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Protein ubiquitination in auxin signaling and transport

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Chapter 3

Ubiquitination-independent proteasomal degradation of the calcium binding protein PBP1

Felipe dos Santos Maraschin, Johan Memelink and Remko Offringa

Summary

In order to regulate physiological functions, eukaryotic cells have developed many control mechanisms for fine-tuning the levels of intracellular proteins. Generally, targeted protein degradation occurs through the specific recognition by the proteasome of proteins that have been marked with ubiquitin chains. Protein labeling with ubiquitin is however emerging as a much more complex signal not only targeting proteins for degradation, but also with roles in the control of subcellular trafficking of proteins. The Arabidopsis calcium binding protein PINOID BINDING PROTEIN 1 (PBP1) was originally identified as an interactor of the PINOID (PID) kinase, which was shown to control its activity in vitro and subcellular localization in vivo. Using an Arabidopsis cell suspension protoplast system we show that PBP1 is a highly unstable, poly-ubiquitinated protein that can be stabilized by inhibition of proteasomal degradation. Co-expression of PID did not affect PBP1 poly-ubiquitination. Surprisingly, substitution of all the lysines in PBP1 blocked poly-ubiquitination, but did not affect its proteasomal degradation. The mutant protein retained all tested wild type functions, such as its interaction with PID, and its subcellular localization. Translational fusions of the lysine-less PBP1 with YELLOW FLUORESCENT PROTEIN (YFP) were ubiquitinated, which indicates that the PBP1 sequence contains a cis-acting motif that is recognized as an ubiquitination signal but not as a degron, since the YFP fusions were not unstable. PBP1 is the first example of a plant protein that is degraded by the proteasome in an ubiquitination-independent pathway. Although the functions of PBP1 ubiquitination remain to be elucidated, our results show that its proteasomal degradation is not dependent on ubiquitination .

INTRODUCTION

In order to regulate physiological functions, eukaryotic cells have developed many control mechanisms for fine-tuning the levels of intracellular proteins. One of these involves post-translational modification by ubiquitination. Ubiquitin (Ub) is a highly conserved 76 amino acid protein that is present in all eukaryotic cells. It is conjugated to cellular proteins through a conserved mechanism involving Ub activating (E1), -conjugating (E2) and -ligating (E3) enzymes. This process, termed ubiquitination, couples the C-terminal glycine residue of Ub to a target lysine residue of the protein substrate, forming an isopeptide bond (Glickman and Ciechanover, 2002). Protein ubiquitination controls many basic cellular processes, such as cell division, signal transduction and DNA repair, by regulating the activity, subcellular localization, and proteasome-mediated degradation of proteins (Pickart and Fushman, 2004). The ubiquitin protein itself contains 7 lysine residues that serve as ubiquitin-linking sites. The 19S regulatory lid of the 26S proteasome recognizes proteins containing a Lys-48 Ub polymer as substrates for degradation by the 20S catalytic core (Baboshina and Haas, 1996; Thrower *et al.*, 2000). Proteins that are polyubiquitinated at Lys-48 are usually unstable and treatment of cells with proteasome inhibitors blocks their degradation enriching the pool of Ub-modified molecules (Lee and Goldberg, 1998). Other ubiquitin conjugations such as mono-ubiquitination, or polymers attached at Lys-11 or Lys-63 are believed to regulate processes such as sub-cellular localization and/or protein activity (Weissman, 2001). About 5% of the Arabidopsis proteome comprises elements of the ubiquitination pathway with a high representation of Ubiquitin Ligases. In plants ubiquitination plays a key role in signal transduction of several phytohormones (Gray *et al.*, 1999; Xu *et al.*, 2002; Guo and Ecker, 2003; Gomi *et al.*, 2004; Jang *et al.*, 2005; Dos Santos *et al.*, 2009) and although all these processes seem to involve the activity of specific ubiquitin ligases, the ubiquitination signal itself has been neglected in scientific studies. Ubiquitin was first discovered as a degradation signal with the best studied examples including mammalian proteins such as the Cdk-inhibitor p27Kip1 which is ubiquitinated by SCF^{SKP2} (Tsvetkov *et al.*, 1999), Sic1 which is ubiquitinated by SCF^{Cdc4} (Petroski and Deshaies, 2003), β -

catenin and I κ B which are ubiquitinated by SCF ^{β -TrCP} (Winston *et al.*, 1999) and p53 which can be ubiquitinated by several E3 ligases (Scheffner *et al.*, 1993; Fang *et al.*, 2000; Xia *et al.*, 2009). Proteasomal p53 degradation occurs via both ubiquitin-dependent and independent ways (Asher and Shaul, 2006), indicating that proteasomal degradation does not necessarily occur via an ubiquitinated intermediate. The removal of the ubiquitin attachment sites by mutation of lysines in a target protein has been shown as an efficient way to assess the relevance of the ubiquitin signal for many unstable proteins degraded independently of ubiquitination, such as p21^{cip1} (Sheaff *et al.*, 2000) and KLF5 (Chen *et al.*, 2007a) revealing the existence, at least in mammalian cells of both ubiquitin-dependent and independent degradation pathways.

Previously, we identified a single EF-hand calcium-binding protein as an interacting protein of the *Arabidopsis thaliana* AGC kinase PINOID (PID), and named it PINOID BINDING PROTEIN 1 (PBP1). PBP1 binds to PID in a calcium-dependent manner and positively regulates PID activity *in vitro*, while inhibiting the auxin-induced calcium-dependent sequestration of PID from the plasma membrane *in vivo*. (Benjamins *et al.*, 2003; Robert-Boisivon, 2008). In our further study on the role of PBP1 as regulator in PID signaling, we tested the biochemical properties of PBP1 by expressing the protein in *Arabidopsis* cell suspension protoplasts. Here we provide evidence that PBP1 is an unstable protein that is poly-ubiquitinated. However, PBP1 degradation by the proteasome is an ubiquitination-independent process. Our results indicate that poly-ubiquitination is not an obligatory signal for PBP1 proteasomal degradation suggesting that plant proteasomes also have diverse mechanisms for recognizing their targets.

MATERIAL AND METHODS

Molecular cloning and constructs

Molecular cloning was performed following standard procedures. The *HA-PBP1* coding region was amplified by PCR from *pET16H-PBP1* (Benjamins *et al.*, 2003) using primers *HAadd* *PBP1* *HindIII* 5'-

GGAAGCTT**GATGTACCCATACGATGTTCCAGATTACGCTATGGCATCTCC**
 TAAAT-CCTC-3' and *PBP1**Xba*I*R* 5'-
GGTCTAGATCAATGCCGGTAAACTCTTCC-3' (HA-tag in bold and restriction
 sites underlined), and the *Hind*III and *Xba*I sites in the primers were used to
 clone the fragment into pART7 (Gleave, 1992), to obtain *p35S::HA-PBP1*.
p35S::HA-PBP1(-K) was obtained by cloning a synthetic *Hind*III/*Bam*HI
 fragment containing the *HA-PBP1* coding region with the lysine K5, K29, K41,
 K49 and K51 codons replaced by the arginine codon (AGA) via Gene Synthesis
 (<http://www.baseclear.com/>) into pART7. To generate His-*HA-PBP1(-K)*, the
 synthetic *HA-PBP1(-K)* coding region was ligated as a *Bam*HI/*Hind*III fragment
 in frame to the His-tag coding region of pET16b (Promega,
www.promega.com). To create C-terminal YFP-*HA* fusions, both *HA-PBP1* and
HA-PBP1(-K) were PCR amplified with *attB* Gateway™ (Invitrogen,
www.invitrogen.com) primers *attB1FHA* 5'-GGGGACAA-
 GTTTGTACAAAAAGCAGGCTTAATGTACCCATACGATGTTCCA-3' and
attB2PBP1R 5'-
 GGGGACCACTTTGTACAAGAAAGCTGGGTCATGCCGGTAAACTCTTCTC
 C-3', removing the original stop codon. Each of the *attB* PCR fragments was
 cloned into *pDONR207* via BP recombination following the manufacturer's
 instructions, and the resulting entry clones were recombined via an LR reaction
 into *pART7-YFP-HA* (C. Galvan Ampudia, unpublished) to generate
p35S::PBP1-YFP-HA and *p35S::PBP1(-K)-YFP-HA*. For construction of the
p35S::5xHis-Ub vector, the cDNA of human ubiquitin was PCR amplified from
 His₆-Ub (Stad *et al.*, 2001) using primers 5'HisUb 5'-
 GGAATTCATGCATCATCATCATCAT-3' and 3'Ub 5'-
 CCCTTACCCACCTCTGAGACGGAGGACC-3' and cloned as a blunt fragment
 into pART7 cut with *Sma*I. Plasmids *p35S::PID-FLAG* (Michniewicz *et al.*, 2007)
p35S::FLAG-GFP, *p35S::GFP* (Dos Santos *et al.*, 2009) *p35S::PID-CFP*
 (C.Galvan-Ampudia, unpublished) were also constructed in pART7.

Protoplast isolation and transformation

Four to six days old *Arabidopsis thaliana* Col-0 cell suspension cultures were diluted 5-fold in auxin-free Cell Medium and incubated at 25°C with shaking (150 rpm) overnight and used for protoplast isolation and transfection, performed as previously described (Schirawski *et al.*, 2000) with minor modifications (Dos Santos *et al.*, 2009). Transfections were performed with 10⁶ cells and 20 µg of the test plasmid DNA. Amounts of the other plasmids are indicated in the figure legends. After transfection the cells were incubated at 25°C in darkness for at least 16h prior to treatments or observation using confocal laser scanning microscopy. Cells were incubated for 1h with 0.1% DMSO (-) or 50 µM MG132 (+) where indicated.

Western blot analysis and immunoprecipitation

Total protein was extracted from pelleted transfected protoplasts with 50 µL cold Extraction Buffer (Tris Buffered Saline, TBS, 1% Triton X-100, 1x Roche Complete Protease Inhibitor Cocktail), and centrifuged for 10 minutes at 20.000 g at 4°C. The 40 µL extract was mixed with 10 µL 5X Laemmli sample buffer and boiled for 5 minutes. Proteins were separated on a 15% SDS-PAGE gel, blotted into a PVDF membrane using semi-dry electrotransfer (BioRad), blocked for 1h with 5 % low-fat dry milk in TBST (TBS, 0.05% Tween20) and probed with HRP-conjugated anti-HA antibodies (1/2000, Roche) for 16h at 4°C. Detection was performed using LumiGLO Detection reagent (Cell Signalling) following the manufacturer's instructions.

For immunoprecipitation, cells were extracted in 500 µL of Extraction Buffer, centrifuged and 50 µL was analysed as 10% input. The remaining volume was mixed end-over-end with 50 µL 50% slurry of anti-HA agarose beads (Roche) for 2 h at 4°C, washed 4x and mixed with 50 µL 2X Laemmli sample buffer for SDS-PAGE and subsequent western blot analysis (see above) using anti-HA antibodies (1/2000, Roche) and P4D1 anti-UB antibodies (1/400, Santa Cruz).

Purification of ubiquitinated proteins via Ni-NTA affinity chromatography

In addition to the indicated test constructs, the protoplasts were co-transfected with 20 µg of the *p35S:His-Ub* plasmid and 16h after transfection the cells were

treated with 50 μ M MG132 for 1h, harvested by centrifugation and frozen in liquid nitrogen. Pellets containing 10^6 harvested cells were resuspended in 100 μ L cold Extraction Buffer (TBS, 1% Triton 100-X, 1x Roche Complete Protease Inhibitor Cocktail, 10 mM N-Ethylmaleimide NEM), vortexed and centrifuged 10 min at 20.000 g at 4°C. Ten microliters of the supernatant was mixed with an equal volume of 2x Laemmli sample buffer, and used as INPUT for Western analysis. The remaining 90 μ L was mixed with 910 μ L of Buffer A (6 M guanidinium-HCl, 0.1 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ pH 8.0, 10 mM imidazole) and used for Ni-affinity chromatography, using Ni-NTA agarose beads (Qiagen) as previously described (Campanero and Flemington, 1997). The eluted proteins were analyzed by western blotting as described above.

***In vitro* pull down experiments**

Crude extracts of *E.coli* cells expressing recombinant proteins were used for *in vitro* GST pull down experiments of His-PBP1 and His-HA-PBP1(-K) with GST alone and GST-PID and GST-WAG2 (C. Gavlan-Ampudia, unpublished) as described (Benamins *et al.*, 2003).

Microscopy

For imaging of transfected protoplasts a Leica DM IRBE confocal laser scanning microscope was used with a 63X water objective, digital zoom and 51% laser intensity. The fluorescence was visualized with an Argon laser for excitation at 514 nm (YFP) and 457 nm (CFP) with 522-532 nm and 471-481 nm band pass emission filters, respectively. Image processing was performed with ImageJ (<http://rsb.info.nih.gov/ij/>).

RESULTS

PBP1 is degraded by the proteasome in a ubiquitination-independent manner

PBP1 was first identified in Arabidopsis as an interactor of the PID kinase (Benamins *et al.*, 2003). PBP1 was also named KRP2 (for KIC-related protein

2) and contains one calcium binding EF-hand motif in common with other related members of a small protein family that includes KIC (KCBP-interacting Calcium binding protein) and the close PBP1 homolog PBP1H/KRP1 (Reddy *et al.*, 2004). The alignment of the three proteins (Figure 1A) shows the high degree of similarity between PBP1 and PBP1H. From the 5 lysines present in the primary PBP1 sequence only one is conserved in all members and another two are present in KIC.

As part of our analysis of the biochemical properties of PBP1 in relation to its interaction with PID, we transfected *Arabidopsis thaliana* cell suspension protoplasts with plasmid *35S::HA-PBP1*. Total protein was extracted from the transfected cells and analyzed by Western blot with a specific antibody against the HA-tag (Figure 1B). The very weak signal observed in untreated cells is significantly enhanced when cells were treated for 1h with the proteasome inhibitor MG132, suggesting that PBP1 is a target for degradation by the proteasome. In addition to the stronger signal representing the full length protein (~16 kDa), a ladder of discrete larger molecular mass bands becomes apparent following MG132 treatment. Immunoprecipitation (IP) with anti-HA and detection with either anti-HA or anti-Ub antibodies shows that these larger molecular mass bands represent ubiquitinated versions of PBP1 (Figure 1C).

Ubiquitin attachment occurs on lysine residues. PBP1 is a relatively small protein with only 5 lysine residues. To confirm that the additional bands observed are due to lysine ubiquitination, we generated a construct encoding a PBP1 variant in which all five lysine residues were substituted by arginines (Figure 1A, arrows). Transfection of the resulting *35S::HA-PBP1-K* construct into protoplasts shows that PBP1-K is still an unstable protein stabilized by MG132 treatment (Figure 2A), but without showing additional higher molecular weight bands representing ubiquitinated forms. These observations indicate that ubiquitination of PBP1 is not a signal for its degradation by the proteasome. PBP1 ubiquitination may serve some function other than signaling proteolysis. Regardless, the example of PBP1 illustrates that observation of poly-ubiquitination and proteasome sensitivity *in vivo* forms insufficient evidence to

conclude that proteasomal degradation of a protein must proceed through an ubiquitinated intermediate.

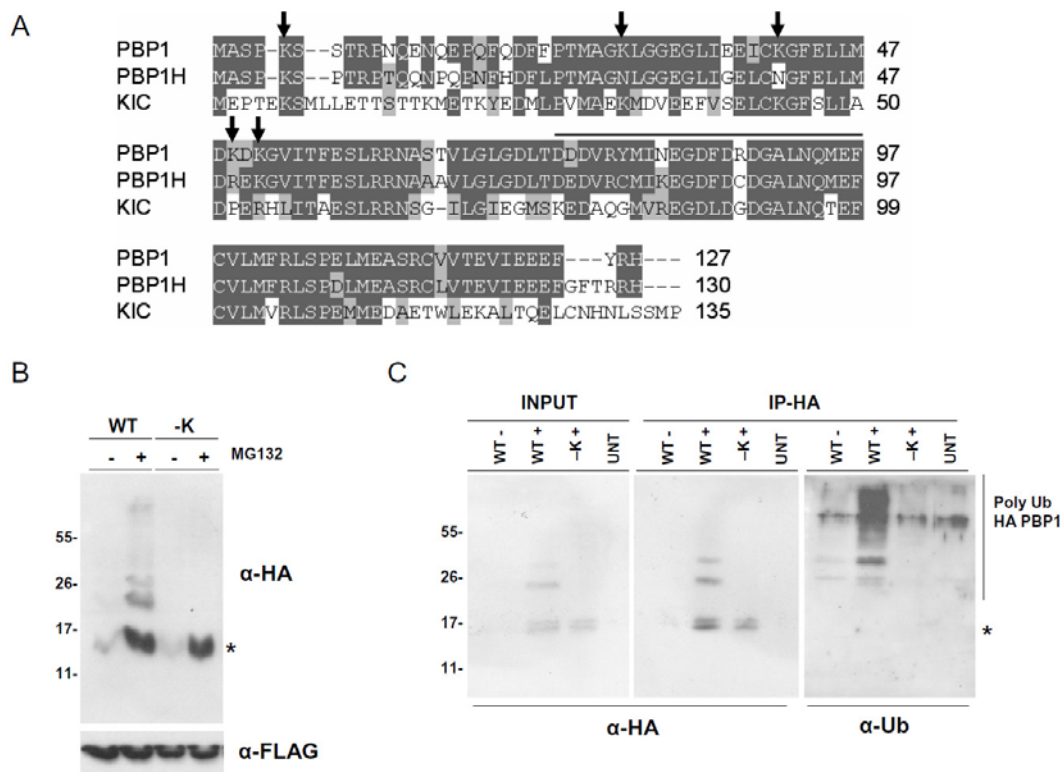


Figure 1: PBP1 is degraded by the proteasome in a ubiquitination-independent manner. (A) Alignment of PBP1, PBP1H and KIC. The lysine (K) residues are indicated by black arrows. The position of the EF-hand calcium binding pocket is indicated with a line. (B) Arabidopsis protoplasts transfected with either *35S::HA-PBP1* (WT) or *35S::HA-PBP1-K* (-K) together with 5 µg *35S::FLAG-GFP*. Sixteen hours after transfection, cells were incubated for 1h with 0.1% DMSO (-) or 50 µM MG132 (+). Total protein extracts (20 µg) were analyzed on western blots using anti-HA (α-HA) or anti-FLAG (α-FLAG) antibodies. (C) Western blot of protein extracts from protoplasts transfected as in B, using anti-HA (α-HA) or anti-Ub (α-Ub) antibodies. Ten percent of the total extract was analyzed as INPUT. The rest was used for immunoprecipitation with anti-HA affinity matrix (IP-HA) prior to Western blot analysis. UNT indicates untransfected control. The position of the poly-ubiquitinated HA-PBP1 is indicated on the right. The asterisk indicates the position of the full length HA-PBP1 protein. Numbers on the left indicate the molecular mass of marker proteins in kDa.

Ubiquitination of PBP1 does not change its functional properties

The interaction of calcium-binding proteins with Ca^{2+} results in a reduced mobility, and therefore these proteins show a double band on a protein gel (Ling

and Zielinski, 1993). Proteins extracted from protoplasts expressing HA-PBP1 also show a double band recognized by anti-HA antibodies (Figure 1C), and interestingly, we found both free and calcium-bound PBP1 to be ubiquitinated, since similar double bands were identified with anti-Ub antibodies. Moreover, PBP1-K retains its ability to bind calcium, since a double band could still be observed (Figure 1C). In order to analyze if ubiquitination of PBP1 is essential for its functionality, we tested binding of PBP1 and PBP1-K to PID, and to another AGC3 kinase, WAG2 (Galvan Robert and Offringa, in prep) in an *in vitro* pull-down assay. GST-PID or GST-WAG2 containing glutathione beads were used to pull down His-PBP1 or His-PBP1-K from total *E.coli* protein extracts. After several washes, the beads were analyzed for bound proteins on a Western blot using anti-His antibodies. Both the wild type and the -K version of PBP1 were found to bind PID and WAG2 with similar affinities (Figure 2A).

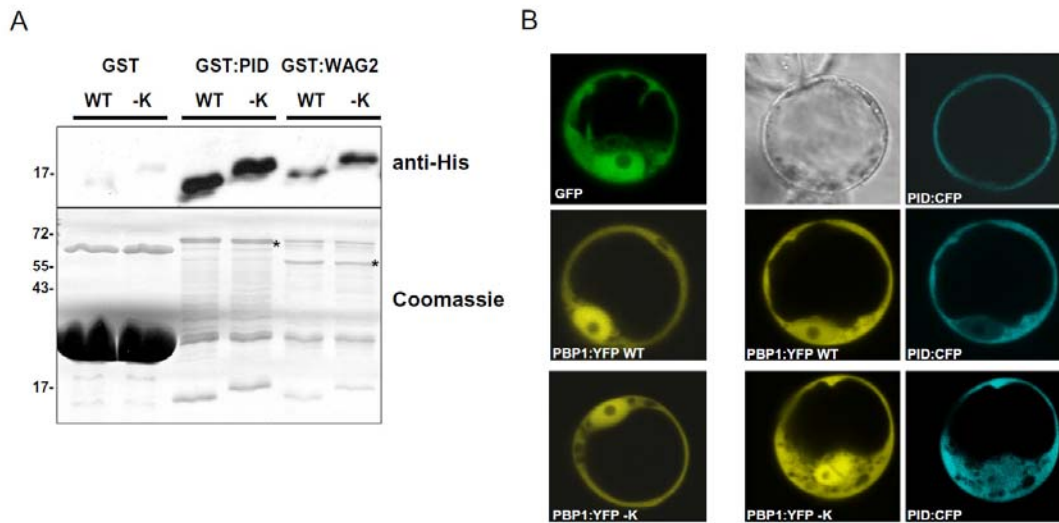


Figure 2: Ubiquitination of PBP1 does not alter its functional properties. (A) *In vitro* pull-down of His-PBP1 (WT) and His-HA-PBP1(-K) with GST, GST:PID or GST:WAG2 bound to glutathione agarose beads. W-His indicates a western blot of the eluates probed with anti-His antibodies. A coomassie stained SDS-PAGE gel of the same samples ran in parallel is depicted below. The asterisks indicate the GST:PID and GST:WAG2 bands. Molecular mass of marker proteins is indicated on the left in kDa. (B, C) Confocal laser scanning microscopy images of Arabidopsis protoplasts transfected with 35S::PID:CFP (B, upper panel), 35S::GFP (C, upper image), 35S::PBP1:YFP-HA (C, middle image) or 35S::PBP1-K:YFP-HA (C, lower image), or co-transfected with 35S::PID:CFP and 35S::PBP1:YFP-HA (B middle panel) or with 35S::PID:CFP and 35S::PBP1-

K:YFP-HA (B, lower panel). In the latter two panels the YFP and CFP signal are shown separately in the left and right images, respectively.

To further test the interaction between PBP1-K and PID *in vivo*, we co-expressed these proteins as respectively YFP and CFP fusions in Arabidopsis protoplasts. Previously, it was observed that PBP1:YFP co-transfection induces the membrane-associated PID:CFP to localize in the cytoplasm (Galvan, Robert, Offringa, in prep). Both the wild type and the -K version of PBP1 are able to sequester PID to the cytoplasm (Figure 2B), indicating that they both are able to bind PID *in vivo*. Our results indicate that the lysine-less PBP1 still retains the tested functional properties of the wild type protein, and that PBP1 ubiquitination is not essential for the protein to bind calcium or PID.

PID and PBP1 are both ubiquitinated and do not affect each other's poly-ubiquitination

We also tested whether the co-expression of PID would affect the ubiquitination status of PBP1 using the modified His-tagged ubiquitin (His-Ub) method for detection of protein ubiquitination (Campanero and Flemington, 1997) which allows identification of *in vivo* ubiquitinated proteins via Nickel-affinity purification. While this assay clearly showed that PID does not have any effect on the ubiquitination of HA-PBP1 (Figure 3, Ni-NTA α -HA panel), we found the PID kinase to be poly-ubiquitinated in Arabidopsis protoplasts (Figure 3, Ni-NTA α -FLAG panel). A single band representing the full length PID-FLAG was detected with the anti-FLAG antibody in the absence of cotransfected His-Ub, indicating basal binding of the abundantly expressed PID-FLAG protein to the Ni-NTA beads during affinity purification. However, only in cells co-expressing His-Ub, an additional smear of slower migrating FLAG tagged protein was detected, representing poly-ubiquitinated PID-FLAG proteins. Co-transfection with *35S::HA-PBP1* does not affect the ubiquitination of PID-FLAG (last lane Ni-NTA α -FLAG panel). These results indicate that PID is ubiquitinated *in vivo* independently of the co-expression of PBP1, and that co-expression of PID does not affect the ubiquitination of PBP1.

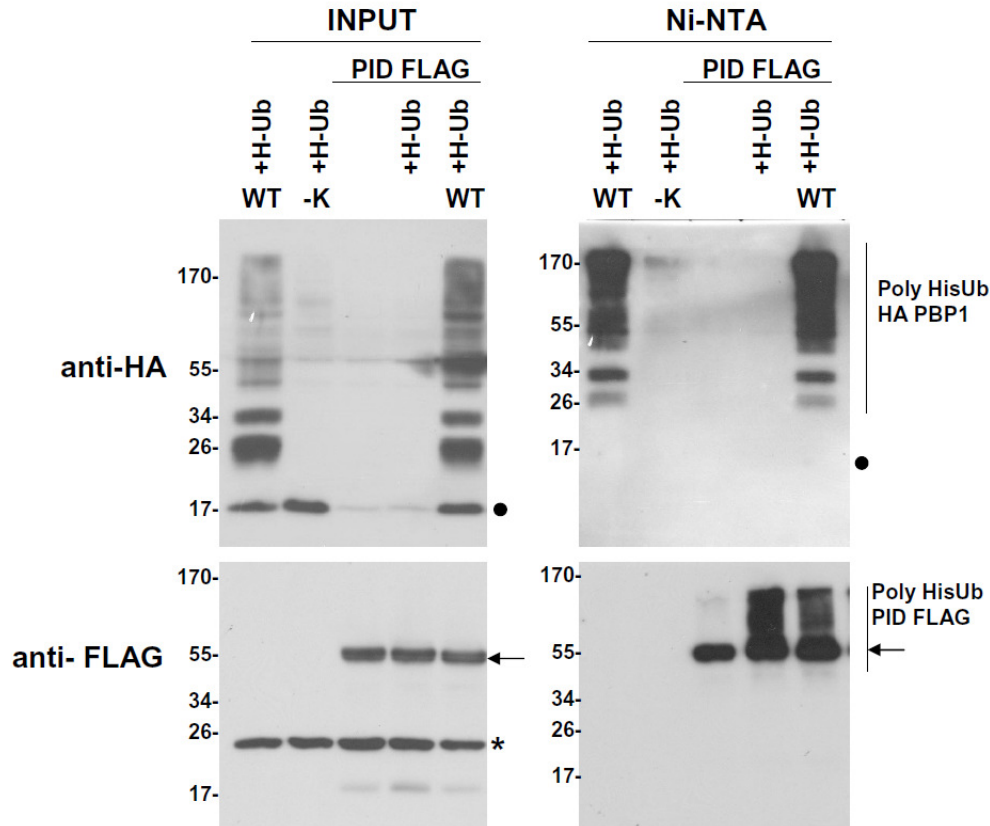


Figure 3: PID and PBP1 are ubiquitinated proteins that do not affect each other's poly-ubiquitination..(A) Western blot analysis of total protein extracts from transfected protoplasts before (INPUT) and after Ni-NTA (Ni-NTA) affinity purification, using anti-HA (upper panels) or anti-FLAG (lower panels) antibodies. Protoplasts were transfected with 20 μ g of plasmid DNA of either *35S::HA-PBP1* (WT) or *35S::HA-PBP1-K* (-K) and *35S::PID-FLAG* (PID-FLAG) or *35S::5xHis-Ub* (H-Ub) where indicated. In all samples 5 μ g *35S::FLAG-GFP* was cotransfected as an internal standard. Sixteen hours after transfection, cells were incubated for 1h with 50 μ M MG132 and harvested in liquid nitrogen. Ten percent of the total extract was analyzed as INPUT and the remaining sample was used to affinity-purify His-Ub tagged proteins with Ni-NTA agarose beads. Poly-ubiquitinated HA-PBP1 and PID-FLAG are indicated. The black dot indicates the full length HA-PBP1, the arrow PID-FLAG, and the asterisk FLAG-GFP.

PBP1 contains a transferable ubiquitination signal

The HA-PBP1 fusion protein is very unstable in protoplasts and is significantly stabilized following treatment with the proteasome inhibitor MG132 (Figure 1A and B). However, when the HA-PBP1-YFP-HA fusion protein was expressed in protoplasts, the fluorescent signal was very strong (Figure 2B and C), and the

fusion protein was produced at high levels without MG132 treatment (Figure 4A). Substitution of the lysines in PBP1 for arginines prevented ubiquitination of HA-PBP1, but did not affect its tested physiological properties, or those of HA-PBP1-YFP-HA (Figure 2B and C). When we used the His-Ub method to analyze the ubiquitination of the PBP1-YFP-HA fusion protein, both HA-PBP1-YFP-HA and HA-PBP1(-K)-YFP-HA appeared as multiple bands before affinity purification (INPUT), with a different pattern of high molecular mass bands in the two samples. Interestingly, after Ni-NTA purification, the HA-PBP1(-K)-YFP-HA version was also recovered in the ubiquitinated pool. These results indicate that one or more of the lysines present in the YFP portion of this fusion protein now serve as substrates for ubiquitination, and that the PBP1(-K) protein can still interact with a E3 ubiquitin ligase and cause ubiquitination of a fused polypeptide. The FLAG-GFP present in all samples was not recovered in the His-Ub tagged fraction, which indicates that GFP (which differs in only one amino acid from YFP) is not ubiquitinated itself. The different patterns of bands observed in the input of the anti-HA blot indicates that different ubiquitin chains are attached to the different proteins due to the difference in the number of Ub attachment sites. These observations suggest that PBP1 contains a transferable ubiquitination signal that can lead to ubiquitination of a lysine-containing amino acid sequence present in *cis*. However, the ubiquitination signal does not function as a degron, since it does not lead an unstable fusion protein.

DISCUSSION

The paradigm in targeted protein degradation is that substrates of the eukaryotic 26S proteasome are ubiquitinated as a prelude to their destruction, and that the primary function of these poly-ubiquitin chains is substrate recognition by the 19S regulatory lid of the proteasome. Several plant proteins were shown to be recruited by E3 ubiquitin ligases, and to be substrates for proteasomal degradation (Gray *et al.*, 2001; Gomi *et al.*, 2004; Thines *et al.*, 2007). For some proteins poly-ubiquitination has been demonstrated (Xie *et al.*, 2002; Saijo *et al.*, 2003; Sasaki *et al.*, 2003; Jang *et al.*, 2005; Dos Santos *et*

al., 2009), and in addition recent high throughput MS analyses have identified multiple plant proteins that are labeled by poly-ubiquitin chains (Maor *et al.*, 2007; Manzano *et al.*, 2008). However, many of these studies lack a detailed analysis of the actual function of the poly-ubiquitin chains.

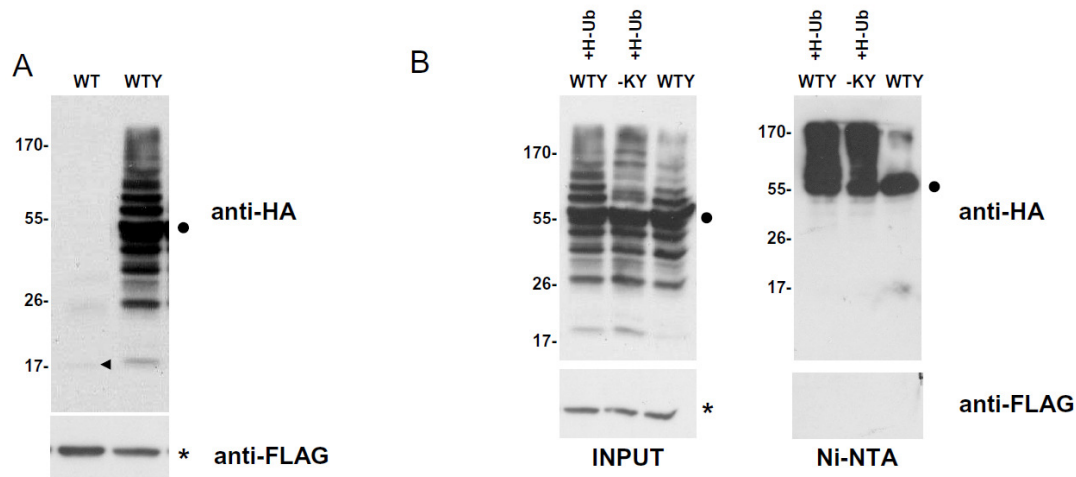


Figure 4: PBP1 contains a transferable ubiquitination signal that does not function as a degron. (A) PBP1 has increased stability when fused to YFP-HA. The arrow indicates the position of HA-PBP1 (WT) and the dot indicates the position of the full length HA-PBP1-YFP-HA (WTY). (B) HA-PBP1(-K) is ubiquitinated when fused to YFP-HA. Arabidopsis protoplasts were transfected with, *35S::HA-PBP1-YFP-HA* (WTY) or *35S::HA-PBP1(-K)-YFP-HA* (-KY), and *p35S::5xHis-Ub* (H-Ub) was co-transfected where indicated. Five micrograms of the plasmid *35S::FLAG-GFP* was added to all transfections as an efficiency control. Western blot analysis of total protein extracts before (INPUT) and after affinity purification (Ni-NTA) was performed with anti-HA (upper panels) or anti-FLAG (lower panels) antibodies. The black dot indicates the position of the full length HA-PBP1-YFP-HA and the asterisk that of FLAG-GFP.

Here we show that the Arabidopsis calcium-binding protein PBP1 is an unstable protein exhibiting proteasome-sensitive turnover and ubiquitination *in vivo*. However, after substituting the lysines for arginines PBP1 remains unstable and its degradation is proteasome-dependent even though it cannot be ubiquitinated. Therefore, PBP1-ubiquitin conjugates are not obligatory intermediates in proteasome-dependent PBP1 turnover. These data do not exclude the possibility that its turnover may be mediated by ubiquitination in some physiological contexts. Alternatively, PBP1 ubiquitination may serve some

function other than signaling proteolysis. Regardless, this example illustrates that observing poly-ubiquitination and proteasome-mediated degradation *in vivo* are insufficient to conclude that protein turnover must proceed through a ubiquitinated intermediate.

Currently, the concept of a linear relationship between ubiquitination and proteasomal degradation knows many exceptions involving other mechanisms than ubiquitination to target proteins to the proteasome (Asher and Shaul, 2005). The ubiquitin-proteasome pathway can be regulated at the level of ubiquitination or at the level of proteasome activity (Glickman and Ciechanover, 2002). Ubiquitination is emerging as an additional regulatory step for the different way proteasomes use to recognize its targets. The proteasome can assume ubiquitin independent recognition of targets via alternative lid configurations such as the REGγ complex (Chen *et al.*, 2007b) showing that proteasome recognition is also a variable and dynamic process. Nonubiquitinated proteins have been reported to be directly recognized by the proteasome in a “degradation by default” mechanism (Asher *et al.*, 2006), via which the degradation occurs unless specific intervention prevents it. The existence of this mechanism suggests that the 20S catalytic core of proteasomes is able to recognize and degrade nonubiquitinated proteins. Examples of proteins degraded via this alternative proteasome pathway are ODC (Ornithine Decarboxylase) (Murakami *et al.*, 1992), p21cip1 (Chen *et al.*, 2007b) and p53/p73 (Asher *et al.*, 2005) for mammalian cells.

To our knowledge, PBP1 is the first plant protein shown to be degraded via a proteasomal ubiquitin-independent process. The presence of ubiquitinated PBP1 suggests that it is a substrate for an E3 ubiquitin ligase. An interesting possibility is that PBP1 may be recruited to the proteasome by being bound to a protein that is itself targeted to the proteasome following ubiquitination. In this case proteasome inhibition may indirectly stabilize PBP1 by affecting the stability of this other protein. Our results indicate that PID is also ubiquitinated, but we have no indications that PID affects the stability or ubiquitination state of PBP1.

The observation that PID is ubiquitinated *in vivo* indicates that the control of protein abundance is an additional step of regulation for the activity of this kinase, which is known to phosphorylate proteins that are themselves ubiquitinated and targeted to the 26S proteasome such as PIN2 (Abas *et al.*, 2006) and BDL (Chapters 2 and 4). Furthermore, the ubiquitination of PID may be regulated through its association with the CSN complex (Chapter 5), something that has been observed for other proteasome targets.

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