AtNBR1 Is a Selective Autophagic Receptor for AtExo70E2 in Arabidopsis

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Selective autophagy is a subcellular process whereby cytoplasmic materials are selectively sequestered into autophagosomes for subsequent delivery to the vacuole for degradation and recycling. Arabidopsis (Arabidopsis thaliana) NBR1 (next to BRCA1 gene 1 protein; AtNBR1) has been proposed to function as a selective autophagy receptor in plants, whereby AtNBR1 anchors the ubiquitinated targets to autophagosomes for degradation. However, the specific cargos of AtNBR1 remain elusive. We previously showed that Arabidopsis exocyst subunit EXO70 family protein E2 (AtExo70E2), a marker for exocyst-positive organelle (EXPO), colocalized with the autophagosome marker Arabidopsis autophagy-related protein8 (AtATG8) and was delivered to the vacuole for degradation upon autophagic induction. Here, through multiple analyses, we demonstrate that AtNBR1 is a selective receptor for AtExo70E2 during autophagy in Arabidopsis. First, two novel loss-of-function nbr1 CRISPR mutants (nbr1-c1 and nbr1-c2) showed an early-senescence phenotype under short-day growth conditions. Second, during autophagic induction, the vacuolar delivery of AtExo70E2 or EXPO was significantly reduced in nbr1 mutants compared to wild-type plants. Third, biochemical and recruitment assays demonstrated that AtNBR1 specifically interacted and recruited AtExo70E2 or its EXPO to AtATG8-positive autophagosomes in a ubiquitin-associated (UBA)-independent manner during autophagy. Taken together, our data indicate that AtNBR1 functions as a selective receptor in mediating vacuolar delivery of AtExo70E2 or EXPO in a UBA-independent manner in plant autophagy.

Autophagy is a subcellular process where cytoplasmic materials are delivered to the lysosome/vacuole for degradation and recycling (Xie and Klionsky, 2007; Marshall and Vierstra, 2018). In the process of macro-autophagy (hereafter autophagy), a double-membrane organelle termed the autophagosome is formed to sequester a part of the cytoplasm and subsequently fuses with a lysosome or vacuole to deliver the contents for degradation (Mizushima, 2007; Yang and Klionsky, 2010; Zeng et al., 2019). For the delivery of specific cargos, selective autophagy is carried out whereby distinct cargo receptors recruit their specific cargos to the developing autophagosome via an interaction with membrane-associated LC3/ATG8 (Stolz et al., 2014). This process is important for quality control and cellular homeostasis (Kraft et al., 2009; Johansen and Lamark, 2011).

In mammalian cells, various autophagy cargo receptors or adapters have been identified, including p62, BRCA1 gene 1 protein (NBR1), NDP52, and NIX/Bnip3L (Pankiv et al., 2007; Kirkin et al., 2009a; Thurston et al., 2009; Gao et al., 2015). p62 is the most well-characterized autophagy cargo receptor in selective autophagy and contains a Phox and Bem1p (PB1) domain, a ZZ-type finger domain, a LC3-interacting region motif, and a ubiquitin-associated (UBA) domain (Lamark et al., 2009). p62 functions in the docking
of ubiquitinated substrates via an interaction between its UBA domain and a polyubiquitin chain on the substrates (Lamark et al., 2009; Johansen and Lamark, 2011). Known substrates of p62 included protein aggregates, midbody rings, mitochondria, peroxisomes, and pathogens (Johansen and Lamark, 2011). The mammalian NBR1 is another cargo receptor with similar domains as p62 and functions in recruiting aggregates and peroxisomes for degradation (Kirkin et al., 2009a; Deosaran et al., 2013). Ubiquitin plays an important role in p62/NBR1-mediated selective autophagy, in which the ubiquitinated substrates are recruited via a p62/NBR1-ubiquitin interaction and delivered to the autophagosome for degradation (Kirkin et al., 2009b).

The plant NBR1 homologs, including Arabidopsis (Arabidopsis thaliana) AtNBR1 and Nicotiana tabacum NtJoka2, have been suggested to play a conserved role in selective autophagy in plants because of their similar domain organization as well as their binding ability to ATG8 and ubiquitin (Svenning et al., 2011; Zientara-Rytter et al., 2011; Floyd et al., 2012; Zientara-Rytter and Sirko, 2014); however, their specific substrates in plants remain elusive. Interestingly, AtNBR1 was shown to bind viral protein CaMV P4 (Hafrén et al., 2017) and NtJoka2 localized to host-pathogen interaction sites during plant defense (Dagdas et al., 2018), indicating the possible involvement of AtNBR1 and NtJoka2 in plant immunity. AtNBR1 might also function in stress tolerance because nbr1 mutants are hypersensitive to abiotic stress (Zhou et al., 2013), albeit different results exist (Rodríguez et al., 2014). In addition, and different to the key role of mammalian p62 and NBR1 in organellophagy (Anding and Baehrecke, 2017), AtNBR1 is not necessary for autophagic clearance of peroxisomes (Jung et al., 2019; Young et al., 2019), indicating possible unique functions of AtNBR1 and NtJoka2 in plants.

We previously identified and characterized a double-membrane organelle termed the exocyst-positive organelle (EXPO), which may be involved in mediating unconventional protein secretion in plants (Wang et al., 2010; Ding et al., 2014a). Interestingly, AtExo70E2-GFP-positive EXPOs were largely separated from the yellow fluorescent protein (YFP)-ATG8e-positive autophagosomes, but upon autophagic induction, EXPO and autophagosome were colocalized and delivered to vacuoles for degradation in transgenic Arabidopsis plants (Lin et al., 2015). However, exactly how AtExo70E2/EXPO is recognized by the autophagic machinery for vacuolar delivery and turnover, remains unknown.

In this study, we demonstrate that the Arabidopsis AtNBR1 functions as a selective autophagy receptor for AtExo70E2 during autophagy in Arabidopsis, which is supported by multiple evidences. First, two newly generated loss-of-function nbr1 CRISPR (nbr1-c1 and nbr1-c2) mutants showed an early-senescence phenotype under short-day growth conditions. Second, during autophagic induction, the vacuolar delivery of AtExo70E2 or EXPO was significantly reduced in nbr1 mutants compared to wild-type plants. Third, biochemical and recruitment assays demonstrated that AtNBR1 specifically interacted with and recruited AtExo70E2 to AtATG8-positive autophagosomes during autophagy. Taken together, we provide a working model underlying AtNBR1-mediated selective autophagy of AtExo70E2 or EXPO via AtATG8-AtNBR1-AtExo70E2 interactions in Arabidopsis.

**RESULTS**

Newly Created nbr1 CRISPR Mutants Exhibit an Early-Senescence Phenotype

To study the possible functions of AtNBR1 in plants, we first generated two new nbr1 mutants using the CRISPR–Cas9 system as described in Gao et al. (2016). We designed three guide RNAs with the target sites on the second exon of AtNBR1 genomic DNA. After three generations’ screening, we obtained two Cas9-free nbr1 mutants, respectively named nbr1-c1 and nbr1-c2 (Fig. 1A). Sanger sequencing revealed a 5-bp deletion in the second exon of nbr1-c1, which leads to a premature stop codon in the resulting translated peptide at amino acid 132. The nbr1-c2 mutant carries a 1-bp insertion and an 8-bp deletion in the second exon, which results in a premature stop codon at amino acid 151 with an introduction of foreign polypeptides of 20 amino acid residues from amino acids 132 to 151 (Fig. 1B). Immunoblot analysis of the endogenous NBR1 with a commercial AtNBR1 antibody anti-AtNBR1 (AS14 2805) suggested that AtNBR1 was knocked out in both nbr1-c1 and nbr1-c2 (Fig. 1C).

We next carried out phenotypic analysis of nbr1-c1 and nbr1-c2 mutants in rich soil. We found that nbr1 mutants exhibit an early-senescence phenotype compared to the wild type Col-0. The early-senescence phenotype was observed under short-day (8-h-light/16-h-dark cycles) conditions. Under short-day conditions, yellowing leaves were observed in ~9-week-old nbr1-c2 and atg7-2, whereas wild-type plants remained green and healthy (Fig. 1D). Further analysis of plant biomass showed reduced biomass of nbr1-c2 under short-day conditions compared to the wild type (Fig. 1E). To further confirm the senescence phenotype of nbr1 mutants, we also performed an identical phenotype analysis on the previously published T-DNA insertional mutant nbr1-2 (Zhou et al., 2013; Hafrén et al., 2017) alongside the two newly generated nbr1 CRISPR mutants nbr1-c1 and nbr1-c2. Similar results of early-senescence phenotypes were observed in both nbr1-c1 and nbr1-c2 under short-day conditions, whereas the nbr1-2 and wild type remained green and healthy (Supplemental Fig. S1A). Interestingly, nbr1-2 may represent a weak mutant because a smaller band of NBR1 can still be detected by immunoblot analysis with the newly generated anti-AtNBR1 (homemade [hm]; Supplemental Fig. S1, C and D),...
Autophagy Induction AtNBR1 Forms Ring-Like Structures upon which could explain the normal phenotype of nbr1-2. Taken together, null nbr1 mutants exhibited an early-senescence phenotype under short-day conditions, which is a typical phenotype of various Arabidopsis atg mutants, including atg5-1 (Thompson et al., 2005) and atg7 (Doelling et al., 2002; Thompson et al., 2005; Chung et al., 2010).

AtNBR1 Mediates Selective Autophagy of AtExo70E2

To investigate the dynamics and subcellular localization of AtNBR1 in plant autophagy, we first generated transgenic Arabidopsis plants expressing fluorescent protein-tagged AtNBR1 under the control of the ubiquitin (UBQ) promoter. As shown in Supplemental Figure S2A, both C-terminal XFP-tagged AtNBR1 (AtNBR1-GFP and AtNBR1-RFP) and N-terminal XFP-tagged AtNBR1 (YFP-AtNBR1 and mCherry-AtNBR1) showed cytosolic and punctate patterns in root cells of these transgenic plants (Fig. 2A, a; Supplemental Fig. S2A). To determine if AtNBR1 responds to autophagic induction and is involved in the autophagy pathway, we next treated the transgenic YFP-AtNBR1 plants with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) to induce autophagy. As shown in Figure 2, upon autophagic induction by BTH treatment for 6 h, the number of AtNBR1-GFP puncta increased (Fig. 2B) and numerous ring-like structures labeled by XFP-tagged AtNBR1 were observed (Fig. 2A, b; Supplemental Fig. S2A). In addition, in cells treated with both BTH and Concanamycin A (Con A), a V-ATPase inhibitor to prevent vacuolar degradation, numerous YFP-AtNBR1 punctae were found inside the vacuole (Fig. 2A, c). These results indicate that AtNBR1 is responsive to autophagic induction to form ring-like structures that locate to the vacuole in root cells of transgenic plants.

To further investigate the possible relationship between the AtNBR1-positive structures and autophagosomes in plant autophagy, we next generated double transgenic Arabidopsis plants coexpressing AtNBR1-GFP and the autophagosome marker mCherry-AtATG8f. As shown in Figure 2C, a, only a few AtNBR1-GFP puncta colocalized with mCherry-AtATG8f in root cells of the double transgenic plants. However, upon autophagic induction by BTH treatment, AtNBR1-GFP largely colocalized with mCherry-AtATG8f on the ring-like structures (Fig. 2C, b), whereas the colocalization ratio between AtNBR1-GFP and mCherry-AtATG8f was significantly increased to ~78% (ratio of mCherry-AtATG8f colocalized atNBR1-GFP puncta/total atNBR1-GFP punctae; Fig. 2D). In addition, after combined BTH and Con A treatment, colocalized AtNBR1-GFP and mCherry-AtATG8f punctae were also observed inside the vacuole (Fig. 2C, c).

To determine whether the subcellular localization of XFP-tagged AtNBR1 was affected as for endogenous AtNBR1 in Arabidopsis, we also generated an AtNBR1 antibody. Immunoblot analysis using protein isolated from 5-d-old wild-type and YFP-AtNBR1-transgenic
seedlings showed that anti-AtNBR1 (hm) recognized both the YFP-AtNBR1 fusion and endogenous AtNBR1 (Supplemental Fig. S1B). Immunofluorescent labeling in root cells of transgenic YFP-AtNBR1 plants showed that anti-AtNBR1 signals were largely colocalized with YFP-AtNBR1 punctae, demonstrating high specificity of anti-AtNBR1 (hm; Supplemental Fig. S3A). Further immunofluorescent labeling in root cells of transgenic YFP-AtATG8e plants showed that anti-AtNBR1 (hm) signals partially overlapped with YFP-AtATG8e signals under normal conditions (Supplemental Fig. S3B, a). However, upon autophagic induction by BTH treatment, anti-AtNBR1 (hm) signals largely overlapped with those of YFP-AtATG8e (Supplemental Fig. S3B, b), indicating a cytosolic translocation of AtNBR1 to autophagosomes in autophagy conditions. To further confirm the general autophagy response of AtNBR1, we also tested if AtNBR1 was sensitive to nitrogen (N) starvation, which is a well-defined TOR-dependent, autophagy-induction condition (Yoshimoto et al., 2004; Pu et al., 2017). The colocalization ratio of NBR1 and ATG8 was increased under N starvation (Supplemental Fig. S2, C, b, and E), albeit the YFP-NBR1 punctae showed no obvious increase (Supplemental Fig. S2, B, b, and D). In addition, AtNBR1-GFP and mCherry-AtATG8f colocalizing punctae were also observed inside the vacuole in root cells subjected to N starvation and Conc A treatment (Supplemental Fig. S2C, b). Similar results were also obtained using anti-AtATG8e (Zhuang et al., 2013) and transgenic YFP-AtATG8e plants (Supplemental Fig. S3C, c). As shown in Supplemental Figure S3D, the anti-AtATG8e signals partially overlapped

Figure 2. AtNBR1 shows both a cytosolic and a punctate localization pattern, and responds to autophagy induction. A, Confocal imaging of root cells of 5-d-old transgenic Arabidopsis seedlings expressing YFP-AtNBR1 upon mock treatment (a), autophagic induction by BTH treatment for 6 h (b), BTH and Conc A treatment for 6 h (c), or Conc A treatment for 6 h (d). Arrows indicate examples of ring-like structures with enlargements provided in the insets, whereas arrowheads indicate examples of YFP-NBR1 positive dots. Scale bars = 10 μm. Images were 5× enlarged in the insets. B, Quantification of the number of total and vacuolar YFP-AtNBR1 puncta from A. The results were obtained from >10 individual seedlings. Error bars represent the SD of puncta per field. C, Roots of 5-d-old transgenic Arabidopsis seedlings expressing AtNBR1-GFP and mCherry-AtATG8f were subjected to mock (a), BTH treatment for 6 h (b), BTH and Conc A treatment for 6 h (c), and Conc A treatment for 6 h (d), followed by confocal imaging. Arrowheads indicate examples of AtNBR1-GFP positive dots with enlargements provided in the insets, whereas arrows indicate examples of ring-like structures with enlargements provided in the insets. Scale bars = 10 μm. Inset images are 5× enlarged. D, Quantification of colocalization ratio between mCherry-AtATG8 and AtNBR1-GFP punctae from C. Error bars represent the SD from >10 individual seedlings used for the quantification. Asterisks indicate significant difference by two-tailed Student’s t test (**P < 0.01 and ***P < 0.001).
with AtNBR1-GFP signals in normal conditions whereas their colocalization percentage was significantly increased from 11% to 34% (ratio of anti-AtATG8e colocalized AtNBR1-GFP punctae/total AtNBR1-GFP punctae) upon autophagic induction (Supplemental Fig. S3C, a and b). In addition, AtNBR1 did not colocalize with known markers of the endomembrane system, including the endoplasmic reticulum (ER), the Golgi apparatus, and prevacuolar compartment (Supplemental Fig. S4, A and B). Taken together, these results demonstrated that YFP-AtNBR1 colocalized with the endogenous AtNBR1 upon autophagic induction in Arabidopsis, indicating the involvement of AtNBR1 in autophagy.

**AtNBR1-Mediated Degradation of AtExo70E2-GFP or EXPO in Arabidopsis**

We have previously shown that AtExo70E2-GFP-positive EXPOs are distinct from YFP-AtATG8-positive autophagosomes under normal conditions, and interestingly, upon autophagic induction, these two markers colocalized and were delivered to the vacuole for degradation (Lin et al., 2015). However, the mechanism underlying vacuolar delivery of AtExo70E2-GFP and EXPO in autophagy remains elusive.

AtNBR1 has been suggested to function as an autophagy cargo receptor in Arabidopsis (Svenning et al., 2011; Zhou et al., 2013; Hafren et al., 2017). We thus hypothesized that AtNBR1 might function as a selective autophagy cargo receptor for AtExo70E2-GFP in autophagy-induction conditions.

To test this hypothesis, we first carried out immunofluorescent labeling studies to establish the relationship between AtExo70E2-GFP and AtNBR1 in autophagy. As shown in Figure 3A, a, the AtExo70E2-GFP signals were largely separate from the anti-AtNBR1 (hm) signals under normal conditions. By contrast, upon autophagy induction, a much higher (10%) degree of colocalization between these two signals was observed (Fig. 3A, b). Similar results were also obtained when using transgenic plants coexpressing AtExo70E2-GFP and mCherry-AtNBR1 (Fig. 3B). Under normal conditions (Mock), mCherry-AtNBR1 colocalized with AtExo70E2-GFP in a few small punctae (Fig. 3B, a); however, upon autophagic induction with BTH treatment, AtExo70E2-GFP signals were colocalized with mCherry-AtNBR1 on ring-like structures (Fig. 3B, b). These ring-like structures are autophagosomes, as they colocalized with the autophagosome marker mCherry-AtATG8f (Fig. 2B, b). In addition, AtExo70E2-GFP punctae signals also largely colocalized with mCherry-AtNBR1 inside the vacuole in cells treated with both BTH and Conc A (Fig. 3B, c). We further confirmed the observation using N starvation as the autophagy-induction condition. Under N starvation, AtExo70E2-GFP showed colocalization with mCherry-AtNBR1 on the ring-like structure in the cytosol (Supplemental Fig. S5A, b) and also inside the vacuole with Conc A (Supplemental Fig. S5A, c). Similarly, colocalization of AtExo70E2-GFP with mCherry-ATG8e can also be observed under N starvation.

**Figure 3.** AtNBR1 is colocalized with the EXPO marker AtExo70E2 in Arabidopsis. A, Immunofluorescent labeling with the AtNBR1 antibody in root cells of 5-d-old seedlings of transgenic Arabidopsis expressing the AtExo70E2-GFP. Five-day-old seedlings were subjected to mock (a) or autophagic induction with BTH and Conc A (b) treatment for 6 h, followed by immunofluorescent labeling with anti-NBR1 and confocal imaging. B, Transgenic Arabidopsis plants coexpressing AtExo70E2-GFP and mCherry-AtNBR1 were subjected to mock (a), BTH treatment for 6 h (b), and BTH and Conc A treatment for 6 h (c), followed by confocal imaging analysis. Arrowheads indicate examples of colocalization between AtExo70E2-GFP and mCherry-AtNBR1 in dots with enlargements provided in the insets, whereas arrows indicate examples of colocalization between AtExo70E2-GFP and mCherry-AtNBR1 in ring-like structures with enlargements provided in the insets. Scale bars = 10 μm. Inset images mages are 5× enlarged.
starvation (Supplemental Fig. S5B, b and c). Taken together, AtExo70E2-GFP colocalized with AtNBR1 on autophagosomes and these proteins were delivered together to the vacuole under autophagy conditions, supporting the possible involvement of AtNBR1 in mediating vacuolar delivery of AtExo70E2-GFP.

Based on the working model for a selective autophagy cargo receptor, if AtNBR1 were the cargo receptor for AtExo70E2-GFP and its EXPO in plant-selective diating vacuolar delivery of AtExo70E2-GFP upon onset of autophagy; and biochemically, AtNBR1 would serve as a linker to bring AtExo70E2 and AtATG8 together for recruitment of AtExo70E2 to autophagosomes via AtATG8.

We next studied the effect of knock-out nbr1 mutants on vacuolar delivery of AtExo70E2-GFP or EXPO in autophagy. We first transformed the EXPO marker AtExo70E2-GFP and autophagosome marker mCherry-AtATG8e into the nbr1-c2 mutant. We previously showed that the EXPO marker AtExo70E2-GFP colocalized with the autophagosome marker mCherry-AtATG8 and was delivered together to the vacuole for degradation upon autophagic induction by BTH and Conc A treatment (Lin et al., 2015). Indeed, as shown in Figure 4A, in transgenic plants coexpressing these two markers, autophagic induction by BTH and Conc A treatment resulted in colocalization of these two markers in the vacuole in the wild-type background. In contrast, when an identical experiment was performed in the nbr1 mutants expressing these two markers, BTH and Conc A treatment resulted in vacuolar localization of mCherry-AtATG8 but with few AtExo70E2-GFP signals, indicating that AtNBR1 depletion prevented vacuolar delivery of AtExo70E2-GFP without affecting vacuolar delivery of mCherry-AtATG8 under autophagy conditions (Fig. 4A; Supplemental Fig. S6). In the wild-type plant, ~30% mCherry-AtATG8e punctate/ring-like structures colocalized with AtExo70E2-GFP, whereas <5% of the colocalization was detected in the nbr1 mutants (Fig. 4B). Similar results were also observed in transgenic AtExo70E2-GFP/nbr1-c1 and AtExo70E2-GFP/nbr1-c2 plants. As shown in Figure 4, C and D, upon autophagy induction with BTH and Conc A treatment, the vacuolar delivery of AtExo70E2-GFP was significantly reduced in nbr1 mutants compared with those in wild type (ratio of fluorescent signal inside vacuole/total: nbr1-c1, 26%; nbr1-c2, 11%; wild type 44%; Fig. 4, C and D). Further GFP cleavage assays using GFP antibody and protein extracts from AtExo70E2-GFP and AtExo70E2-GFP/nbr1-c2 plants with or without BTH treatment showed that much lower GFP core (ratio of free GFP/AtExo70E2-GFP and free GFP intensity: wild type, 0.37; nbr1-c2, 0.15) was detected in AtExo70E2-GFP/nbr1-c2 after BTH treatment (Fig. 4E). These immune detection results are consistent with the confocal observations. Taken together, these results demonstrate that NBR1 is essential for vacuolar delivery of AtExo70E2-GFP/EXPO under autophagy conditions, indicating that, genetically, NBR1 may function as a selective autophagy cargo receptor for AtExo70E2-GFP/EXPO in plant autophagy.

AtNBR1-Mediated Autophagic Degradation of AtExo70E2-GFP Occurs via Direct Interaction with AtExo70E2 in a UBA-Independent Manner

Biochemically, AtNBR1 was proposed to function as an autophagic receptor by interacting with both AtATG8 and ubiquitinated targets (Svenning et al., 2011). Because AtNBR1 is required for vacuolar delivery of AtExo70E2-GFP during autophagy induction, we thus hypothesized that AtNBR1 functions as a selective autophagy receptor for AtExo70E2 during autophagy, in which AtNBR1 recruits AtExo70E2 to AtATG8-positive autophagosomes through an AtATG8-AtNBR1-AtExo70E2 interaction (Fig. 5A), a model being tested in this study.

To study the possible interaction among AtExo70E2, AtATG8e, and AtNBR1, we first carried out a communoprecipitation (co-IP) assay using protein extracts from Arabidopsis cells coexpressing YFP-AtNBR1 and AtExo70E2-4xHA or YFP-AtATG8e and AtExo70E2-4xHA. The co-IP assay showed that AtExo70E2-4xHA was immunoprecipitated by both YFP-AtNBR1 and YFP-AtATG8e, indicating the AtExo70E2 interacts with both YFP-AtNBR1 and YFP-AtATG8e directly or indirectly (Fig. 5B). Further tripartite interaction analysis via the yeast two-hybrid (Y2H) system showed that a direct interaction exists between AtNBR1 and AtExo70E2 but not between AtExo70E2 and AtATG8e (Fig. 5C). In addition, we performed photobleaching-fluorescence resonance energy transfer (FRET) analysis (Fig. 5D). The obtained FRET efficiency results demonstrated the direct interaction between AtNBR1 and AtExo70E2 as well as AtATG8e and AtNBR1, but not between AtExo70E2 and AtATG8e (Fig. 5E).

To further demonstrate the working model (Fig. 5A), we next adopted a Calnexin-mCherry-AtATG8e (CNX-mCherry-AtATG8e) recruitment assay, in which the ER-localized CNX-mCherry-AtATG8e would be used to recruit its interaction partner upon their transient coexpression in Arabidopsis protoplasts (Fig. 5F). As shown in Figure 5G, when coexpressed with the ER-localized CNX-mCherry-AtATG8e in Arabidopsis protoplasts, YFP-AtNBR1 fully colocalized with CNX-mCherry-AtATG8e in the ER (Fig. 5G, b), whereas AtExo70E2-CFP mainly exhibited punctate patterns that were separated from the ER pattern of CNX-mCherry-AtATG8e (Fig. 5G, a), demonstrating that AtATG8e recruits AtNBR1 but not AtExo70E2. However, when both YFP-AtNBR1 and AtExo70E2-CFP were coexpressed together with CNX-mCherry-AtATG8e in Arabidopsis protoplasts, AtExo70E2-CFP showed ER-like patterns and colocalized with both YFP-AtNBR1 and CNX-mCherry-AtATG8e (Fig. 5G, c). Taken together, these data further support the working model that AtNBR1 functions as a bridge to anchor AtExo70E2 to the autophagosome via interaction with both AtATG8 and AtExo70E2.
Deletion of AtNBR1 impairs vacuolar localization and degradation of AtExo70E2 upon autophagic induction. A, Five-day-old seedlings of Col-0 and nbr1-c2 coexpressing the EXPO marker AtExo70E2-GFP and mCherry-AtATG8e were subjected to BTH and Conc A treatment for 6 h, followed by confocal imaging analysis. Scale bars = 10 μm. B, Quantification of the colocalization between AtExo70E2-GFP and mCherry-AtATG8e in Col-0 and nbr1-c2 background. Error bars represent the SD from at least 10 individual seedlings used for the quantification. Asterisks indicate significant difference by two-tailed Student’s t test (**P < 0.01 and ***P < 0.001). C, Vacuolar delivery of AtExo70E2 is impaired in nbr1-c1 and nbr1-c2 mutants. Five-day-old seedlings were subjected to BTH and Conc A treatment for 6 h (early stage) and 8 h (late stage), followed by confocal imaging analysis. Scale bars = 10 μm. D, Quantification of AtExo70E2-GFP fluorescence signal inside the vacuole under autophagy-induction conditions in Col-0, nbr1-c1, and nbr1-c2 mutants. Signal distribution of AtExo70E2-GFP in transgenic plants from 6-h BTH and Conc A treatment were quantified using the software ImageJ. Error bars represent the SD from at least 10 individual seedlings used for the quantification. Asterisks indicate significant difference by two-tailed Student’s t test (**P < 0.01 and ***P < 0.001). E, Immunoblot detection of the vacuolar delivery of GFP core in AtExo70E2-GFP/Col-0 and AtExo70E2-GFP/nbr1-c2 before/after autophagic induction by BTH treatment. Total proteins were subjected to immunoblot analysis with GFP antibodies. Values below the blots represent the ratio of free GFP intensity to the combinatorial intensity of both AtExo70E2-GFP and free GFP in individual lanes quantified by the software ImageJ.
Figure 5. AtNBR1 interacts with AtExo70E2 and recruits AtExo70E2 to AtATG8-positive autophagosomes. A, Working model of AtNBR1-mediated recruitment of AtExo70E2 to AtATG8-positive autophagosome upon autophagic induction. B, Co-IP analysis of interaction between AtExo70E2 and AtNBR1 as well as AtExo70E2 and AtATG8e. Arabidopsis protoplasts expressing YFP (lane 1), YFP-AtATG8e (lane 2), or YFP-AtNBR1 (lane 3) with AtExo70E2-4xHA were subjected to protein extraction and IP with GFP-trap followed by immunoblot analysis with the indicated antibodies. Arrowheads indicate the corresponding detected proteins. Arrows indicate AtExo70E2-4xHA proteins immunoprecipitated by YFP-AtATG8e and YFP-AtNBR1. C, Y2H analysis of interactions...
DISCUSSION

AtNBR1 was previously suggested to function as a selective receptor for ubiquitinated cargos via interaction of the C-terminal UBA domain of AtNBR1 with UBQ (Svenning et al., 2011). To determine which domain(s), including the UBA domain of AtNBR1, interacted with AtExo70E2, we generated three truncated forms of AtNBR1 (D1-YFP to D3-YFP) for interaction assays (Fig. 6A). We then performed a Calnexin-mCherry-X (X refers to the truncated forms of AtNBR1) recruitment assay, in which the ER-localized CNX-mCherry-X would recruit AtExo70E2-GFP to the ER if the truncated forms of AtNBR1 can interact with AtExo70E2. When coexpressed with CNX-mCherry-AtNBR1 and CNX-mCherry-D1 (truncated AtNBR1 with two UBA domains deletion), AtExo70E2-GFP showed ER-like patterns and colocalized with CNX-mCherry-AtNBR1 or CNX-mCherry-D1 in the ER (Fig. 6B). However, when coexpressed with CNX-mCherry-D2 (truncated AtNBR1 harboring PB1 domain only) and CNX-mCherry-D3 (truncated AtNBR1 with two UBA domains only), AtExo70E2-GFP showed punctate patterns separate from CNX-mCherry-D2 and CNX-mCherry-D3 (Fig. 6B). We also performed both co-IP and Y2H assays using these AtNBR1 truncations to identify which domain of AtNBR1 is responsible for its interaction with AtExo70E2. We carried out co-IP assays using protein extracts from Arabidopsis cells coexpressing D1-GFP and AtExo70E2-4xHA, D2-GFP and AtExo70E2-4xHA, or D3-GFP and AtExo70E2-4xHA. The co-IP assay showed that AtExo70E2-4xHA was immunoprecipitated by D1-GFP (Fig. 6C) indicating that the two UBA domains of AtNBR1 are not necessary for interaction with AtExo70E2. In addition, the co-IP assay also showed that AtExo70E2-4xHA cannot be immunoprecipitated by D2-GFP or D3-GFP (Fig. 6C), suggesting that the PB1 domain or the UBA domains of AtNBR1 did not interact with AtExo70E2. In addition, the Y2H assay further confirmed that the truncated AtNBR1 without the UBA domains still interacted with AtExo70E2, demonstrating that AtNBR1 interacts with AtExo70E2 in a UBA-independent manner.

In this study we have provided multiple lines of evidence supporting the conclusion that AtNBR1 functions as the selective autophagy receptor for AtExo70E2 and its EXPD during autophagy in Arabidopsis. We showed that, genetically, AtNBR1 is required for vacuolar delivery of AtExo70E2 for turnover during autophagy (Fig. 4). At the molecular level, AtNBR1 serves as a linker to bring the AtExo70E2 and/or its EXPD together with the AtATG8-positive autophagosome via AtATG8-AtNBR1-AtExo70E2 interaction in a UBA-domain-independent manner (Figs. 5 and 6). We have thus provided insight into the function of AtNBR1 as a selective autophagy receptor and its underlying mechanism in recruiting the cargo AtExo70E2 in plant autophagy.

In plants, studies on selective autophagy are quite limited and only a few autophagy cargo receptors have been reported, for example AtNBR1 and AtSec62 (Svenning et al., 2011; Hu et al., 2020). AtNBR1 has been the most-studied putative selective autophagy cargo receptor in plants but its native cargos remain largely unknown. Several previous studies on the plant NBR1 homologs have shown their binding to both AtATG8 homologs and UBQ (Svenning et al., 2011; Zientara-Rytter et al., 2011; Zientara-Rytter and Sirko, 2014) as well as revealed their possible involvement in plant defense against pathogens (Hafren et al., 2017; Dagdas et al., 2018) and stress tolerance (Zhou et al., 2013). In addition, and distinct from the essential roles of mammalian NBR1 homologs in organellophagy (Anding et al., 2011, 2015), AtNBR1 was not required for autophagic clearance of peroxisomes (Jung et al., 2019; Young et al., 2019), indicating possible unique functions of AtNBR1 in plants.

We previously showed that the AtExo70E2-positive EXPD may be involved in unconventional protein secretion (Wang et al., 2010; Ding et al., 2012, 2014b). Under normal conditions, the EXPD marker AtExo70E2 was distinct from the autophagosomal marker AtATG8e/f, indicating that EXPD and autophagosome are distinct organelles (Lin et al., 2015). However, under autophagy induction conditions, AtExo70E2 colocalized with AtATG8 and is delivered to the vacuole for degradation via an unknown mechanism (Lin et al., 2015). Therefore, this study provides a working model underlying the observation of AtExo70E2 and/or EXPD delivery into the vacuole via AtNBR1-mediated selective autophagy for degradation during plant autophagy (Fig. 7). In wild-type plants, AtNBR1 is the substrate for autophagy and is
recruited to the newly formed autophagosome by interacting with AtATG8-PE on the autophagosomal membrane, for subsequent delivery to the vacuole for degradation upon autophagosome-vacuole fusion (Fig. 7, i; Svenning et al., 2011). Two possibilities exist for AtNBR1-mediated selective autophagy of AtExo70E2 and/or EXPO in transgenic plants coexpressing AtExo70E2 and AtNBR1: (Fig. 7, ii) AtExo70E2-positive EXPO may be recruited to autophagosomes via AtExo70E2-AtNBR1 interaction for subsequent delivery to the vacuole for turnover; or (Fig. 7, iii) AtExo70E2 may be recruited to the phagophore membrane via AtExo70E2-AtNBR1-AtATG8 interaction, and then delivered into the vacuole for degradation. In nbr1 mutants expressing AtExo70E2 and AtATG8 (Fig. 7, iv), vacuolar delivery of AtExo70E2 is defective whereas AtATG8 reaches the vacuole normally in autophagy conditions. It will be of interest in future study to investigate whether degradation of AtExo70E2-GFP is affected in other autophagic mutants such as atg5 and atg7, which will require the generation and analysis of AtExo70E2-GFP/atg5 and AtExo70E2-GFP/atg7 seeds with good GFP signals for further confocal imaging and immunoblot analysis.

In selective autophagy, ubiquitination has been known to play a key role in protein degradation. For example, NBR1 and p62 in mammalian cells interact with UBQ to mediate the delivery of the ubiquitinated substrates to autophagosomes for degradation (Kirkin et al., 2009a). Similarly, the C-terminal UBA domains of AtNBR1 were shown to bind to UBQs albeit with unknown substrates/cargos (Svenning et al., 2011). Interestingly in this study, the binding of AtNBR1 to its cargo AtExo70E2 was in a UBA-domain-independent manner (Fig. 6). These results demonstrated that interaction between the receptor AtNBR1 and the cargo does not involve the UBA domains of the receptor. It will be interesting to perform precise mapping of the domain involved in the interaction with AtExo70E2 in future study. Indeed, several recent studies presented similar conclusions: Deletion of UBA2 (the main UBQ-binding domain) of AtNBR1 does not affect its binding to full-length CaMV P4 (Hafren et al., 2017); RPS6 and AP2M are suggested to interact with AtNBR1 independent

**Figure 6.** AtNBR1 binds to AtExo70E2 in a UBA-independent manner. A, Full-length AtNBR1 and its truncations used in the interaction assay with AtExo70E2. B, Calnexin (CNX)-mCherry-AtNBR1 and CNX-mCherry-D1 recruit AtExo70E2-GFP to the ER whereas CNX-mCherry-D2 and CNX-mCherry-D3 cannot recruit AtExo70E2-GFP to the ER. Constructs were transiently expressed in Arabidopsis protoplasts for 12 h before confocal observation. Scale bars = 10 μm. C, Co-IP analysis of the interaction between AtExo70E2 and AtNBR1 truncations. Arabidopsis protoplasts coexpressing YFP (lane 1), D1-YFP (lane 2), D2-YFP (lane 3), or D3-YFP (lane 4) with AtExo70E2-4xHA were subjected to protein extraction and IP with GFP-trap, followed by immunoblot analysis with the indicated antibodies. Arrowheads indicate the corresponding detected proteins. Arrow indicates AtExo70E2-4xHA proteins immunoprecipitated by D1-YFP. D, Y2H analysis of the interaction between the two UBA domains of AtNBR1 and AtExo70E2. Yeast cells transformed with plasmids were grown on synthetic complete medium lacking Leu, Trp, His (SD-3) for interaction assays. 3-Amino-1,2,4-triazole (3-AT) is used to suppress the background self-activation of the binding domain genes.
of the UBA domain (Tarnowski et al., 2020); and in AtNBR1-mediated aggrephagy, the major population of substrate aggregation-prone GFP-FL2 SP is not ubiquitinated (Jung et al., 2019). Taken together, this study provides a new example of AtNBR1-mediated cargo recognition in plant-selective autophagy.

The exocyst components have been known to involve in autophagy in yeast and mammalian cells (Pecenková et al., 2017). In yeast, multiple exocyst subunits (Sec3, Sec5, Sec6, Sec8, and Sec10) are required for the trafficking of Atg9 vesicle to the preautophagosomal structure during the expansion of the autophagosome, indicating the role of the exocyst in tethering the autophagy machinery to the isolation membrane (Farré and Subramani, 2011; Singh et al., 2019). In mammalian cells, RalB binds to Exo84 and induces the assembly of the protein complex containing active ULK1 and Beclin1-VPS34 PISP kinase on the exocyst for the formation of isolated membrane to initiate autophagy (Bodemann et al., 2011). Similarly, exocysts in plants may also play roles in autophagy as the majority of AtExo70 homologs contain AtATG8-interacting motifs (Kulich et al., 2013) and AtExo70B1 is required for autophagy-mediated delivery of anthocyanin to the vacuole in Arabidopsis (Cvrčková and Zárský, 2013).

However, it remains largely elusive as to whether and how the exocyst subunits would be turned over via the autophagic pathways in yeast, animal cells, and plants. A recent study showed that autophagy induction by flg22 or BTH treatment results in vacuolar delivery of AtExo70B2 for degradation via direct AtExo70B2-AtATG8 (ATATG8-interacting motif) interaction (Teh et al., 2019), indicating that autophagy may regulate exocyst homeostasis in plants. Indeed, in addition to AtExo70E2, CNX-mCherry-AtNBR1 can also recruit other exocyst subunits including AtSec5 and AtExo84 (Supplemental Fig. S7), indicating the possible role of AtNBR1-mediated selective autophagy for regulating exocyst homeostasis in plants. In addition, AtExo70B2 and AtExo70E2 are recruited to autophagosomes via two distinct mechanisms: direct AtExo70B2-AtATG8 binding (Teh et al., 2019) and AtExo70E2-AtNBR1-AtATG8 interaction (this study), respectively. It would be interesting to determine how other exocyst components are recruited to autophagosomes as well as their physiological significance in plants in future studies.

Autophagy is considered to function in the nutrient recycling, and plant autophagy mutants (e.g. atg2, atg5, and atg7) often show early-senescence phenotypes under nutrient-deficient conditions (Wang et al., 2011). Studies have shown that autophagy-related genes play important roles in regulating cell death in plants (Qi et al., 2020). For example, atg2 and atg5 exhibited accelerated salicylic acid signaling-dependent programmed cell death in senescence and immunity

Figure 7. Working model of AtNBR1-mediated selective autophagy of AtExo70E2 and EXPO. Upon autophagic induction, in wild-type plants, (i) AtNBR1 is recruited to the newly formed autophagosome by interacting with the AtATG8-PE form on the autophagosomal membrane, and then delivered to the vacuole for degradation upon autophagosome-vacuole fusion (Svenning et al., 2011) in transgenic plants coexpressing AtExo70E2 and AtNBR1. (ii) AtExo70E2-positive EXPO may be recruited to autophagosomes via AtExo70E2-AtNBR1 interaction for subsequent delivery to the vacuole for turnover, or (iii) AtExo70E2 may be recruited to the phagophore membrane via AtExo70E2-AtNBR1 interaction, and then delivered into the vacuole for degradation. (iv) In nbr1 mutants expressing AtExo70E2 and AtATG8, vacuolar delivery of AtExo70E2 is defective whereas AtATG8 reaches the vacuole normally.
(Yoshimoto et al., 2009). Similarly, silencing ATG6 leads to enhanced cell death after viral infection in Arabidopsis (Liu et al., 2005). In this study, the nbr1 CRISPR mutants showed early-senescence phenotypes (Fig. 1D); however, the mechanisms underlying these early-senescence phenotypes remain elusive. Such a phenotype could be caused either by a defect in the cargo degradation mediated by selective autophagy involving exocyst components or by a defect in plant cell death regulated by salicylic acid signaling and autophagy. To determine the causes underlying the early-death regulated by salicylic acid signaling and autophagy or cargo degradation mediated by selective autophagy in plants. However, the biological significance of AtNBR1 in plant autophagy signaling.

In conclusion, in this study we have demonstrated that AtNBR1 mediates the selective autophagy of AtExo70E2 and/or EXPO via a unique mechanism of UBA-independent AtATG8-AtNBR1-AtExo70E2 interaction in Arabidopsis, thus providing a new example of native cargo for AtNBR1 in Arabidopsis during selective autophagy in plants. However, the biological significance of AtExo70E2/EXPO degradation remains to be investigated, including the specific binding mechanism of AtNBR1 with AtExo70E2, and the possible role of AtNBR1 in plant autophagy signaling.

**CONCLUSION**

In conclusion, in this study we have demonstrated that AtNBR1 mediates the selective autophagy of AtExo70E2 and/or EXPO via a unique mechanism of UBA-independent AtATG8-AtNBR1-AtExo70E2 interaction in Arabidopsis, thus providing a new example of native cargo for AtNBR1 in Arabidopsis during selective autophagy in plants. However, the biological significance of AtExo70E2/EXPO degradation remains to be investigated, including the specific binding mechanism of AtNBR1 with AtExo70E2, and the possible role of AtNBR1 in plant autophagy signaling.

**MATERIALS AND METHODS**

**Plasmid Construction**

To obtain AtNBR1 and truncation forms of AtNBR1 with different fluorescent tags (Cerulean/GFP/YFP/RFP/mCherry), the coding sequence with/without stop codon was synthesized with primers AtNBR1FF and AtNBR1RF followed by double restriction enzyme digestion (New England Biolabs), and then ligation into the digested pBI212 (Ampicillin [Amp]) vectors with different tags. For transient expression, the truncated form of AtNBR1 was cloned into the pBI212 (Amp) vectors containing YFP tags under the UBQ10 promoter. For Calnexin-mCherry-X recruitment assay, Calnexin and mCherry-ATG8e coding sequences were amplified and cloned into the pBI212 backbone containing the UBQ promoter (Zhuang et al., 2013) to generate the pBI212/UBQ-CNX-mCh-AtNBR1, pBI212/UBQ-CNX-mCh-D1, pBI212/UBQ-CNX-mCh-D2, and pBI212/UBQ-CNX-mCh-D3 constructs accordingly. For Y2H analysis, the CDS of AtNBR1 gene was cloned into the pGBK7 (Kanamycin) and pGADT7 (Amp) vectors. All constructs were confirmed by Sanger sequencing. Primers used for plasmid construction are listed in Supplemental Table S1.

**Plant Materials and Growth and Treatment Conditions**

All plants used in this study are in the Arabidopsis (Arabidopsis thaliana) Columbia-0 (Col-0) background. Seeds were sterilized and germinated on a solid one-half strength Murashige and Skoog (MS) medium with 0.8% (w/v) phyto agar and 1% (w/v) Suc (pH 5.7; Wang et al., 2018). The plates were kept at 4°C for 2 d before being moved to the growth chamber. Seedlings were grown at 22°C under long-day conditions (16-h light/8-h dark) or short-day conditions (8-h light/16-h dark). For drug treatments, 5-d-old seedlings were transferred in liquid one-half strength MS with 100 µM of BTH and/or 0.5 µM of Conc A for 6 h followed by confocal imaging. For N-starvation treatments, 5-d-old seedlings were transferred in liquid one-half strength MS without N in dark for 24 h or in liquid one-half strength MS without N in dark for 18 h and an additional 6 h with a concentration of 0.5 µM of Conc A followed by confocal imaging. The nbr1-c1 and nbr1-c2 mutants were generated using the CRISPR system. The synthetic guide RNA guide sequence from the second exon of AtNBR1 genes was designed with the web tool CRISPR-P2.0 (http://crispr.hzau.edu.cn/CRISPR2/; Liu et al., 2017). Three guide sequences with a predicted high on-score value and a low off-target site were selected (Supplemental Table S1). Complementary DNA sequences were synthesized, annealed, and recombined within pUA66-Cas9 plasmid. The cassette was then cloned into the pCAMBIA1300 vector. Col-0 plants were transformed with the CRISPR constructs by floral dipping (Clough and Bent, 1998). The nbr1-c1 and nbr1-c2 plants were identified at the T2 stage. Cas9-free mutants from T3 seeds were screened on hygromycin (50 µg mL⁻¹) plates and confirmed by PCR.

To generate the transgenic plants expressing AtNBR1, all the resulting constructs were introduced into Agrobacterium tumefaciens strain GV3101 and transformed into wild-type Col-0 by floral dip. The genome-edited candidate plants were screened by sequencing. Homozygous CRISPR mutants were generated by self-crossing of the candidate mutants and verified by sequencing. The transformants were selected according to the antibiotic markers associated with corresponding vectors used. Transgenic plants expressing either mCherry-AtATG8 or AtExo70E2-GFP were generated as described in Zhuang et al. (2013) and Lin et al. (2015). To generate the double transgenic lines, AtNBR1-GFP and mCherry-AtATG8, AtExo70E2-GFP and mCherry-AtNBR1, and AtExo70E2-GFP and mCherry-AtATG8 were first generated and then respectively crossed.

**Transient Expression in Protoplasts and Confocal Imaging**

Transient expression of proteins with fluorescent tags in Arabidopsis Plant System Biology Dark-type protoplasts was performed as described in Miao and Jiang (2007). Cells were observed by using a 63×NA1.20 (water) objective, the GFP/YFP signal was excited by 488-nm laser, the mCherry/RFP signal was excited by 552 nm, and the CF signal was excited by 405 nm. The corresponding channels were used in a sequential scanning mode to acquire images with the Leica software (Leica Application Suite X 3.5.5.19976) and processed using Photoshop (Adobe).

**Antibodies, Protein Isolation, and Immunoblot Analysis**

The synthetic peptide KCKDSEERTQGRKPVNLE was ordered from GenScript Biotech. The peptide sequences correspond to the N-terminal region of AtNBR1 between the PB1 and ZZ. The synthetic peptides were conjugated

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with keyhole limpet hemocyanin and used to immunize Rats at the animal core facility of the Chinese University of Hong Kong. Antibodies were purified from Rats serum with cyanogen bromide-activated Sepharose 4B column (Sigma-Aldrich) conjugated with the peptides.

Five-day-old seedlings of wild-type, mutant, and transgenic lines were ground in liquid N and extracted in ice-cold lysis buffer at 4°C, and centrifuged at 7000 × g for 5 min at 4°C as described in Lin et al. (2015). Then the supernatant proteins were boiled in SDS sample buffer at 95°C and separated on 10% SDS-PAGE gels. Proteins were transferred to membranes, blocked, and then immunoblotted with various antibodies including anti-Fru-1,6-bisphosphatase (cF1P; catalog no. AS04 043; Agrisera), anti-GFP (catalog no. 633281; Clontech), anti-HA (catalog no. ab18181; Abcam), anti-AtnBR1 (catalog no. AS14 2805; Agrisera), and the newly generated anti-AtnBR1 (hm) antibody. Quantification of the relative intensity was conducted using the software ImageJ (https://imagej.nih.gov/ij/).

**Immunoprecipitations**

Protein extraction and immunoprecipitation (IP) were performed as described in Cai et al. (2012) and Zhuang et al. (2013). Transformed protoplasts were first mixed with 3-fold volume 250-mu solution and then harvested by centrifugation at 10000 × g for 3 min. The protoplasts were further resuspended in IP buffer (50 mM of Tris-HCl at pH 7.4, 150 mM of NaCl, 0.5 mM of EDTA, 1% (v/v) Triton X-100, 5% (v/v) glycerol, and 1× Complete Protease Inhibitor Cocktail) and lysed by passing through a 1-ml syringe with needle. Total cell lysates were centrifuged at 4°C, 7000 × g for 5 min. The supernatant samples were then incubated with GTP-TRAP Agarose Beads (ChromoTek) for 4 h at 4°C in IP buffer in a top-to-end rotator, followed by washing five times with ice-cold washing buffer. The resulted protein was boiled in 1× SDS loading dye and separated using SDS-PAGE for immunoblot detection by the corresponding antibodies.

**Immunofluorescence Labeling in Arabidopsis Roots**

Immunofluorescent labeling in transgenic plants was performed as described in Shen et al. (2018). The roots of 5–6-old seedlings were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline supplemented with 0.1% (v/v) Triton X-100. The fixed samples were digested by 2% (w/v) droselase, permeabilized by 3% (v/v) Nonidet P-40 plus 10% (v/v) DMSO, and then blocked with 3% (w/v) bovine serum albumin. The roots were then incubated with primary antibody at 4°C overnight, followed by washing with phosphate buffered saline, and probed with secondary antibody for confocal observation. Anti-EMP12, -VSR, and -calreticulin antibodies were used as described in the literature (Tse et al., 2004; Gao et al., 2012; Chung et al., 2018).

**Y2H Assay**

Y2H analysis was performed according to the manufacturer’s instructions (Matchmaker GAL4 Two-Hybrid System 3; Clontech). Pairs of active domain and binding domain were cotransformed into yeast AH109 by way of heat shock (MacDonald, 2001). Positive transformants were selected on synthetic drop-out (50 μl) medium without Trp and Leu (SDΔ2). For detection of interactive proteins, positive colonies were dropped on SD medium lacking His, Trp, and Leu (SDΔ3) and SD medium lacking Ade, His, Trp, and Leu (SDΔ4). Each experiment was repeated three times independently and similar results were obtained.

**FRET Analysis**

FRET acceptor bleaching analysis was conducted on a SP8 Confocal System (Leica) according to the manufacturer’s instructions. Transgenic protoplasts expressing various Cerulean and eYFP fusions were used for photobleaching (514-nm laser) at the speed of 200 in the bidirectional mode. The intensity of Cerulean donor fluorescence was documented before and after photobleaching a region of interest of eYFP fusions to <10% of its initial intensity. FRET efficiency was calculated as FRET efficiency = 100 × (Dpost-Dpre)/Dpost, where Dpre and Dpost is the fluorescence intensity of the donor bleaching before and after bleaching, respectively. At least 20 protoplasts were used for FRET efficiency quantification and analysis. Cerulean-linker-eYFP fusion was used as a positive control that has a FRET efficiency of 30% to 40%. Free Cerulean and eYFP were coexpressed as a negative control that has a FRET efficiency of <5%.

**Quantification and Statistical Analysis**

Sample numbers and the number of biological replicates for each experiment are indicated in the figure legends or as indicated above. Data are presented as mean values ± sd. Two-tailed Student’s t test was used when data met criteria for parametric analysis. Differences in means were considered statistically significant at P < 0.05. Significance levels are: *P* < 0.05; **P** < 0.05; and ***P*** < 0.01.

**Accession Numbers**

Sequence data from this article could be found at The Arabidopsis Information Resource (www.arabidopsis.org) database under the following accession numbers: AtnBR1 (AT4G24690), AtnExo70E2 (AT5G61010), AtnATG8e (AT2G43170), AtnATG8f (AT4G16520), AtnExo84A (At1g10385), AtnSec5b (ATG21170), AtnExo70A1 (At5g03540), AtnSec10 (At5g12370), AtnSec8 (At3g10380), and AtnExo70B2 (At1g07000).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Phenotypic analysis of nbr1 CRISPR mutants and nbr1 T-DNA insertional mutant nbr1-2.

**Supplemental Figure S2.** AtnBR1 responds to autophagic induction.

**Supplemental Figure S3.** Colocalization of AtnBR1 with autophagosomal marker AtnATG8e.

**Supplemental Figure S4.** AtnBR1 signals are separated from other known organelle markers in Arabidopsis.

**Supplemental Figure S5.** Colocalization of AtnExo70E2-GFP with mCherry-AtnBR1 or mCherry-ATG8e under N-starvation conditions.

**Supplemental Figure S6.** Vacular delivery of mCherry-ATG8e is not affected in the nbr1-2 mutant.

**Supplemental Figure S7.** CNX-mCherry-AtnBR1 recruits exocytosis complex components.

**Supplemental Table S1.** Primers used in this study

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