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Elan W. Silverblatt-Buser, '12
Melissa A. Frick, '12
C. Rabeler
Nicholas J. Kaplinsky
Swarthmore College, nkaplin1@swarthmore.edu

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Genetic Interactions Between BOB1 and Multiple 26S Proteasome Subunits Suggest a Role for Proteostasis in Regulating Arabidopsis Development

Elan W. Silverblatt-Buser,1 Melissa A. Frick,1 Christina Rabeler, and Nicholas J. Kaplinsky2
Department of Biology, Swarthmore College, Swarthmore, PA 19081
ORCID ID: 0000-0002-2402-2728 (M.A.F.)

ABSTRACT
Protein folding and degradation are both required for protein quality control, an essential cellular activity that underlies normal growth and development. We investigated how BOB1, an Arabidopsis thaliana small heat shock protein, maintains normal plant development. bob1 mutants exhibit organ polarity defects and have expanded domains of KNOX gene expression. Some of these phenotypes are ecotype specific suggesting that other genes function to modify them. Using a genetic approach we identified an interaction between BOB1 and FIL, a gene required for abaxial organ identity. We also performed an EMS enhancer screen using the bob1-3 allele to identify pathways that are sensitized by a loss of BOB1 function. This screen identified genetic, but not physical, interactions between BOB1 and the proteasome subunit RPT2a. Two other proteasome subunits, RPN1a and RPN8a, also interact genetically with BOB1. Both BOB1 and the BOB1-interacting proteasome subunits had previously been shown to interact genetically with the transcriptional enhancers AS1 and AS2, genes known to regulate both organ polarity and KNOX gene expression. Our results suggest a model in which BOB1 mediated protein folding and proteasome mediated protein degradation form a functional proteostasis module required for ensuring normal plant development.

KEYWORDS
BOB1
NudC
proteasome
plant
development
proteostasis
AS1
AS2
FIL

Protein homeostasis (proteostasis) is a fundamental prerequisite for cellular function and, by extension, for growth and development in multicellular organisms. Proteostasis is established and maintained through the interplay of two core cellular processes, protein folding and protein degradation. Co- and post-translational protein folding are facilitated by protein chaperones while most regulated protein degradation is performed by the 26S proteasome (26SP). Protein chaperones are a diverse group of proteins, many are encoded by evolutionarily conserved heat shock protein (HSP) genes. The activity and the functional importance of both HSP chaperones and the 26SP are well established in plants and include a wide range of roles in plant growth and development (Ishiguro et al. 2002; Queitsch et al. 2002; Ueda 2004; Jenik and Barton 2005; Perez et al. 2009; Wang et al. 2009; Ueda et al. 2011). Both processes have been extensively studied but the importance of interactions between HSP mediated protein folding and protein degradation by the 26SP has not been characterized in much detail.

Protein misfolding is predicted to occur at appreciable rates (2–9% of all cellular proteins) even under normal conditions (Drummond and Wilke 2008). In the absence of sufficient chaperone or proteasome activity, misfolded proteins accumulate in the cytoplasm and can form cytotoxic aggregates. The first line of defense against the formation of these aggregates is the small HSPs (sHSPs). sHSPs are protein chaperones that bind to and prevent the irreversible aggregation of misfolded proteins in an ATP independent manner (Basha et al. 2012). As their name suggests, sHSPs are small proteins (<40 kD). They contain an
alpha-crystallin domain (ACD), have the ability to inhibit protein aggregation in vitro, and localize to cytoplasmic heat shock granules in heat stressed plant cells (Hasbeck et al. 2005; Siddique et al. 2008; Wallace et al. 2015). BOB1 is a non-canonical Arabidopsis sHSP that exhibits all of these characteristics. It is required for organismal thermotolerance and contains a NudC domain that is predicted to have structural homology with ACD-containing sHSPs (Garcia-Ranea et al. 2002; Perez et al. 2009). As is true for BOB1, A. nidulans, C. elegans, and human NudC proteins have also been shown to have in vitro chaperone activity using model substrates (Chiu et al. 1997; Dawe et al. 2001; Fairclough et al. 2009; Zheng et al. 2011). In vivo, human NudC proteins function as Hsp90 co-chaperones and their client proteins have been systematically identified (Taipale et al. 2014). It is, of course, possible that NudC proteins could have additional uncharacterized functions in addition to their demonstrated chaperone activity. BOB1 is an essential gene in Arabidopsis and NudC loss of function mutations are also lethal in A. nidulans, C. elegans and Drosophila.

Analysis of the arrested globular embryos of null alleles of BOB1 (bob1-1 and bob1-2) revealed early and severe developmental defects including an expanded apical meristem and associated STM expression. STM, a KNOX gene essential for shoot meristem function, is normally expressed only in the central domain of the apical half of the Arabidopsis embryo. In bob1 null mutants STM expression expands into the lateral apical domains of the embryo. The expansion of the meristem is accompanied by a lack of cotyledon development and an associated loss of expression of genes normally expressed in lateral organs (Jurkuta et al. 2009). These results demonstrate that BOB1 negatively regulates KNOX gene expression.

BOB1 is also required for post-embryonic development. The hypomorphic bob1-3 allele exhibits pleiotropic developmental defects. bob1-3 plants have short roots, small serrated leaves, short branched inflorescences, and inflorescence and floral meristem defects that result in pin-formed meristems and floral organ number defects (Perez et al. 2009). Many of these phenotypes are reminiscent of mutants defective in auxin signaling or transport. The serrations on the margins of bob1-3 leaves are dependent on PIN1 activity, supporting the idea that BOB1 is required for auxin mediated developmental patterning (Jurkuta et al. 2009; Kaplinsky 2009). However, the molecular mechanisms by which this sHSP affects plant development are not self-evident from any of these studies.

BOB1 interacts genetically with both AS1 and AS2, providing a clue about a developmental pathway that requires BOB1 activity. AS1 and AS2 are transcriptional regulators that play roles in establishing meristem boundaries by repressing KNOX expression as well as reinforcing ab-adaxial polarity during leaf development (Iwakawa et al. 2002; Iwasaki et al. 2013; Machida et al. 2015). An allele of BOB1 called cal-1 was identified in an as1 enhancer screen (Ishibashi et al. 2012). Surprisingly, cal-1 has the same mutation as the bob1-3 (G141E) allele. The only viable allele of BOB1 with known phenotypes (Perez et al. 2009). cal-1; as1 and as2 double mutants have abaxialized filamentous leaves and exhibit increased levels of KNOX and ETT expression in their shoot apices. ETT functions to enhance abaxial identity and is a direct target of the AS1-AS2 complex (Iwasaki et al. 2013). The polarity defects in cal-1; as2 plants were suppressed in an ett background suggesting that ETT is downstream of both BOB1 and AS1 and AS2 (Ishibashi et al. 2012).

The aim of this study was to identify BOB1 dependent developmental pathways in order to understand the requirement for this sHSP in ensuring normal development. We used a genetic approach, reasoning that bob1-3 enhancers would be caused by mutations in genes and pathways that are sensitive to reductions in BOB1 activity.

### MATERIALS AND METHODS

#### Plant stocks and growth conditions

bob1-3 and bob1-1 were both back crossed into Ler background six times before being used to analyze TH phenotypes (both alleles) and for the EMS mutagenesis (bob1-3). TH plants in both Col-0 and Ler backgrounds were generated by crossing bob1-3 homozygotes to bob1-1 heterozygotes. Seeds produced by these crosses segregate bob1-3/bob1-1 (TH phenotype) and bob1-3/+ (WT phenotype) plants in a 1:1 ratio. fil mutants and proteasome subunit T-DNA insertion lines were obtained from the ABRC (File S1). Plants were grown on soil under standard long-day greenhouse conditions with supplemental lighting. Plants grown on plates were grown on 0.5x Murashige and Skoog (0.5x MS) media containing 1% sucrose at 22°C under constant light conditions in E-30B growth chambers (Percival Scientific).

#### EMS mutagenesis

5000 bob1-3 seeds in a Ler background were soaked in a 0.2% EMS solution on a rocker for 12 hr. They were then rinsed eight times with water and planted on soil. M1 Seeds were collected from pools of 4-5 M0 self-fertilized plants.

#### Leaf shape measurements

The fifth and sixth leaves from three week old plants were flattened, taped to white paper with transparent packing tape, and scanned at 600dpi using a Canon iR-ADV C5530. The scanned images were used to measure the length and mid-length width of each leaf lamina as well as the depth of the deepest serration on each leaf using ImageJ (Schneider et al. 2012).

#### BOM1 mapping and cloning

DNA from 538 mutant individuals in a bom1 mapping population was prepared using a DNase Plant Maxi Kit (Qiagen). A NEBNext DNA library prep set for Illumina (NEB) was used to prepare the sequencing library that was sequenced using an Illumina HiSeq (Illumina). Reads were mapped to the TAIR9 reference genome using SHORE and bom1 was mapped using SHOREmap (Schneeberger et al. 2009).

#### RT-PCR

RNA from five day old seedlings grown in 0.5x MS liquid media was prepared using RNeasy plant mini kits (Qiagen). It was quantified using a Nanodrop ND-1000 (Thermo Scientific) and reverse transcribed using M-MuLV reverse transcriptase (NEB). The following primers were used for PCR reactions with a 50°C annealing temperature and 35 cycles:<br/>

**ACTIN-F, 5'GACCTGACTTATGATACCGATGGGCCC3'; ACTIN-R, 5'CAGCACGTTAGAAGGCTTTGAGGCC3'; RPT2a-F, 5'ATCCATGGAAGACCCATG3'; RPT2a-R 5'TTACATGTAGAGGCCCTTCG3'**

#### MG132 treatment

MG132 (Cayman Chemical) was resuspended at 100 mM in DMSO before being added to MS agar (1%) media. Col-O, bob1-3, bom, rpt2a-2, and bob1-3; bom seeds were plated and stratified at 4°C for two days. The plates were then transferred to a 22°C incubator in a vertical orientation, exposed to light for five hours, and then wrapped in two layers of aluminum foil. After five days of growth the plates were scanned using an Expression 1600 scanner (Epson) and hypocotyl lengths were measured using ImageJ (Schneider et al. 2012).
RESULTS

BOB1 phenotypes are different in Ler and Col-O genetic backgrounds

bob1-3 phenotypes include small plants with short roots, serrated leaf margins, and abnormal floral organ numbers (Perez et al. 2009). These phenotypes are very similar in Col-O and Ler ecotypes. By reducing the dosage of BOB1 we uncovered other ecotype specific phenotypic differences. We combined the bob1-1 null allele and the bob1-3 partial loss of function allele to create bob1-1/bob1-3 trans-heterozygotes (THs). In a Ler background these plants had inflorescence phenotypes that were markedly different from the pin-formed meristems that develop in bob1 TH plants in a Col-O background (Perez et al. 2009). Several types of lateral organs are produced by Ler TH inflorescences, often on the same plant. Most Ler TH inflorescence meristems produce a series of relatively normal flowers followed by increasingly abnormal flowers and then finally filamentous structures and arrested primordia. Based on their placement on the flank of the inflorescence meristem these filamentous structures appear to be derived from flowers. On some plants the filaments are bare (Figure 1A, C) while on other plants they terminate in stigmatic papillae, consistent with a floral derivation (Figure 1B, D). A second class of Ler TH inflorescences terminates in a mixture of structures including isolated carpels, leaf like structures with ectopic ovules on their margins, and filamentous organs (Figure 1E). Intermediate flowers (between the relatively normal early flowers and the terminated meristems) exhibit severe polarity defects including visible external ovules (Figure 1F). Finally, Ler TH plants can occasionally develop fasciated meristems with strap like stems (Figure 1G).

bob1 THs in a Col-O background are completely sterile and do not produce any seeds. In contrast, the relatively normal early Ler TH flowers are fertile and set seed. The abnormal inflorescences observed in Ler THs are qualitatively different from what we observed in a Col-O background. These differences suggest the existence of genetic modifiers that affect bob1 phenotypes. We reasoned that identifying these modifiers and other genes that enhance bob1 phenotypes might provide insights into BOB1’s developmental functions.

bob1-3 enhances fil phenotypes

Homozygous bob1-3 mutants never exhibit the filamentous flower phenotype we see in bob1-3/bob1-1 THs. This phenotype, which we...
only observe in a Ler background, is similar to the filament-like structures described in filamentous flower (fil) mutants. fil mutant plants, like bob1 THs, also produce a number of relatively normal flowers before terminating in clusters of filamentous organs. FIL encodes a YABBY gene required for floral meristem identity, flower formation, and flower development (Chen et al. 1999) and has a demonstrated role in establishing ab-adaxial polarity in developing leaves. As is true for BOB1, FIL also functions as a negative regulator of KNOX gene expression (Kumaran et al. 2002; Jurkuta et al. 2009; Ishibashi et al. 2012; Iwasaki et al. 2013). These similarities suggest a functional overlap between BOB1 and FIL.

To investigate whether bob1-3 mutants would enhance the filamentous flower phenotype we crossed it with intermediate (fil-2) and strong (fil-5) fil alleles in a Ler background. The petals of bob1-3 flowers are morphologically normal although bob1-3 flowers often have more than four petals per flower (Perez et al. 2009). In contrast, fil-5 mutant flowers have small twisted petals (Figure 2A). Wild type and bob1-3 plants never produce filamentous flowers. fil-2 and fil-5 plants that are wild type or heterozygous for bob1-3 produce more than 50 normal flowers before producing filamentous organs. fil-2 and fil-5 plants homozygous for bob1-3 never produced more than 12 normal flowers before making filamentous organs. In these double mutants, the small number of early flowers produced look like fil flowers while all later flowers are converted into filamentous organs (Figure 2A, B). This phenotype suggests that the filamentous organ phenotypes seen in bob1 THs may be due to disruptions of a developmental pathway that involves YABBY mediated establishment of organ polarity.

**bom1 is a bob1-3 enhancer**

In addition to investigating genetic interactions between BOB1 and FIL we also undertook a non-targeted approach to discover genes that interact genetically with BOB1. Since Ler THs are fertile we decided to...
Figure 3  bom1 developmental phenotypes. Size and leaf serration phenotypes in three (A) and five (B) week old plants. Height differences in mature, dried plants (C). Rosette widths and inflorescence heights were measured in four week old and mature plants, respectively. Leaf lamina length/width and serration/leaf width ratios were measured for leaves five and six in three week old plants. Letters indicate significant differences among genotypes (Bonferroni corrected 2-tailed unpaired t-tests, \( P < 0.05 \)) (D). bob1-3; bom1 phenotypes include abaxial leaf spurs (E), stem fasciation (F), and abaxialized flowers with visible external ectopic ovules (G). Scale bars are 1cm in A and 2cm in C. Error bars are +/- SD.
screen for modifiers in a Ler background. We used ethylmethanesulfonate (EMS) to mutagenize bob1-3 plants. M₁ plants were planted, allowed to self-fertilize, and M₂ progeny were screened for phenotypes similar to those observed in bob1-3/bob1-1 TH plants. We screened for multiple phenotypes including reduced plant size, defects in floral organ polarity, fasciated stems, and abaxial leaf spurs. Putative mutants were crossed to wild-type plants, the F₁ progeny were self-fertilized, and F₂ plants were grown out and genotyped to verify that their phenotypes were bob1-3 dependent and not expressed in wild-type plants. We screened a total of 414 M₂ pools and identified five bob1-3 dependent mutants, one of which is described here.

**bobber modifier (bom1)** was identified as a bob1-3 modifier that enhances several bob1-3 phenotypes in a manner resembling bob1 TH phenotypes. These include small plant size and heavily serrated, narrow leaves. bom1 behaves as a single recessive mutation in a bob1-3 background. We backcrossed bom1 to bob1-3 in Col-O and wild-type Col-O plants six times before phenotypic analysis to remove unlinked EMS-induced mutations. All further analyses described in this paper were performed in a backcrossed Col-O background.

Both bob1-3 and bom1 single mutants have serrated and narrow leaves. bob1-3 mutant plants have smaller rosette diameters and shorter inflorescences compared to wild-type plants (Perez et al. 2009). This is in contrast to bom1 mutant plants whose rosette diameters and shoot heights are larger than those of Col-O plants. bob1-3; bom1 leaves are narrower and more serrated than those of either single mutant and the rosette diameter and shoot height of double mutant plants are smaller than those of Col-O, bob1-3, or bom1 plants (Figure 3A-D). These epistatic genetic interactions suggest that BOB1 and BOM1 function in a shared biological pathway (Both et al. 2009).

Similar to bob1 THs, bob1-3; bom1 double mutants also have abaxial leaf spurs, fasciated stems, and their inflorescences terminate in clusters of abaxialized flowers. These flowers lack obvious sepals, petals, or stamens. The carpeloid organs that are produced at inflorescence termini have exposed ovules on their margins (Figure 3E-G). We occasionally observe tiny leaf spurs in bob1-3 in a Col-O background but they are never as pronounced as those seen in bob1-3; bom1 plants.

**bom1 is an allele of RPT2a**

We simultaneously mapped and cloned BOM1 using a next-generation sequencing approach (Schneeberger et al. 2009). bob1-3; bom1 plants in a Ler background (in which the mutation was generated) were crossed to bob1-3 plants in a Col-O background. The F₁ progeny were self-fertilized and the resulting F₂ plants segregated the bob1-3; bom1 phenotype in a bob1-3 background. DNA was extracted from 548 bob1-3; bom1 plants, pooled, and sequenced to 82x coverage. The SHORE and SHOREmap software packages (Schneeberger et al. 2009) were used to identify Col/Ler SNP allele frequencies across the entire Arabidopsis genome. Two regions of enrichment were identified. As expected, a region of enrichment of Col SNP alleles in the Bob1 region of chromosome 5 is consistent with the Col origin of the bob1-3 allele. Enrichment of Ler SNPs was observed only on chromosome 4 with a peak at 14.3 Mb (Figure 4A). We decided to focus on G → A / C → T mutations (characteristic of EMS mutagenesis) that resulted in non-synonymous changes as candidate mutations. The closest non-synonymous G → A mutation to the mapping peak was a mutation that results in a G395E amino acid change in AT4G29040. AT4G29040 encodes the RPT2a subunit of the 19S regulatory particle of the 26S proteasome. The mutation in the bom1 allele is located close to the AAA-ATPase domain of RPT2a and affects a glycine residue that is invariant among plants (Arabidopsis), animals (humans and flies), and fungi (Saccharomyces pombe) (Figure 4B).

In order to confirm that bob1-3; bom1 phenotypes are caused by a mutation in RPT2a we performed a complementation test using rpt2a-2 (SALK_005596), a null allele of RPT2a that was first described as halted root-2 (hr2-2) (Ueda 2004). rpt2a-2 was crossed to a bob1-3; bom1/+ plant. F₁ plants were genotyped and a bob1-3/+; rpt2a-2/bom1 plant was self-fertilized. If bom1 is an allele of RPT2a we would expect 1/4 of the F₂ plants to exhibit the double mutant phenotypes while if bom1 is not an allele of RPT2a we would expect 1/16 of the F₂ plants to exhibit the double mutant phenotypes. 12 out of 43 plants in the F₂ population had bob1-3; bom1 double-mutant phenotypes. These plants were genotyped for bob1-3, bom1, and rpt2a-2. As expected, all plants were homozygous for bob1-3 and we identified bom1 and rpt2a-2 homozygotes as well as bom1/rpt2a-2 plants among the plants with double mutant phenotypes. This lack of complementation shows that the bom1 enhancement of bob1-3 phenotypes is caused by a mutation in RPT2a. The increased size of bom1 plants (Figure 3) is also consistent with reports that rpt2a mutants are larger than wild-type plants (Kurepa et al. 2009; Lee et al. 2011).

Three other RPT2a alleles have been described in addition to bom1 and rpt2a-2. rpt2a-1 is the original halted root-1 (hr-1) allele identified in a Wassilewskija background and has a 13bp deletion in its first intron (Ueda 2004). Three T-DNA insertional alleles, rpt2a-2 (hr2-2), rpt2a-3, and rpt2a-4 are all in a Col-O background. No full-length RNA transcripts accumulate in any of these T-DNA alleles making it likely that they are all loss of function alleles (Lee et al. 2011). We propose that bom1 be designated as rpt2a-5.

The phenotypes of the rpt2a-2; bob1-3 and bom1; bob1-3 double mutants generated during the complementation test were very similar. This suggests that the G395E mutation is a strong allele with phenotypic effects similar to the T-DNA insertional alleles. To characterize the bom1 allele of RPT2a further we investigated whether RPT2a RNA accumulates in bom1 mutants. rpt2a-2 is an RNA null and, as expected, there was no detectable RPT2a RNA in rpt2a-2 mutants. Full-length RPT2a RNA was detectable in bom1 single mutants and in bob1-3; bom1 double mutants at levels similar to wild type and bob1-3 plants (Figure 4C).

**bom exhibits MG132 hyposensitivity**

The rpt2a2 null mutant as well as mutations in other proteasome regulatory particle subunits such as rpn10 and rps12 are, paradoxically, less sensitive to the proteasome inhibitor MG132 than wild-type plants (Kurepa et al. 2008). To determine if bom1 shares this phenotype we measured hypocotyl elongation in MG132-treated etiolated seedlings. bom1 and rpt2a-2 mutants both exhibited increased MG132 tolerance compared to either Col-O or bob1-3 plants (Figure 5A). This suggests that the G395E point mutation in bom1 results in a similar defect as the rpt2a-2 null allele. To investigate whether a loss of BOB1 would affect the MG132 hyposensitivity observed in bom1 we also grew bob1-3; bom1 double mutants at 400 μM MG132. At this concentration we observe a nearly complete inhibition of hypocotyl elongation in Col-O and bob1-3 plants. The bob1-3; bom1 double mutants and bom1 single mutants exhibited similar levels of MG132 tolerance, demonstrating that a loss of BOB1 function does not affect this aspect of rpt2a mutant phenotypes (Figure 5B).
Cloning and characterization of bom1. BOM1 was simultaneously mapped and cloned using a NGS approach. bom1 and bob1-3 were generated in Ler and Col-O backgrounds, respectively, and their positions can be seen as peaks of enrichment in Ler and Col SNPs (A). A protein lineup of BOM1/RPT2a. The G395E mutation in bom1 is highlighted in red (B). RT-PCR was used to amplify full length RPT2a transcripts from mRNA isolated from the indicated genotypes (C).
BOB1 interacts genetically with multiple proteasome subunits

To determine if a physical interaction underlies the genetic interaction between BOB1 and BOM1 we cloned cDNAs of both genes into BiFC vectors and tested their interaction using transient tobacco leaf transformation. The basic functional unit for sHSPs is a dimer that in turn can be incorporated into higher order structures (Basha et al. 2012). Consistent with this, BOB1 homodimerization can be detected using BiFC in two different combinations of BiFC constructs, (BOB1::CCFP & NYFP::BOB1, CYFP::BOB1 & NYFP::BOB1). In contrast, there was no evidence of a physical interaction between BOB1 and BOM1 in any of the BiFC construct orientations (Figure 6). It is possible that this result is a false negative and it is not possible to detect interactions between BOB1 and RPT2a using BiFC. However, based on the genetic interactions between BOB1 and several other proteasome subunits (see below), we suspect that the genetic interactions between BOB1 and the proteasome are not mediated by direct physical interactions.

An alternative explanation for the genetic interaction between BOB1 and BOM1 is a more general interaction between BOB1 mediated protein folding and 26S proteasome mediated protein degradation. To test whether the interaction is specific to BOM1 or whether it is a more general genetic interaction we made crosses between bob1-3 plants and BOM1 in any of the BiFC construct orientations (File S1). As expected, bob1-3; rpt2a-2 double mutants exhibit a strong synergistic phenotype. In this experiment rpt2a-2 single mutants were not significantly larger than wild type Col-O plants as has been previously reported (Kurepa et al. 2009) and as we observed for bom1 (Figure 7). This discrepancy could be due to variability in our greenhouse growth conditions or the altered stress tolerance levels observed in proteasome mutants (Kurepa et al. 2008; Wang et al. 2009). We did not follow up on this observation.

In addition to RPT2a, T-DNA insertions in RPN1a and RPN8a exhibited strong enhancement of bob1-3 phenotypes. Double mutants between bob1-3 and all of these proteasome mutants were smaller than either corresponding single mutant or wild type plants. Double mutant leaves were also narrower than either single mutant. They often lacked any serrations on their margins (73% of bob1-3; rpt2a-2 leaves, 26% of bob1-3; rpn1a leaves, and 55% of bob1-3; rpn8a leaves) but when serrations were present, they were deeper than the serrations of single mutant or wild type leaves (Figure 7).

DISCUSSION

We found that mutations in the proteasome subunits RPT2a, RPN1a, and RPN8a enhance bob1-3 developmental phenotypes. These interactions are interesting because BOB1 encodes a protein chaperone that prevents the aggregation of misfolded proteins and the proteasome is responsible for regulated protein degradation. The interaction between these core cellular pathways in Arabidopsis highlights the importance of proteostasis for normal development. In addition to slow growth, bob1; rpt2a double mutants have narrow, deeply serrated leaves, abaxial leaf spurs, and flowers with disrupted polarity. The floral phenotypes of this double mutant and bob1 TH plants are similar to those of mutants in genes that regulate ab-adaxial polarity. Consistent with this, we also uncovered a genetic interaction between BOB1 and FIL, a
YABBY with demonstrated roles in promoting abaxial identity (Siegfried et al. 1999).

The interactions we have discovered define a genetic network that connects proteostasis to the AS1-AS2 developmental pathway (Figure 8). The network consists of three sets of genetic interactions. The first is a proteostasis module defined by genetic interactions between BOB1 and the 26SP (this work) and rests on the assumption that BOB1’s chaperone activities are responsible for the interactions. The second set of genetic interactions is between BOB1 and AS1-AS2 (Ishibashi et al. 2012). Finally, the third consists of genetic interactions among RPT2a, RPN1a, and RPN8a (the three proteasome subunits that interact genetically with BOB1) and AS1-AS2 (Huang et al. 2006). Double mutants among these groups of genes (BOB1, 26SP, and AS1-AS2) all produce similar phenotypes and all of these genes are required for the repression of KNOX genes.

The AS1-AS2 complex directly represses KNOX gene expression (Guo et al. 2008; Machida et al. 2015). Repression of KNOX gene expression has also been demonstrated for RPN8a and RPT2a. Mutant alleles of both of these genes exhibit abaxial leaf spurs, similar to those observed in BOB1 THs and bob1-3;bom1 mutants, in which KNOX
genes are ectopically expressed. This shows that, as is true for BOB1, the 26SP can also function to negatively regulate KNOX expression (Huang and Huang 2007; Jurkuta et al. 2009; Ishibashi et al. 2012). A plausible explanation for these observations is that BOB1/26SP mediated proteostasis is required for normal AS1-AS2 function (Guo et al. 2008; Machida et al. 2015).

In addition to the regulation of KNOX genes, AS1-AS2 also functions in establishing and maintaining organ polarity in

Figure 7 Plant growth phenotypes of bob1-3; 26SP double mutants. Three week old plants (A). Rosette widths were measured in four week old plants. Leaf lamina length/width and the serration/leaf width ratios of serrated leaves were measured for leaves five and six in three week old plants (B). * indicates significant differences between each double mutant and the corresponding single mutants as well as Col-0 plants (One-way ANOVA with post-hoc Tukey HSD Test, P < 0.05). Error bars are +/- SD. The scale bar in A is 1 cm.
Arabidopsis (Iwasaki et al. 2013; Machida et al. 2015). rpn2a, rpn1a, and rpn8a mutants all enhance the abaxialization phenotypes observed in as2 mutants, demonstrating a requirement for 26SP function in the specification of organ polarity (Huang et al. 2006). Establishment of abaxial identity also requires FIL, another gene we have shown interacts genetically with BOB1. Supporting this connection among BOB1 and polarity genes is the observation that the establishment of leaf polarity is sensitive to high temperatures. as1 and as2 mutants produce leaves with disrupted adaxial-abaxial polarity and one class of these leaves, called lotus leaves, occurs more frequently at high temperatures in an er (i.e., Ler) background (Xu et al. 2003; Qi et al. 2004). There is a significant overlap between the machinery required for maintaining proteostasis under normal conditions and the machinery required for responding to heat stress (Albanèse et al. 2006). This suggests that, at high temperatures, the machinery required for developmental proteostasis could be titrated away by stress induced misfolded proteins, uncovering phenotypes in cellular pathways such as the AS1-AS2 pathway.

Plant hormones are important regulators of plant development and the ubiquitin-26SP system is integral to most plant hormone signal transduction pathways. In response to the presence of hormones, negative regulators are degraded by the 26SP, enabling rapid signal transduction (Santner and Estelle 2010). Our results suggest that, in addition to these very specific roles, the 26SP may also be required for ensuring normal development more generally by maintaining proteostasis in concert with protein chaperones.

The limitation of this work is that all of the interactions we have discovered are genetic. They do not provide direct mechanistic insights into the developmental requirements for BOB1 activity. BOB1 may have undiscovered functions in addition to chaperone activity and it is conceivable that the in vitro assays used to demonstrate this activity do not accurately reflect BOB1’s cellular functions. The discovery of new BOB1 functions could change our interpretation of our interaction data. Even without a full understanding of the mechanisms that underlie the genetic interactions between BOB1 and the 26SP, their possible importance is highlighted by their conservation between plants and animals. An integrated C. elegans physical and genetic interaction network identified interactions supported by phenotypic and expression correlation data between nud-1, the BOB1 homolog, and multiple proteasome subunits including rpi-2, rpn-1, and rpn-8 (Gunsalus et al. 2005). AS1 and AS2 form a heterodimeric complex that directly binds to the promoters of target genes (Guo et al. 2008). One explanation for our results is that this physical interaction requires BOB1 and 26SP mediated proteostasis. In the absence of this quality control mechanism the AS1-AS2 complex would not function normally. FRET and yeast 2-hybrid approaches have been used to assay the AS1-AS2 interaction making it possible to test this hypothesis directly (Xu et al. 2003; Rast and Simon 2012).

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