membrane was blocked with Western Blocker™ Solution for HRP detection systems (Sigma) for 1 h. The same solution was used for primary (Anti-GFP, Roche) and secondary (anti mouse-HRP, Sigma) antibody dilutions. The blot was incubated with primary antibody at 4 °C overnight, and with secondary antibody at RT for 2 h. After incubation with antibodies, the membrane was washed 3 times with PBS and signals were detected with chemiluminescence (Luminata Forte Western HRP substrate, Merck) followed by exposure to autoradiographic films (Hyperfilm, Amersham Pharmacia Biotech). Finally, the membrane was stained with 0.1% Coomassie R-250 in 40% methanol and 10% acetic acid, and destained in 40% methanol and 10% acetic acid.

Immunoprecipitation of BPM1-GFP from L104 seedlings

Seven-day-old seedlings were grown on MS medium supplemented with 2% sucrose, at 24 °C in long day conditions

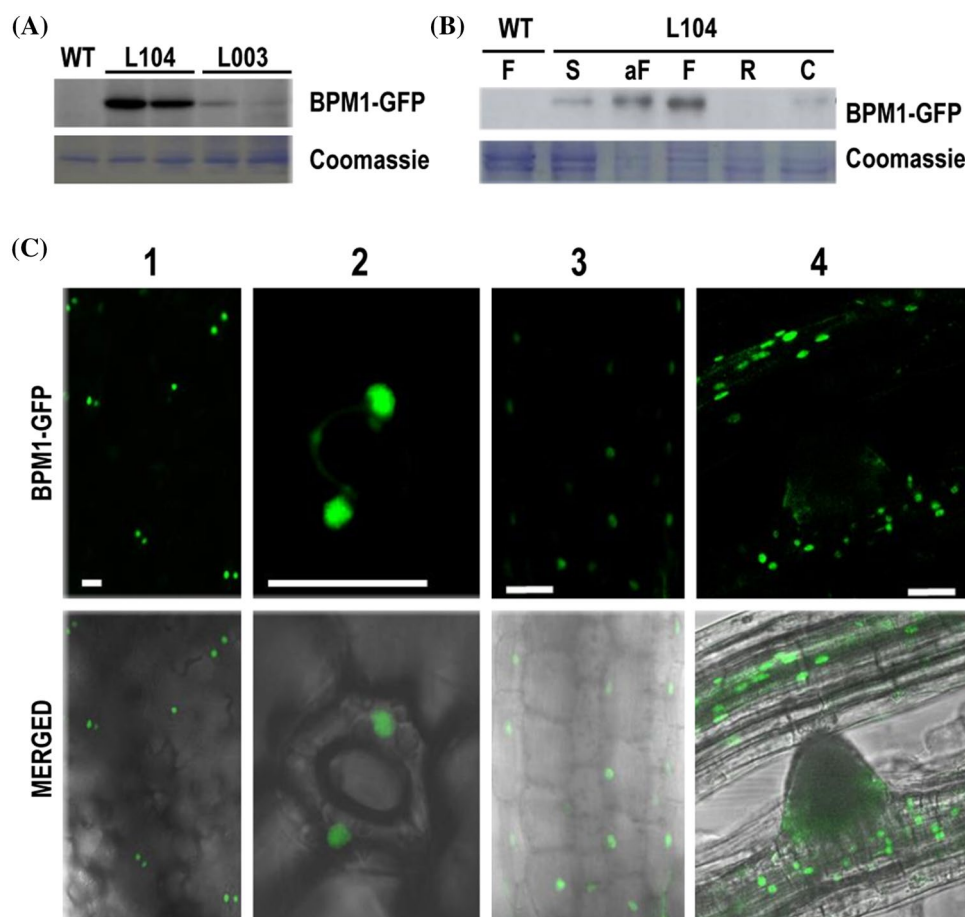


Fig. 1 BPM1 protein expression profile in *A. thaliana* BPM1 over-expression lines. **a** Accumulation of BPM1-GFP protein was verified in BPM1 overexpression lines (L104 and L003). BPM1 overexpressors were grown in standard conditions and 12 day-old seedling were sampled for protein extraction and immunodetection. Two biological replicates (seed stocks) per overexpression line are shown. **b** BPM1 protein accumulates in siliques (S) abnormal flowers (aF), normal flowers (F), rosette (R) or cauline (C) leaves of 12-week-old BPM1-overexpressing plants. BPM1 overexpressors were grown in standard conditions and tissues of 12-week-old plants were sampled for protein

extraction and immunodetection. Whole protein extracts were immunoblotted with anti-GFP monoclonal antibody (upper panels in a, b). For loading control, proteins were stained with Coomassie on PVDF membranes (lower panels in a, b). **c** BPM1-GFP protein accumulates in guard cells (1, 2) hypocotyl (3) and in root (4) of 12-day-old seedlings of BPM1 overexpression lines. Lines L003 (1 and 2) and L104 (3 and 4) are shown. Images were obtained by confocal microscopy. Fluorescent and merged (bright field and BPM1-GFP signal) images are shown. Scale bar = 25 μ m

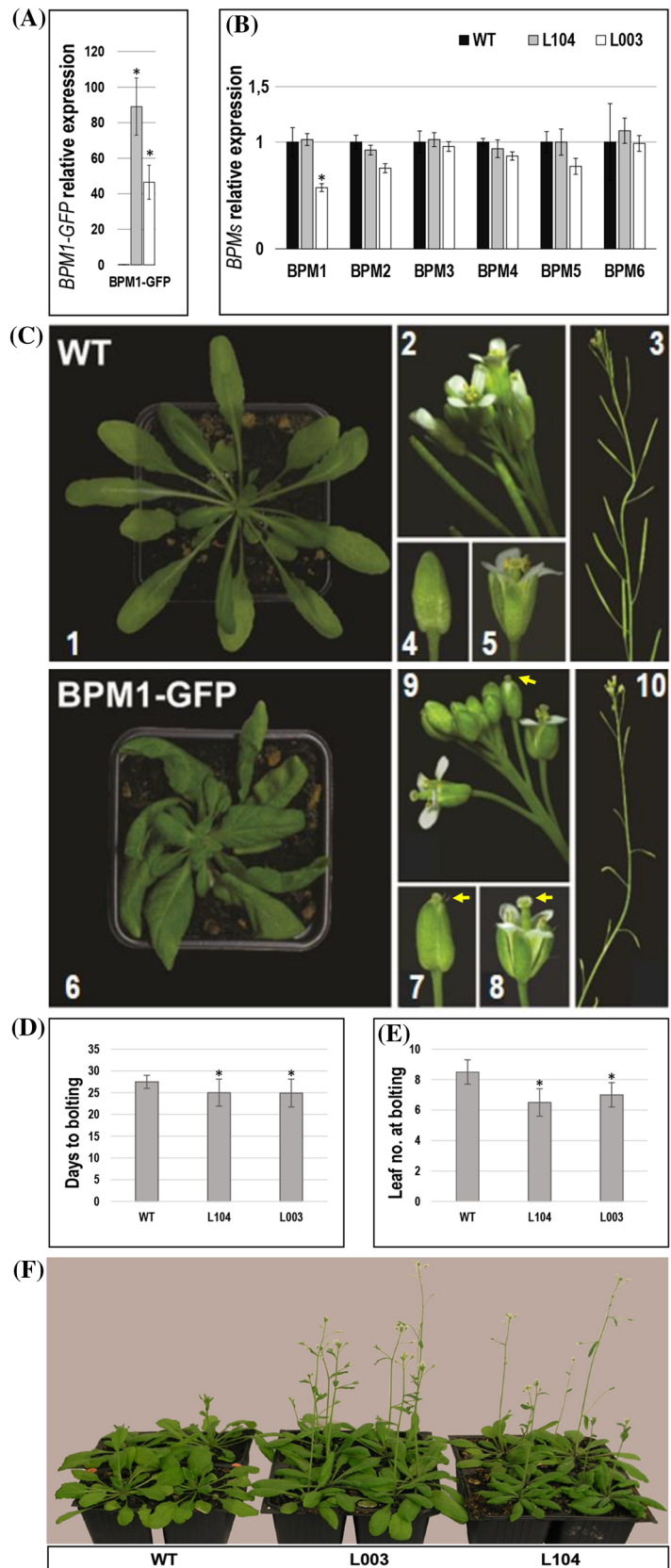
(8 h day/16 h light, 43 μ mol/m² s). Seedlings (450 mg) were frozen in liquid nitrogen and grinded in a chilled mortar. Proteins were extracted using 2 mL of PEB50 buffer (50 mM HEPES, 50 mM KCl, 2.5 mM MgCl₂, 1 mM EDTA, 0.1% TritonX-100, 1 mM DTT and 1 mM NaF) supplemented with cOmplete™ Protease Inhibitor Cocktail (Roche). Four μ g of anti-GFP antibody (Roche) and 10 mg of prepared Protein A Sepharose beads (GE Healthcare) were added to protein extract, followed by incubation under gentle agitation for 16 h at 4 °C. Beads with bound protein were washed three times in 600 μ L PEB50 buffer, denatured in SDS loading buffer at 80 °C for 10 min and analyzed by Western blotting. Each sample was loaded in duplicates on 12% SDS–polyacrylamide gel. After electrophoresis, proteins were transferred on PVDF membrane and blocked.

Membrane was cut in two parts across protein marker line. One part was used for immunodetection with monoclonal Ubiquitin antibody (P4D1) (Santa Cruz Biotechnology), and another part with anti-GFP monoclonal antibody (Roche). Secondary anti-mouse IgG–Peroxidase antibody (Sigma Aldrich) and Immobilon Forte Western HRP substrate (Merck) were used for detection.

MG132, MLN4924 and CHX treatments

For MG132 and MLN4924 treatments, 4-day-old seedlings overexpressing BPM1 germinated on MS germination plates were vacuum infiltrated and incubated for 2 h in MES buffer (pH 5.7) containing 100 μ M MG132 or 50 μ M MLN4924 dissolved in 0.2% dimethyl sulfoxide (DMSO) or 0.2%

Fig. 2 Gene expression profiles and phenotypic characteristics of *A. thaliana* BPM1 overexpression lines. **a** Expression levels of *BPM1-GFP* are significantly increased in BPM1-overexpressors. **b** Expression levels of endogenous *BPM* genes (*BPM1-6*) in BPM1 overexpression lines. Seedlings of wild-type Col-0 (WT; control) and BPM1-overexpressors (L104 and L003) were grown in standard conditions and sampled for gene expression analysis. Expression levels of *BPM1-GFP* and *BPMs* were examined using quantitative RT-PCR analysis. Expression of *BPM1-GFP* (in **a**) and *BPMs* (in **b**) was normalized to expression of *RHIP1* and *TIP4.1* genes and calibrated to wild type expression of *BPM1* (*BPM1.1* and *BPM1.3*) or wild type expression of *BPMs*, respectively. Expression values are shown as mean fold change \pm SD. **c** BPM1 overexpression lines exhibit altered plant growth and approach herkogamy with pronounced stigma exertion. Wild type (WT) plant: rosette phenotype of 8-week-old plant (1), inflorescence (2, 3), flower bud (4) and flowers (5) of 12-week-old plant. BPM1-GFP overexpressing line (L104): curved rosette phenotype of 8-week-old plant (6) flower bud (7), flower (8) and inflorescence (9, 10) of 12-week-old plant. Arrows point to stigma exertion. **d–f** BPM1 overexpressors exhibit an early flowering phenotype, with significantly lower number of days to bolting (**d**) and significantly lower number of leaves at time of bolting (**e**) in L104 and L003 compared to wild type (shown as mean value \pm SD). **f** Inflorescences of wild type and BPM1 overexpression lines (L104 and L003) 6 weeks after germination. For all quantitative data, asterisks indicate statistically significant differences between means of tested sample and control at $P < 0.05$ (Student's *t* test). Similar results were obtained in three independent experiments



DMSO. After incubation, the seedlings were thoroughly washed to remove residual MG132, MLN4924 or DMSO. For protein analysis, seedlings were frozen in liquid nitrogen, ground and whole proteins were extracted.

To examine BPM1 protein stability, seedlings were treated with the translational inhibitor cycloheximide (CHX) as described by Gilkerson et al. (2016). Seedlings were grown on MS medium for 6 days at 24 °C and incubated with CHX (0.2 g/L) for 1 and 3 h at 24 °C and 37 °C. After sampling, seedlings were frozen in liquid nitrogen, homogenized and whole proteins and RNA were extracted and used for immunoblot analysis.

Yeast-two-hybrid screen

Full length CDS sequences of BPM1.1 and BPM1.2 were fused to the GAL4-binding domain of pGBT9 or pGBKT7, respectively and AtCUL3A was cloned as fusion to the GAL4 activation domain of pGAD424 (Clontech). For yeast-two-hybrid screens the yeast host strain Hfc7 was used (Feilotter et al. 1994). Transformants were selected on synthetic SD-Trp-Leu media (SD-T-L; Clontech) and interactions were tested on SD-Trp-Leu-His (SD-T-L-H) supplemented with 10 mM 3-Amino-1,2,4-Triazol, allowing growth for 2 days at 30 °C.

Pollen viability test

Pollen grain viability was assessed by using fluorescein diacetate (FDA 10 mg/mL in acetone). FDA was added to hydrated pollen on a glass slide at a final concentration of 0.2 mg/mL. Sample was incubated for 5 min in dark and observations were made with an Axiovert 200 M fluorescent microscope (Zeiss) and filter set 13 (excitation BP 470/20 and emission BP 505-530).

Multiple sequence alignments

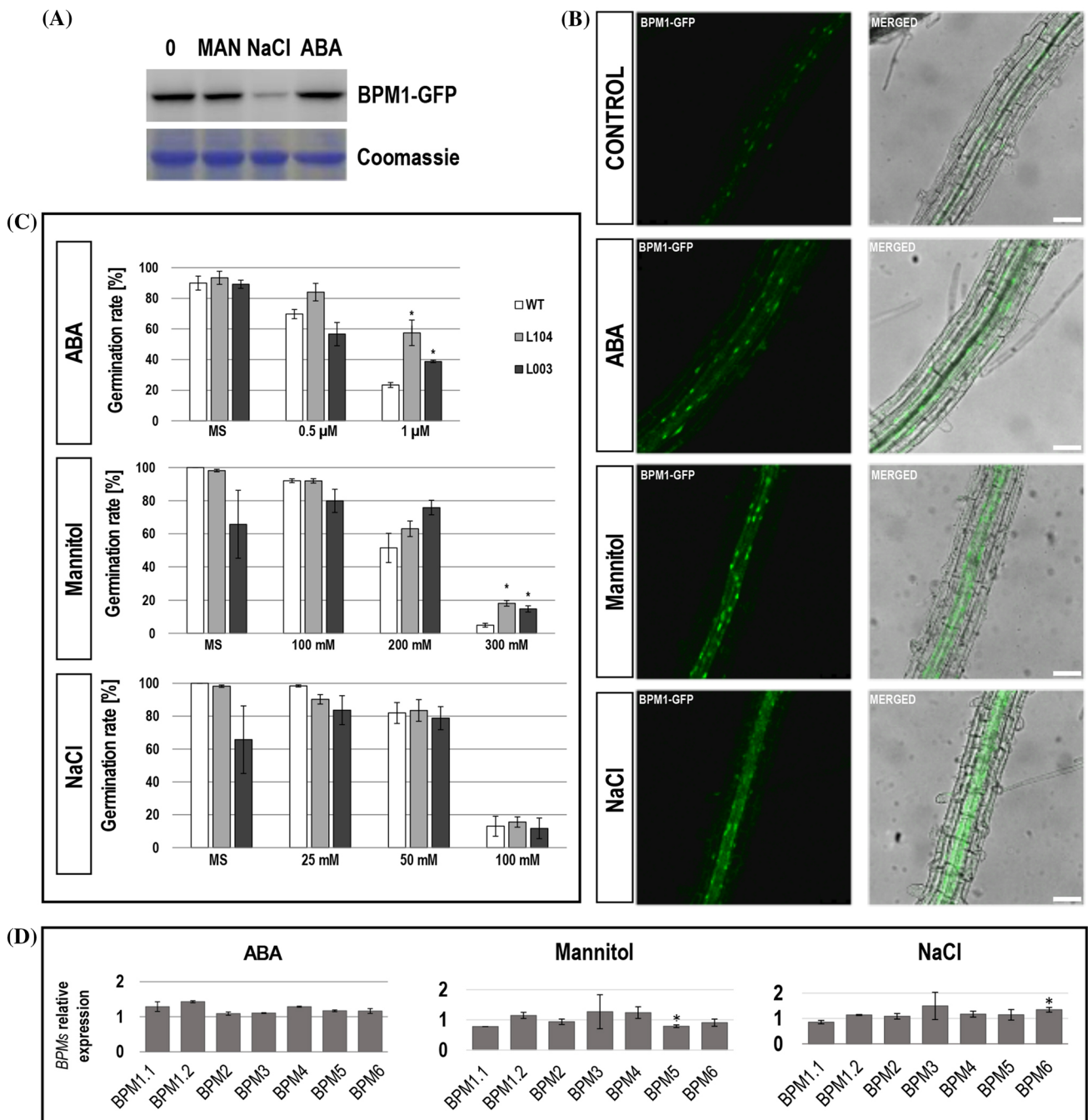
For multiple sequence alignments of BTB and MATH domains, sequences of 16 known BPM proteins (www.uniprot.org) were used: BPM1.1 (Q8L765), BPM1.2 (A0A178UFC0), BPM2.1 (A0A1I9LQF8), BPM2.2 (F4JAT1), BPM2.3 (A0A1I9LQF9), BPM2.4 (A0A1I9LQG0), BPM2.5 (A0A178VH59), BPM3.1 (A0A1P8AZW8), BPM3.2 (A0A178W0L2), BPM3.3 (A0A178VZP7), BPM4.1 (Q9SRV1), BPM4.2 (A0A178V9U9), BPM5.1 (Q1EBV6), BPM6.1 (A1L4W5), BPM6.2 (A0A1I9LS60) and BPM6.3 (A0A1I9LS59). The putative MATH and BTB domain sequences were pooled

Fig. 3 BPM1-GFP protein stability and germination rate of BPM1 overexpressors increase under ABA and osmotic stress. **a** BPM1 protein is degraded after exposure to NaCl-induced salt stress but remains stable after ABA treatment and mannitol-induced osmotic stress. Twelve-day old seedlings were exposed for 6 h to either 50 μM ABA, 150 mM NaCl, 300 mM mannitol (MAN) or mock solution (liquid MS; control) and sampled for protein extraction. Whole protein extracts were immunoblotted with anti-GFP monoclonal antibody (upper panel). For loading control, proteins were stained with Coomassie on PVDF membranes (lower panel). **b** BPM1-GFP accumulates in root cell nuclei after exposure to ABA and osmotic stress but diminishes and translocates to root vasculature after exposure to salt stress. Twelve-day old seedlings of BPM1 overexpression lines (L104 and L003) were exposed for 6 h to either 50 μM ABA, 150 mM NaCl or 300 mM mannitol (MAN) and immediately analyzed by confocal microscopy. Seedlings incubated in mock solution (liquid MS; control) were used as control. Fluorescent and merged (bright field and BPM1-GFP signal) images of L104 are shown. Scale bar=50 μm. **c** BPM1 overexpression lines germinate better under ABA and mannitol-induced osmotic stress compared to wild type. BPM1 overexpressors and wild type are equally susceptible to NaCl-induced salt stress. Seeds of wild type (WT) and BPM1 overexpressors (L104 and L003) were germinated on MS medium supplemented with varying concentrations of ABA, mannitol or NaCl (denoted on the graphs) and germination rates (percentage of seeds with radicle emergence) were examined after 2 days. **d** Endogenous *BPM5* expression decreases after treatment with mannitol (middle) and *BPM6* expression increases after treatment with NaCl (right). Other endogenous *BPMs* do not significantly change in response to ABA (left), mannitol or NaCl treatment. Seedlings of wild-type Col-0 were exposed for 3 h to either 50 μM ABA, 300 mM mannitol, 150 mM NaCl or mock solution (liquid MS; control) and sampled for gene expression analysis. Expression levels of BPM genes under different treatments were examined using quantitative RT-PCR analysis. Expression of BPM genes was normalized to expression of *RHIP1* and *TIP4.1* genes or only *RHIP1* gene. For all treatments, expression of each individual BPM gene was calibrated to expression of that gene in untreated control, which was taken as 1. Expression values are shown as mean fold change ± SD. For all quantitative data, asterisks indicate statistically significant differences between means of tested sample and control at $P < 0.05$ (Student's *t* test). Similar results were obtained in three independent experiments

according to Pfam database, aligned using ClustalX v.2.0 (Larkin et al. 2007) and alignments were displayed using Jalview v.2 (Waterhouse et al. 2009).

Statistical analysis

Experiments were replicated at least three times on independent samples. Expression levels of *BPM* genes, *HsfA3*, time to bolting, no. of leaves at bolting and germination rate were analyzed by two-tailed *T* test between means for wild type and transgenic lines. The effects of treatments on gene expression were analyzed by two-tailed *T*-test between means for control and treated samples. Differences with a *P* value of < 0.05 or < 0.01 were regarded as significant.



Results and discussion

Diversity of BPM proteins in Arabidopsis

The *Arabidopsis* genome comprises six MATH-BTB genes (called *BPM1-6*) encoding for at least 16 different BPM protein isoforms, according to available databases. *Arabidopsis* BPM proteins are presumed to play a role in targeted protein degradation. In this process, the MATH domain recognizes specific substrate proteins whereas the BTB domain binds CUL3 of the E3 ubiquitin ligase complex,

which ubiquitinates and thereby designates proteins for proteasomal degradation (Genschik et al. 2013). Multiple alignments of BPMs amino acid sequences show high similarity (84–100%) within the MATH domain responsible for substrate recognition (Fig. S1a). This is in accordance with the finding that MATH domain recognizes specific amino acid consensus sequence (ϕ - π -S-X-S/T where ϕ is nonpolar; π is polar and X is any amino acid) in substrate proteins (Morimoto et al. 2017). However, the BTB domain is marked by surprisingly high variability (30–100% similarity, Fig. S1b) suggesting that BPM proteins may have additional

roles, not only those coupled with CUL3 E3 ligase. One such role could be interaction with proteins outside the Cullin3 pathway.

The *BPM1* gene encodes for two proteins, BPM1.1 and BPM1.2. In this work, to generate BPM1-overexpressing lines, we used the shorter variant (BPM1.1) which is missing the BPM1.2-specific sequence of 35 amino acids positioned within the BTB domain (Fig. S1c). Yeast-two hybrid screens showed that both protein variants interacted with Cul3A with no observable differences in interaction affinity (Fig. S2).

Phenotypic and molecular characterization of transgenic *Arabidopsis* plants with BPM1 overexpression

Several overexpressors of BPM proteins have been regenerated, namely the GFP-fused BPM2 and BPM4 (Morimoto et al. 2017), MYC-fused BPM3 (Lechner et al. 2011), and HA-fused BPM3 and BPM5 (Julian et al. 2019). Additionally, three different lines with BPM knockdown have been described to date (Lechner et al. 2011; Chen et al. 2013; Morimoto et al. 2017), and each was shown to possess some unique features, presumably due to uneven downregulation of individual *BPM* genes. Here, we generated *Arabidopsis* plants expressing *BPM1-GFP* under strong constitutive 35S promoter. Though all primary transformants (T1) expressed full length *BPM1-GFP* mRNA, recombinant BPM1-GFP protein was detected by fluorescent microscopy in only two (lines L003 and L104) among more than a hundred T1 lines inspected. The difficulty of detecting BPM proteins *in planta* has been reported previously. For instance, MYC-fused BPM3 could not be detected despite the high transgene expression levels (Lechner et al. 2011), implying rapid turnover and high instability of this protein family. Therefore, even though an occasional fluorescent BPM1-GFP signal was detected in several T2 and T3 seedlings of other BPM1-overexpressing lines, only the lines with significant accumulation of BPM1-GFP recombinant protein (Fig. 1a; L003 and L104) were used for further experiments.

Confocal microscopy and immunodetection showed that *BPM1* overexpressing lines do not accumulate the protein in a constitutive manner (Fig. 1b, c). In adult transgenic plants, accumulation of recombinant BPM1-GFP protein resembled endogenous *BPM1* gene expression pattern (Weber and Hellmann 2009; Lechner et al. 2011), showing highest abundance in siliques, flowers, and cauline leaves (Fig. 1b). Furthermore, the recombinant BPM1-GFP protein was most detectable in guard cells, hypocotyl and root of 4-day-old seedlings (Fig. 1c). Consistent with previous results (Lechner et al. 2011; Leljck Levanic et al. 2012; Morimoto et al. 2017), BPM1-GFP localized mainly in the nucleus, except in root elongation zone where it was additionally detected in

differentially-sized mobile cytoplasmic particles of unknown origin and function (Online Resource 1).

Both BPM1-overexpressing lines (L104 and L003) showed significant increase of *BPM1-GFP* transcripts relative to endogenous *BPM1* transcripts in wild type (Fig. 2a). Line L104 and L003 show 89 and 46 times higher *BPM1* expression, respectively. Substantial BPM1 overexpression had no impact on expression of endogenous *BPM* genes in line L104, while in line L003 expression of endogenous *BPM1* gene was significantly reduced (Fig. 2b).

BPM1 overexpression induced developmental and physiological alterations in transgenic *Arabidopsis* plants compared to wild type, including rosette growth and development (Fig. 2c) and earlier flowering (Fig. 2d–f). Plants overexpressing BPM1 had smaller and more compact rosette with leaves that curved counter clockwise, shorter petioles and wider leaf blades that curled under (adaxialized) (Fig. 2c). The strongest phenotype was observed in flowers which showed exaggerated opening and approach herkogamy where the stigmas were prematurely elongated and positioned above the anthers (arrows in Fig. 2c). This spatial separation of male and female reproductive organs is not typical for *A. thaliana* and is most likely the cause of silique shortening (Fig. 2c, panel 10) and lower seed production in our transgenic lines. Stigma exertion was always pronounced in flowers produced immediately after the transition from vegetative to reproductive phase and became less apparent as the plants grew older. Still, overexpressors produced shorter siliques with less seeds or total lack of seed in some siliques. All alterations were more obvious in line L104 than in L003. Shorter siliques are described in the amiR-*bpm* mutant which, together with stigma exertion, exhibits reduced pollen viability (Lechner et al. 2011). Here, BPM1 overexpressors developed viable pollen (Fig. S3) and no defects in pollen growth and development were observed. Another BPM knockdown line described by Chen et al. (2013; *6xami-bpm*) produces bigger seeds enriched in fatty acids. We estimated the seed size of BPM1 overexpressors but noticed no change compared to wild type. Another important phenotypic trait of BPM1 overexpressors was early flowering phenotype (Fig. 2d–f), with a significant decrease in both the time to bolting and leaf number at bolting of BPM1 overexpressors compared to wild type plants (Fig. 2d, e) at long day conditions.

BPM1 overexpressors show increased transgenic protein stability and germination rates in stress conditions

The signal transduction pathway governed by ABA regulates early developmental programs such as seed dormancy, germination and seedling growth. Besides these functions, ABA also acts as an important signaling molecule during

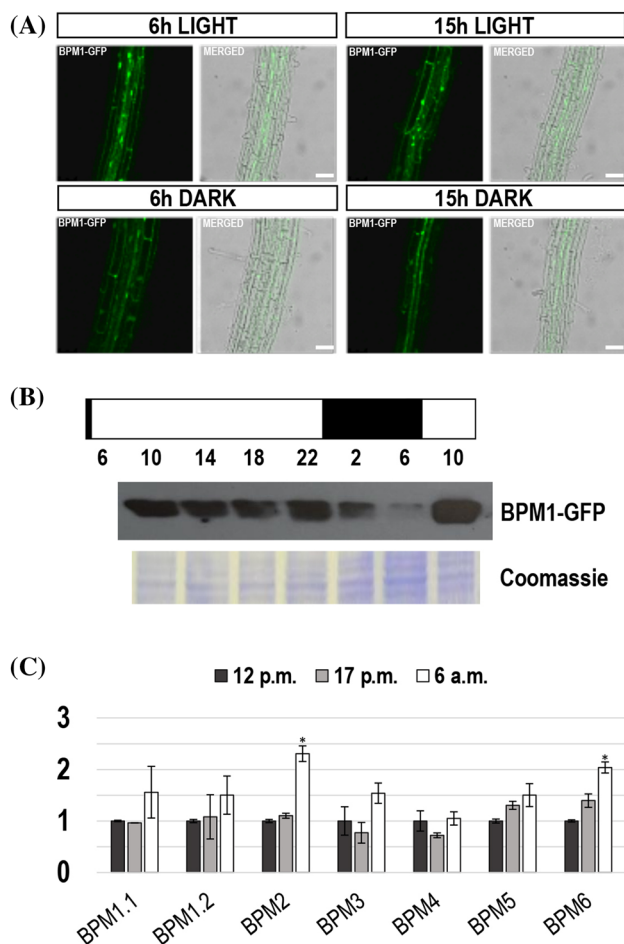


Fig. 4 BPM1-GFP protein stability is susceptible to daily rhythm changes. **a** BPM1-GFP protein accumulates in root epidermal cell nuclei during light exposure and in stele during prolonged dark exposure. Twelve-day-old seedlings of BPM1 overexpression lines were incubated in either dark or light for 6 h (left) or 15 h (right) and immediately analyzed by confocal microscopy. Fluorescent and merged (bright field and BPM1-GFP signal) images are shown. Scale bar = 50 μm. **b** BPM1 protein levels drop during nighttime. Twelve-day-old seedlings of BPM1 overexpression lines were cultivated in 16 h day/8 h night regime with the dark period beginning at 11 p.m. and ending at 7 a.m. (represented by black color in the schematic diagram). Seedlings were sampled every 4 h for protein extraction. Whole protein extracts were immunoblotted with anti-GFP monoclonal antibody (upper panel). For loading control, proteins were stained with Coomassie on PVDF membranes (lower panel). **c** Expression levels of endogenous *BPM2* and *BPM6* remain stable during the day and significantly increase at the end of the dark period. A similar trend is observed for all *BPMs*. Seedlings of wild-type Col-0 were grown in standard growth conditions and sampled for gene expression analysis at 12 p.m., 5 p.m. and 6 a.m. (near the end of the dark period). Expression levels of *BPM* genes were examined using quantitative RT-PCR analysis. Expression of *BPM* genes was normalized to expression of *RHIP1* gene and for each individual *BPM* gene, the expression at 5 p.m. and 6 a.m. was calibrated to expression of that gene at 12 p.m., which was taken as 1. Expression values are shown as mean fold change ± SD. Asterisks indicate statistically significant differences between means of tested sample and control at $P < 0.05$ (Student's *t* test). Similar results were obtained in two independent experiments

plant response to environmental stress (Sharp and LeNoble 2002; Zhu 2002; Hubbard et al. 2010) and affects expression of more than 1300 genes mediating dramatic changes in plant physiology (Hoth et al. 2002; Sah et al. 2016). Through regulation of HB6 and DREB2A transcription factors and protein phosphatases type 2C, BPMs have been described as important factors in ABA signaling and stress tolerance (Lechner et al. 2011; Morimoto et al. 2017; Julian et al. 2019). To examine whether stress conditions impact the physiology of BPM1-overexpressors, we first tested transgenic BPM1 protein stability in response to ABA, mannitol and NaCl treatment. Exposure to salt stress for 6 h decreased BPM1 protein levels, while exposure to mannitol and ABA treatment had no effect on BPM1 protein levels (Fig. 3a). Additionally, exposure of BPM1-overexpressing seedlings to ABA, mannitol and NaCl showed accumulation of BPM1 in root cell nuclei under ABA and mannitol-induced osmotic stress, as opposed to a more dispersed protein presence along the root stele under NaCl-induced salt stress (Fig. 3b).

Next, we examined ABA and abiotic stress impact on germination of BPM1 overexpressors. Although seeds of BPM1 overexpressors generally showed a slight delay in radicle protrusion compared to wild type, this discrepancy was no longer observable 48 h after imbibition (Fig. S4). Therefore, we selected the 48 h time point as suitable for the germination assay. Seeds of wild type and BPM1 overexpressors were germinated on varying concentrations of ABA, mannitol and NaCl and germination rates were measured. Again, a trend could be observed in BPM1 overexpressors' response to ABA and mannitol-induced stress as opposed to salt stress (Fig. 3c). BPM1 overexpressors showed higher germination rates under ABA and mannitol-induced stress compared to wild type, whereas no difference was observed under salt stress. For instance, only 23% wild type seeds germinated when treated with 1 μM ABA, while this number reached 39–58% for BPM1-overexpressing lines.

In our study, neither ABA nor mannitol or NaCl treatment had significant influence on expression of most endogenous *BPM* genes (Fig. 3d), the only exceptions being *BPM5* with slightly decreased expression after mannitol treatment, and *BPM6* with slightly increased expression after NaCl treatment. Overall, this reflects the currently available expression data (Genevestigator; EFP Browser) showing very low perturbations in expression of *BPMs* during different experimental conditions.

Altogether, resistance to ABA during germination and enhanced germination ability upon osmotic stress support a possible role of BPM1 in drought response. This corroborates previous findings showing that lines with down-regulated *BPMs* are more drought-sensitive than wild type (Lechner et al. 2011; Morimoto et al. 2017).

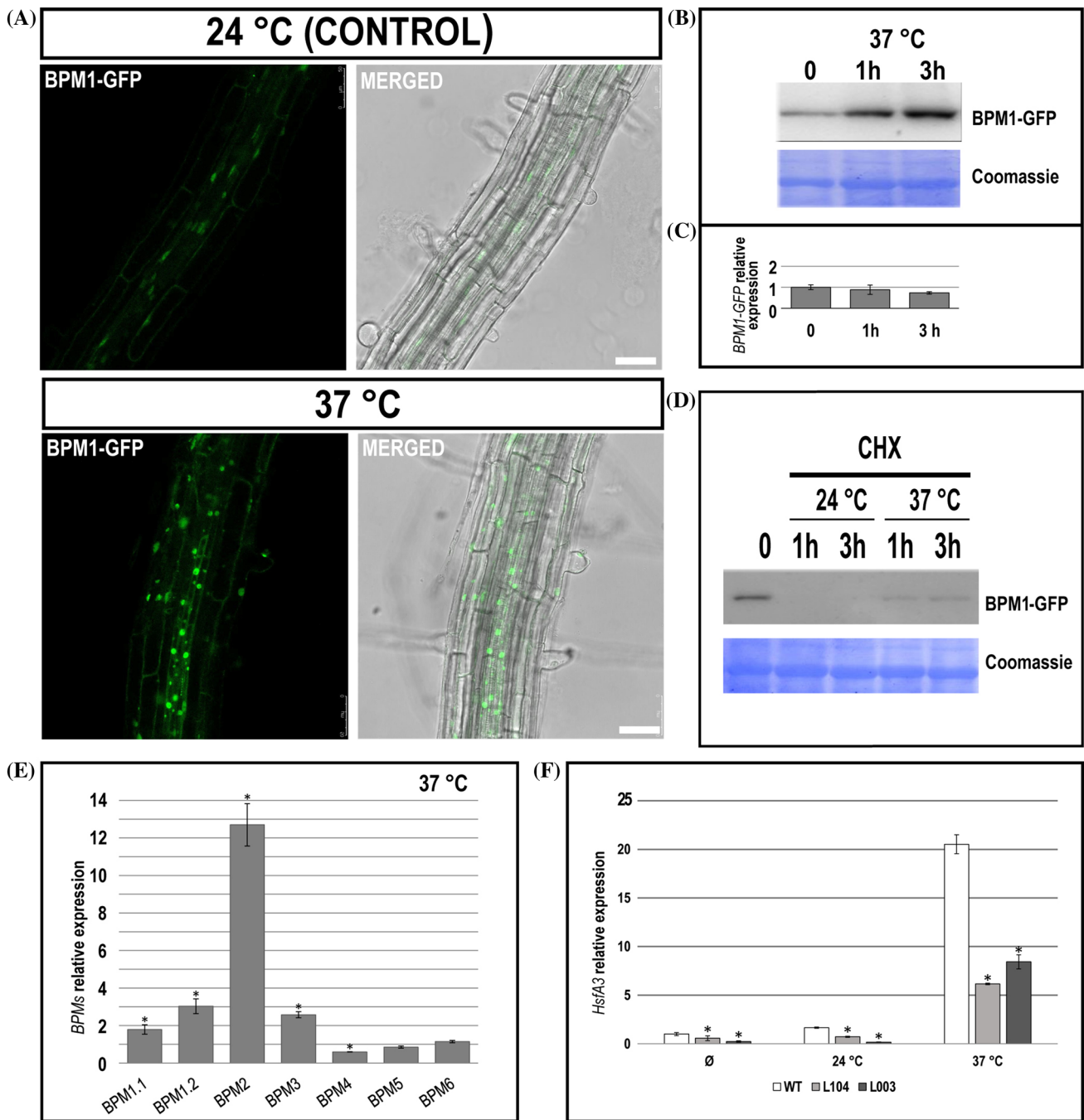
Environmental conditions influence BPM1 protein stability

By mediating proteolysis of different transcription factors, BPMs are involved in sensing environmental changes, such as daily rhythm oscillations, temperature fluctuation and abiotic stress (Henriksson et al. 2005; Sakuma et al. 2006; Cernac et al. 2006; Chen et al. 2015). However, nothing is known about the stability of BPM proteins during these external changes. Here, we tested BPM1 protein stability during normal daily rhythm (16 h day and 8 h dark, 24 °C) and under elevated temperature. Accumulation of BPM1-GFP showed different patterns after exposure to light or dark (Fig. 4a). Both 6 h and 15 h of exposure to light caused a characteristic accumulation of BPM1-GFP in root epidermal cell nuclei of 12-day-old transgenic seedlings. On the other hand, incubation in the dark first showed a dispersion of GFP signal (6 h), then translocation of signal into and along the root stele (15 h). Finally, prolonged dark incubation (24 h) resulted in BPM1-GFP accumulation in xylem (Fig. S5). Additionally, Western analysis showed consistent accumulation of BPM1 during the day and reduction of BPM1 protein levels in the dark, with BPM1 levels reaching their minimum at the end of the dark period, at 6 a.m. (Fig. 4b). Gene expression analysis of endogenous *BPMs* in wild type seedlings showed differences in expression during different times of day (Fig. 4c). Wild type seedlings were sampled at 12 p.m., 5 p.m. (periods of light) and 6 a.m. (end of the dark period) and expression of each endogenous *BPM* gene was estimated relative to the 12 p.m. value. The expression of endogenous *BPM* genes remained stable during the day and showed a tendency to increase at the end of the dark period, but with statistical significance only for *BPM2* and *BPM6*. The apparent increase in expression of *BPMs* at the end of the dark period (at 6 a.m.) correlates with degradation of BPM1-GFP during the dark period. Hypothetically, if protein levels of all BPMs drop during the night and rise up again at daylight, there would indeed be an increased need for synthesis of *BPM* proteins at the end of the dark period, reflected by a rise in gene expression. Nevertheless, the photoperiod-dependent change in stability of BPM1-GFP is interesting in light of a previously proposed model, by which BPM1 participates in photoperiod-dependent regulation of flowering. According to the authors, BPM1 induces the degradation of the transcription inhibitor MYB56, thereby relieving the negative effects of MYB56 on the promoter of the FT gene, a major activator of flowering (Chen et al. 2015). In the future, it would be interesting to see whether BPM1 protein stability plays a role in this molecular mechanism.

Next, we analyzed the effects of elevated temperature on stability of recombinant BPM1. Transgenic seedlings incubated at 37 °C showed extensive accumulation

Fig. 5 BPM1-GFP protein is stabilized at elevated temperatures. **a** ▶ BPM1-GFP protein accumulates in root cell nuclei after exposure to 37 °C. Twelve-day-old seedlings of BPM1 overexpression lines (L104 and L003) were incubated for 6 h at 24 °C (control) and 37 °C in the dark and immediately analyzed by confocal microscopy. Fluorescent and merged (bright field and BPM1-GFP signal) images of L104 are shown. Scale bar=50 μm. **b** BPM1-GFP accumulates after exposure to 37 °C. Six-day-old seedlings were sampled before treatment (0) and 1 and 3 h after incubation at 37 °C in the dark. **c** Expression of *BPM1-GFP* transgene remains unchanged at 37 °C. Six-day-old seedlings of BPM1-GFP overexpressing line L104 were sampled before treatment (0) and 1 and 3 h after incubation at 37 °C. *BPM1-GFP* transgene expression was analyzed by quantitative RT-PCR, normalized to RHIP1 and calibrated to control (0; at 24 °C) which was taken as 1. **d** BPM1 protein is stabilized at 37 °C in conditions of inhibited protein synthesis. Six-day-old seedlings of BPM1 overexpression line L104 were sampled before treatment (0) and 1 and 3 h after treatment with protein synthesis inhibitor cycloheximide (CHX, 0.2 mg/mL) at either 24 °C or 37 °C. Whole protein extracts were immunoblotted with anti-GFP monoclonal antibody (upper panel in b and d). For loading control, proteins were stained with Coomassie on PVDF membranes (lower panel in b and d). **e** Expression levels of most endogenous *BPM* genes (*BPM1.1*, *1.2*, *2-4*) significantly change after heat treatment, with the highest increase measured for *BPM2*. Seedlings of wild-type Col-0 were incubated for 3 h at 37 °C or 24 °C (untreated control) and sampled for gene expression analysis using quantitative RT-PCR. Expression of each individual *BPM* gene was normalized to expression of RHIP1 gene and calibrated to expression of that same *BPM* gene in untreated control, which was taken as 1. Expression values are shown as mean fold change ± SD. Asterisks indicate statistically significant differences between means of treated and control (untreated) sample at $P < 0.05$ (Student's *t* test). Similar results were obtained in three independent experiments. **f** Expression profile of *HsfA3* in BPM1-overexpression lines. The increase in expression of *HsfA3* in response to heat treatment is less severe in BPM1 overexpression lines compared to wild type. Seedlings of wild-type Col-0 (WT) and BPM1-overexpressors (L104 and L003) were sampled for gene expression analysis prior to heat treatment (Ø; control), after 3 h of no heat treatment (24 °C) and after 3 h of heat treatment (37 °C). Expression levels of *HsfA3* were examined using quantitative RT-PCR analysis, normalized to expression of *RHIP1* gene and calibrated to expression of *HsfA3* in the wild type control sample. The calibrator value was taken as 1 (shown as the first bar on the left). Expression values are shown as mean fold change ± S.D. Asterisks indicate statistically significant differences between means of wild type and BPM1-overexpression lines for each sample type (Ø, 24 °C and 37 °C) at $P < 0.05$ (Student's *t* test). Similar results were obtained in two independent experiments

of BPM1-GFP in root cell nuclei compared to control (Fig. 5a), and an overall increase in protein levels as shown by anti-GFP immunoblotting using whole protein extracts of treated seedlings (Fig. 5b). To exclude the possibility that heat-induced BPM1 accumulation was caused by enhanced transgene expression, we performed RT-qPCR analysis and found that relative expression levels of *BPM1-GFP* transgene remained unchanged at 37 °C (Fig. 5c). Therefore, accumulation of BPM1 is most likely regulated at the level of protein synthesis and/or degradation. To further test this hypothesis, transgenic seedlings incubated at 24 °C and 37 °C were treated with protein synthesis inhibitor CHX and BPM1-GFP protein levels were quantified. Protein



synthesis was blocked by CHX and BPM1-GFP was not detectable after only 1 h at 24 °C (Fig. 5d). However, when CHX-treated seedlings were incubated at 37 °C, the BPM1-GFP signal remained detectable for as long as 3 h of incubation, reflecting enhanced stability of already synthesized BPM1-GFP at higher temperatures. Taken together, these results show that environmental temperature appears to have an important role in regulating BPM1 protein stability. In wild type plants, RT-qPCR analysis showed that 3 h of incubation at 37 °C induced the expression of endogenous

BPM1, *BPM2* and *BPM3*, with the strongest increase measured for *BPM2* (Fig. 5e). Expression of *BPM4* was slightly decreased, while expression of *BPM5* and *BPM6* remained unchanged.

These results are particularly interesting in light of recent findings regarding DREB2A, a key transcription factor that controls the response to dehydration and heat stress by activating many stress-inducible target genes (Sakuma et al. 2006). *DREB2A* expression itself is induced by dehydration or elevated temperature and *DREB2A* protein levels

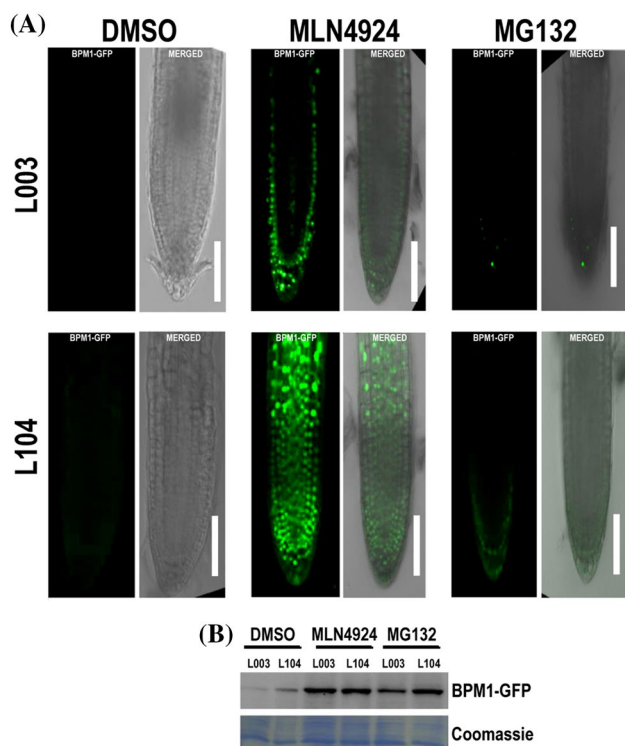


Fig. 6 The neddylation inhibitor MLN4924 and proteasome inhibitor MG132 stabilize BPM1 protein *in planta*. **a** BPM1-GFP accumulates in roots of BPM1 overexpression lines after treatment with MLN4924 and MG132. Four-day-old seedlings of lines overexpressing BPM1-GFP (L003 and L104) were treated for 2 h with either 50 μ M MLN4924, 100 μ M MG132 or mock solution (0.2% DMSO; control) and root tips were subjected to confocal microscopy. Fluorescent and merged (bright field and BPM1-GFP signal) images are shown. Scale bar = 100 μ m. **b** BPM1-GFP accumulates in BPM1 overexpression lines after treatment with MLN4924 and MG132. Twelve-day-old seedlings of BPM1 overexpressors (L003 and L104) were treated with 0.2% DMSO, 50 μ M MLN4924 or 100 μ M MG132 and sampled for protein extraction and immunodetection. Whole protein extracts were immunoblotted with anti-GFP monoclonal antibody (upper panel). For loading control, proteins were stained with Coomassie on PVDF membranes (lower panel)

are markedly increased immediately after exposure to heat stress, with an apparent drop after a few hours (Morimoto et al. 2013). Moreover, DREB2A protein degradation by the 26S proteasome is mediated by DRIP1/2 and CUL3-BPMs (Morimoto et al. 2013, 2017). BPM1-6 proteins were shown to interact with DREB2A in yeast two hybrid, as well as *in planta* and research on BPM2 protein showed formation of BPM2-DREB2A complex after exposure to heat (Morimoto et al. 2017). Therefore, the substantial (12-fold) increase in wild type *BPM2* expression caused by elevated temperature reported here could reflect the increase in demand for BPM2, as a negative regulator of DREB2A upon heat stress (Morimoto et al. 2017). To indirectly test whether DREB2A levels changed in plants overexpressing BPM1, we performed RT-qPCR analysis of *HsfA3*, a gene

encoding a heat-shock transcription factor and shown to be up-regulated by DREB2A in conditions of heat stress (Sakuma et al. 2006). Seedlings of wild type and BPM1 overexpression lines (L104 and L1003) were incubated at 37 $^{\circ}$ C and *HsfA3* expression levels were estimated relative to wild type levels prior to heat treatment. As expected, in wild type plants *HsfA3* levels significantly increased (20-fold) after heat exposure (Fig. 5f). The *HsfA3* levels also increased in BPM1 overexpression lines after heat treatment but this increase was significantly lower compared to wild type (only sixfold in L104 and eightfold in L003). Additionally, even when no heat treatment was applied (24 $^{\circ}$ C), BPM1 overexpressors showed decreased expression levels of *HsfA3* compared to wild type plants. This result indicates that DREB2A protein levels could be decreased in BPM1 overexpressors, possibly through the BPM1-mediated proteolysis of excess DREB2A in heat stress conditions. This potential role of BPM1 in heat stress response presents an intriguing topic for further research.

To test whether degradation of BPM1-GFP is mediated by the ubiquitin proteasome pathway, BPM1 overexpressing seedlings were treated with the proteasome inhibitor MG132 and Cullin neddylation inhibitor MLN4924 (Palombella et al. 1994; Hakenjos et al. 2011). Both inhibitors induced accumulation of BPM1-GFP in root tips of transgenic seedlings (Fig. 6a; lines L003 and L104), with stronger effect observed for the Cullin neddylation inhibitor MLN4924. Furthermore, whole protein extraction of treated seedlings followed by immunodetection of BPM1-GFP showed higher overall accumulation of transgenic protein compared to untreated control (Fig. 6b). This indicates an important role of proteasome 26S and Cullin E3 ligase in BPM1 degradation. Ubiquitination of BPM1-GFP was tested by immunoprecipitation but only faint traces of anti-ubiquitin signals could be observed in samples of purified BPM1-GFP, indicating but not confirming monoubiquitination of transgenic protein (Fig. S6). Future research should aim to elucidate whether BPM1 is degraded simultaneously with its ubiquitinated targets in a CUL3-dependent manner, or whether another E3 ligase targets BPM1 for ubiquitination and subsequent degradation.

Conclusions

The 6 *BPM* genes in the *A. thaliana* genome encode at least 16 different BPM protein isoforms. To better characterize BPM1 protein, transgenic *A. thaliana* plants overexpressing *BPM1-GFP* gene were regenerated. BPM1 overexpression induced notable morphological and physiological changes such as earlier flowering, reduced seed production and resistance to ABA and osmotic stress during germination.

Environmental conditions substantially influenced BPM1 accumulation in *planta*. BPM1 protein levels dropped during the dark and the protein translocated from root cell nuclei to the root stele after incubation in the dark. Prolonged exposure to heat induced the expression of *BPM1-3* genes and enhanced BPM1 protein stability. Expression of heat-inducible *HsfA3* gene markedly decreased in heat-treated BPM1 overexpressors compared to wild type, supporting an existing hypothesis of BPM proteins acting as negative regulators of transcription factor DREB2A during heat stress. Regarding the apparent stabilization of BPM1 upon heat treatment, several questions remain to be answered. Namely, how does the process of BPM1 stabilization occur, how is it regulated and is there a link connecting the environmentally conditioned stability of BPM1 and its role in targeting transcription factors for ubiquitination and proteasomal degradation?

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Author contributions Nataša Bauer, Pascal Genschik and Esther Lechner contributed to the study conception and design. Material preparation and phenotype analysis were performed by Nataša Bauer, Andreja Škiljaica and Mateja Jagić. Q-RT-PCR analysis were done by Nataša Bauer, Esther Lechner, Kristina Majsec and Andreja Škiljaica. Confocal images were acquired by Nataša Bauer, Esther Lechner and Nenad Malenica. Immunodetections were done by Nataša Bauer, Esther Lechner, Mateja Jagić and Andreja Škiljaica. The first draft was written by Nataša Bauer and improved by all authors. All authors read and approved the final version.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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