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Letter to the Editor

Arabidopsis BAG1 Functions as a Cofactor in Hsc70-Mediated Proteasomal Degradation of Unimported Plastid Proteins

Dear Editor,

Eliminating misfolded or mistargeted proteins is crucial for cell viability because these proteins accumulate as non-specific aggregates, which can be toxic to the cell (Lee et al., 2009; Sroka et al., 2009). Previously, we have shown that in *ppi2* (*plastid protein import 2*) mutant plants, the transcript levels of *Hsc70-4* (one isoform of the Hsc70 family) and *CHIP* (an E3 ligase) were highly upregulated, which ultimately plays crucial roles in proteasomal degradation of unimported plastid proteins (Lee et al., 2009). We also found that, along with those of *Hsc70-4* and *CHIP*, the transcript level of *AtBAG1* (*Arabidopsis thaliana Bcl2-associated athanogene 1*) in the *ppi2* mutant was 2.38-fold higher than that in the wild-type (Lee et al., 2009). In mammalian cells, BAG proteins play multiple roles in protein homeostasis, especially as nucleotide exchange factors of Hsc70, thereby contributing to protein quality control (Alberti et al., 2003; Doukhanina et al., 2006). Therefore, in this study, we examined the role of AtBAG in Hsc70-4-mediated protein quality control in the cytosol.

Arabidopsis contains seven isoforms of BAG proteins. Among these, AtBAG1–4 comprise a subfamily of proteins that are predicted to possess UBL (ubiquitin-like) and BAG domains. AtBAG5–7 comprise another subfamily containing a unique IQ domain (for calmodulin binding) as well as a BAG domain (Doukhanina et al., 2006). First, we determined the subcellular localization of GFP-tagged AtBAG proteins. AtBAG1–3 are mainly localized in the cytosol, but AtBAG4 is localized in both the cytosol and the nucleus (Figure 1A, Supplemental Figure 1A and 1B). To exclude the possibility that the nuclear localization of AtBAG4 is due to an intrinsic characteristic of GFP, we subjected *T7-AtBAG4*-transformed protoplasts to immunostaining with anti-T7 antibody, which confirmed the nuclear localization of AtBAG4 (Supplemental Figure 1B). GFP-AtBAG5 and -6 were present as punctate spots, the identity of which remains to be elucidated (Supplemental Figure 1C and 1D). Consistent with a previous report, GFP-AtBAG7 largely colocalized with the ER marker BiP-RFP (Supplemental Figure 1E) (Williams et al., 2010). To examine the functional relationship between Hsc70 and AtBAG proteins, we performed a coimmunoprecipitation assay and found that GFP-tagged AtBAG1–4 interacted with T7-Hsc70-4 (Figure 1B and Supplemental Figure 2A). Moreover, GFP-AtBAG1 and AtBAG4 bound to endogenous Hsc70 (Figure 1C and Supplemental Figure 2B). In mammalian cells, the BAG domain of BAG proteins interacts with the ATPase domain of Hsc70 (Alberti et al., 2003). Likewise, we found that GFP-AtBAG1ΔBAG and GFP-AtBAG4ΔBAG, in which the BAG domains were deleted, lost the ability to interact with endogenous Hsc70 (Figure 1C and Supplemental Figure 2B). Interestingly, overexpressed GFP-AtBAG5 and GFP-AtBAG7 did not interact with endogenous Hsc70 (Supplemental Figure 2C). Thus, among the seven *Arabidopsis* BAG proteins, AtBAG1–4 may share functional relationships with Hsc70 in the cytosol.

Next, we examined the contribution of AtBAG1 to the degradation of unimported chloroplast precursors in *Arabidopsis* protoplasts. First, we confirmed the significant upregulation of *AtBAG1* in the *ppi2* mutant by quantitative RT–PCR, which confirmed the previous microarray data (Figure 1D) (Lee et al., 2009). Many studies have revealed that overexpressing chaperones or co-chaperones such as Hsp70/Hsp90 and mammalian BAG1 strongly degrades their substrates (Lee et al., 2009; Sroka et al., 2009; Tillmann et al., 2015). Therefore, in the current study, we tested three types of constructs as the substrates of Hsc70-4 and AtBAG1 (Figure 1E). RbcS-nt:GFP has been used as a model substrate to monitor preprotein targeting to chloroplasts, and the overexpression of Hsc70-4 has little effect on this construct (Lee et al., 2006, 2009, 2015). The construct *O2-GFP* contains the minimal sequence motif directly recognized by Hsc70-4 and is strongly degraded in protoplasts overexpressing *Hsc70-4* (Lee et al., 2009). *RbcS-ntΔT6,7-GFP*, a construct in which the T6 and T7 segments in the RbcS transit peptide were deleted, primarily remains in the cytosol even in the presence of critical sequence motifs required for efficient chloroplast targeting, such as the MLM, FNGLK, and FP/RK motifs (Figure 1E) (Lee et al., 2006, 2015). In this analysis, protoplasts transformed with the indicated constructs were subdivided into two fractions. At 12 h after transformation, one fraction was analyzed with immunoblotting and the other one was subject to semi-quantitative RT–PCR for normalization of the expression levels. We found that the level of GFP protein was not affected by the overexpression of T7-Hsc70-4 or T7-AtBAG1, presumably due to proper folding of GFP in the cytosol (Figure 1F and Supplemental Figure 3A). To confirm this finding, as control, we utilized Δ2GFP-HA, in which the second exon of GFP was deleted, thereby exhibiting defective folding of GFP (Supplemental Figure 4A) (Prasad et al., 2010). Overexpression of T7-AtBAG1 caused degradation of Δ2GFP-HA, indicating that AtBAG1 is specific to misfolded substrates in the cytosol (Supplemental Figure 4B). Consistent with the previous results, overexpressing T7-Hsc70-4 or T7-AtBAG1 had little effect on the import of RbcS-nt:GFP. However, when both constructs were overexpressed simultaneously, the level of imported RbcS-nt:GFP decreased significantly (Figure 1F and

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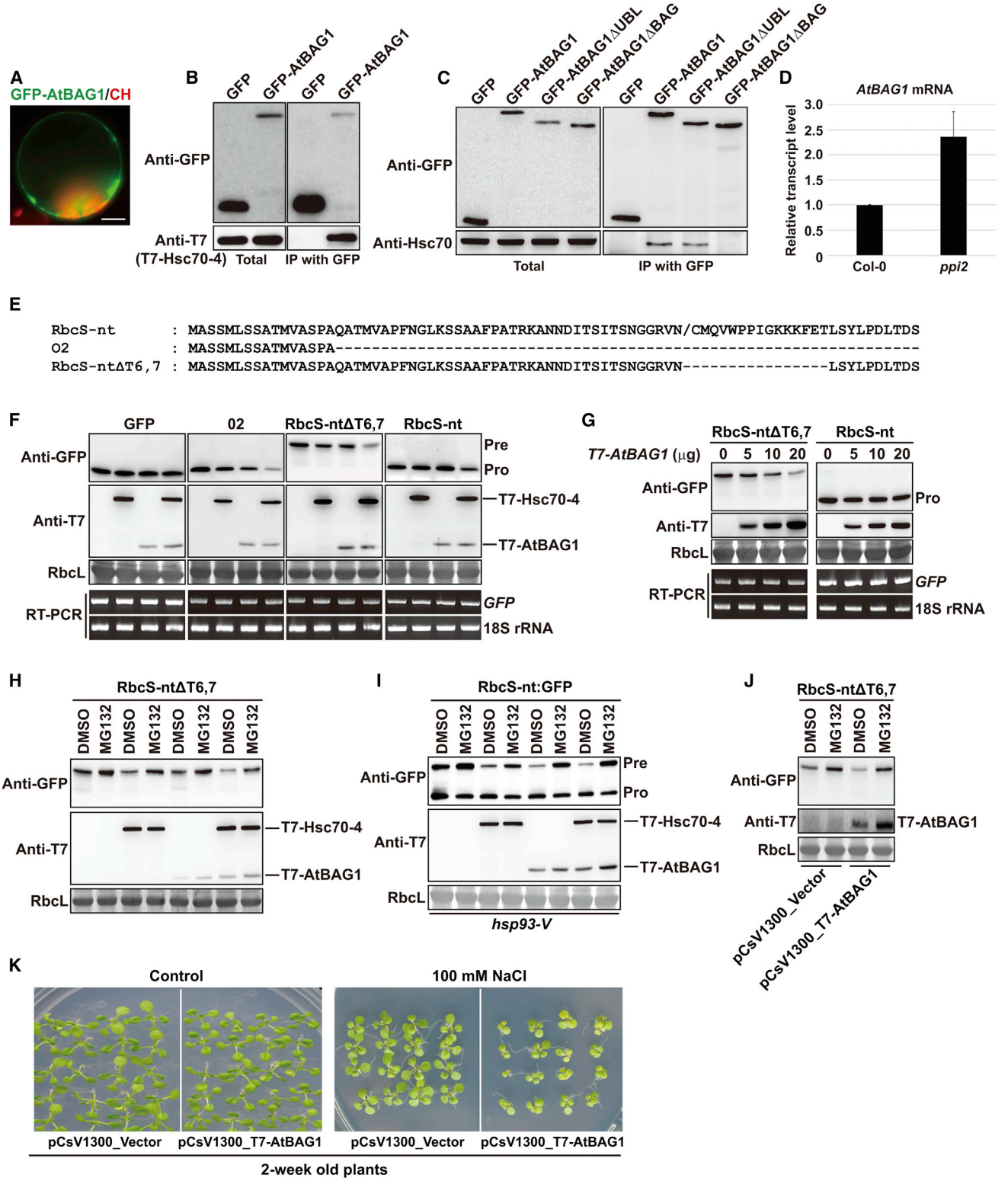

Figure 1. AtBAG1 Is Involved in Hsc70-Mediated Quality Control, and Proper Levels of AtBAG1 Are Critical for Normal Plant Growth.

(A) Subcellular localization of AtBAG1. *GFP-AtBAG1* was transformed into *Arabidopsis* protoplasts, and GFP patterns were examined 12 h after transformation. Scale bar, 20 μm.

(B) Interaction between GFP-AtBAG1 and T7-Hsc70-4. *T7-Hsc70-4* was cotransformed into protoplasts together with *GFP* or *GFP-AtBAG1*. At 12 h after transformation, total protein extracts were subject to immunoprecipitation with anti-GFP antibody followed by immunoblot analysis with anti-GFP and anti-T7 antibodies. Total, 2% of total protein extracts; IP, immunoprecipitation.

(legend continued on next page)

Supplemental Figure 3A). On the other hand, the levels of O2-GFP and RbcS-ntΔT6,7-GFP were significantly reduced with overexpression of T7-Hsc70-4 or T7-AtBAG1 alone. The degradation of these proteins was greater when both T7-Hsc70-4 and T7-AtBAG1 were overexpressed (Figure 1F and Supplemental Figure 3A). Moreover, the level of RbcS-ntΔT6,7:GFP gradually decreased with increasing amounts of T7-AtBAG1 compared with that of RbcS-nt:GFP (Figure 1G and Supplemental Figure 3B). These results indicate that Hsc70-4 and AtBAG1 specifically and cooperatively function in the degradation of misfolded substrates and unimported chloroplast proteins in the cytosol.

Unimported chloroplast proteins are recognized by Hsc70-4 and ubiquitinated by the E3 ligase CHIP for degradation through the 26S proteasome (Lee et al., 2009). The UBL domain of BAG1 recruits the proteasome to the substrates for degradation (Lüders et al., 2000). We therefore investigated the proteasome dependency of AtBAG1-mediated degradation of unimported chloroplast precursors. Degradation of RbcS-ntΔT6,7-GFP by T7-Hsc70-4 or T7-AtBAG1 was strongly blocked by treatment with MG132, an inhibitor of the proteasome (Figure 1H and Supplemental Figure 5A), indicating that AtBAG1-mediated degradation of substrates is proteasome dependent. Quality control mediated by Hsc70-4 and AtBAG1 was evident for the import-incompetent preproteins containing a defective transit peptide. Next, we examined the physiological role of Hsc70 and AtBAG1 in plants with a defect in import machinery. We transformed the wild-type construct *RbcS-nt:GFP* with or without *T7-Hsc70-4* or *T7-AtBAG1* into *hsp93-V* protoplasts and examined the precursor level of RbcS-nt-GFP. In *hsp93-V* plants, import of preproteins is significantly delayed and precursors remain in the cytosol (Lee et al., 2015). Unlike in wild-type protoplasts (Figure 1F and 1G), in *hsp93-V* protoplasts, the precursors of RbcS-nt:GFP were degraded in a proteasome-dependent manner even when introduced into protoplasts alone, and they were degraded at higher levels in the presence of T7-Hsc70-4 or T7-AtBAG1 (Figure 1I and Supplemental Figure 5B). These results indicate that Hsc70-4- and AtBAG-1-mediated degradation of unimported chloroplast preproteins is not limited to substrates with a defective transit peptide, but it is also crucial for eliminating import-competent preproteins in plants defective in chloroplast protein import, such as *ppi2* and *hsp93-V* (Lee et al., 2009). Finally, we analyzed the proteasome dependency of AtBAG1-mediated protein quality control in the transgenic line stably overexpressing T7-AtBAG1 under the control of the strong cassava virus promoter (pCsV). Protoplasts from pCsV1300_T7-AtBAG1 or pCsV1300_Vector plants were transformed with *RbcS-ntΔT6,7-GFP* and incubated with MG132 or DMSO. In both types of protoplasts, RbcS-ntΔT6,7-GFP levels were higher upon MG132 treatment than DMSO treatment. Moreover, the relative fold increase was higher in pCsV1300_T7-AtBAG1 than in pCsV1300_Vector (Figure 1J and Supplemental Figure 5C), confirming that AtBAG1 is actively involved in the quality control of unimported chloroplast proteins.

In the presence of MG132, we observed an increase in T7-AtBAG1 levels, implying that the steady-state level of AtBAG1 is subject to regulation (Figure 1H–1J). To monitor the levels of T7-AtBAG1 protein, protoplasts isolated from transgenic plants were treated with DMSO or MG132. T7-AtBAG1 levels increased in the presence of MG132 (Supplemental Figure 6A), indicating that the level of AtBAG1 itself may be controlled by the proteasome. To further confirm this, protoplasts isolated from pCsV1300_T7-AtBAG1 plants were treated with cycloheximide to block translation, and the levels of T7-AtBAG1 were monitored in the presence of DMSO or MG132 with time (Supplemental Figure 6B and 6C). The level of T7-AtBAG1 was gradually

(C) Interaction between GFP-AtBAG1 and endogenous Hsc70. *GFP*, *GFP-AtBAG1*, *GFP-AtBAG1ΔUBL*, and *GFP-AtBAG1ΔBAG* were transformed into *Arabidopsis* protoplasts. At 12 h after transformation, total protein extracts were subject to immunoprecipitation with anti-GFP antibody followed by immunoblot analysis with anti-GFP and anti-Hsc70 (Lee et al., 2009) antibodies. Total, 2% of total protein extracts; IP, immunoprecipitation.

(D) Upregulation of *AtBAG1* in *ppi2* mutant plants. Total mRNA from 3-week-old wild-type and *ppi2* plants was analyzed by quantitative real-time PCR using *AtBAG1*-specific primers.

(E) Sequences of RbcS-nt, O2, and RbcS-ntΔT6,7.

(F) Hsc70-4 and AtBAG1-mediated degradation of substrates. *GFP*, *O2-GFP*, *RbcS-ntΔT6,7:GFP*, and *RbcS-nt:GFP* were cotransformed with empty vector (*R6*), *T7-Hsc70-4*, *T7-AtBAG1*, or both of these; 5 μg of plasmid DNA was used for all constructs. At 12 h after transformation, total protein extracts isolated from half of the transformed protoplasts were analyzed by immunoblot analysis with anti-GFP and anti-T7 antibodies. Total mRNA from the other half of the transformed protoplasts was subject to semi-quantitative RT–PCR to confirm equal transformation efficiency. RbcL, Rubisco large subunit stained with Coomassie brilliant blue (CBB); Pre, precursor form; Pro, processed form.

(G) Dose-dependent degradation of substrates by T7-AtBAG1. *RbcS-nt:GFP* or *RbcS-ntΔT6,7:GFP* at 5 μg was cotransformed with the indicated amounts of *T7-AtBAG1*. At 12 h after transformation, total protein extracts isolated from half of the transformed protoplasts were analyzed by immunoblot analysis with anti-GFP and anti-T7 antibodies. Total mRNA from the other half of the transformed protoplasts was subject to semi-quantitative RT–PCR to confirm equal transformation efficiency. RbcL, Rubisco large subunit stained with CBB; Pro, processed form.

(H) Proteasome dependency of Hsc70-4- and AtBAG1-mediated degradation of RbcS-ntΔT6,7:GFP. *RbcS-ntΔT6,7:GFP* at 5 μg was cotransformed with 5 μg of empty vector (*R6*), *T7-Hsc70-4*, *T7-AtBAG1*, or both. At 12 h after transformation, transformed protoplasts were treated with DMSO or 20 μM MG132 for 6 h, followed by immunoblot analysis with anti-GFP and anti-T7 antibodies. RbcL, Rubisco large subunit stained with CBB.

(I) Proteasome dependency of Hsc70-4- and AtBAG1-mediated degradation of import-competent RbcS-nt:GFP in *hsp93-V* knockout protoplasts. *RbcS-nt:GFP* (5 μg) was cotransformed with 5 μg of empty vector (*R6*), *T7-Hsc70-4*, *T7-AtBAG1*, or both. At 12 h after transformation, transformed protoplasts were treated with DMSO or 20 μM MG132 for 6 h, followed by immunoblot analysis with anti-GFP and anti-T7 antibodies. RbcL, Rubisco large subunit stained with CBB.

(J) Elevation in proteasome dependency of AtBAG1-mediated degradation of unimported chloroplast proteins in T7-AtBAG1 transgenic protoplasts. Protoplasts isolated from pCsV1300_Vector or pCsV1300_T7-AtBAG1 transgenic plants were transformed with 5 μg of *RbcS-ntΔT6,7:GFP*. At 12 h after transformation, transformed protoplasts were treated with DMSO or 20 μM MG132 for 6 h, followed by immunoblot analysis using anti-GFP and anti-T7 antibodies. RbcL, Rubisco large subunit stained with CBB.

(K) Phenotypes of transgenic plants harboring *pCsV1300_Vector* and *pCsV1300_T7-AtBAG1*. Both transgenic plants were grown on 1/2 Murashige and Skoog medium with or without 100 mM NaCl for 2 weeks.

decreased in DMSO-treated samples, but not in MG132-treated samples, indicating that the level of AtBAG1 itself is under quality control. Next, we examined the effect of AtBAG1 overexpression on plant growth. In normal medium, no visible difference was observed between pCsV1300_Vector and pCsV1300_T7-AtBAG1 transgenic plants until 1 week after planting (Supplemental Figure 7). However, 2-week-old pCsV1300_T7-AtBAG1 plants were smaller than pCsV1300_Vector plants, indicating a delay in growth (Figure 1K). On the other hand, in medium containing 100 mM NaCl, the growth of pCsV1300_T7-AtBAG1 plants was more severely retarded from the early stage of growth than that of pCsV1300_Vector plants (Figure 1K and Supplemental Figure 7). Taken together, these results help confirm the importance of self-regulation of AtBAG1 levels to plant growth.

In summary, we present compelling evidence that an *Arabidopsis* homolog of BAG proteins, AtBAG1, plays a crucial role in Hsc70-4-mediated proteasomal degradation of misfolded and unimported plastid proteins in the cytosol (Supplemental Figure 8). Furthermore, we show that the proper level of AtBAG1 is critical for normal plant growth, especially under adverse environmental conditions.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

D.W.L. and I.H. conceived this project. D.W.L. and S.J.K. performed the coimmunoprecipitation analysis. D.W.L. performed most of the protoplast experiments. D.W.L., S.K., and Y.J.O. generated transgenic plants. B.C. and J.L. performed qRT–PCR. D.W.L. and I.H. wrote the manuscript.

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