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RESEARCH PAPER

Brassinosteroids act as a positive regulator of NBR1-dependent selective autophagy in response to chilling stress in tomato

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Abstract

Autophagy is a highly conserved and regulated catabolic process involved in the degradation of protein aggregates, which plays critical roles in eukaryotes. In plants, multiple molecular processes can induce or suppress autophagy but the mechanism of its regulation by phytohormones is poorly understood. Brassinosteroids (BRs) are steroid phytohormones that play crucial roles in plant response to stresses. Here, we investigate the role of BRs in NBR1-dependent selective autophagy in response to chilling stress in tomato. BRs and their signaling element BZR1 can induce autophagy and accumulation of the selective autophagy receptor NBR1 in tomato under chilling stress. Cold increased the stability of BZR1, which was promoted by BRs. Cold- and BR-induced increased BZR1 stability activated the transcription of several autophagy-related genes (*ATG*s) and *NBR1* genes by directly binding to their promoters, which resulted in selective autophagy. Furthermore, silencing of these *ATG*s or *NBR1* genes resulted in a decreased accumulation of several functional proteins and an increased accumulation of ubiquitinated proteins, subsequently compromising BR-induced cold tolerance. These results strongly suggest that BRs regulate NBR1-dependent selective autophagy in a BZR1-dependent manner in response to chilling stress in tomato.

Keywords: Autophagy, autophagy-related gene, brassinosteroids, brassinazole resistant 1 (BZR1), chilling stress, NBR1, tomato.

Introduction

Numerous economically important plant species, for example, maize, rice, and tomato, show reduced survival at temperatures below 12 °C because of their poor cold acclimation. As an important abiotic stress, low temperature induces photoinhibition, metabolic imbalance-induced oxidative stress, and inhibition of nutrient uptake (Zhu *et al.*, 2007; Takahashi and Murata, 2008; Xia *et al.*, 2018). In addition, cold is associated with decreased water uptake, which results in cellular dehydration (Thomashow, 1999). These aspects of cold stress significantly impact plant responses to cold at the proteomic level. Specifically, cold-induced oxidative stress increases the risk of protein oxidation and protein misfolding, leading to

the aggregation of non-functional proteins. Under cold conditions, protein folding and processing are active, to prevent cold-induced protein denaturation and aggregation (Boston *et al.*, 1996). For example, plants accumulate chaperones, such as HSP70 and LEA proteins, to stabilize and/or re-establish normal protein conformation, thereby maintaining cellular homeostasis when subjected to chilling stress (Anderson *et al.*, 1994; Bremer *et al.*, 2017). However, it seems likely that this is insufficient in most cases; accordingly, the recycling or degrading of denatured or aggregated proteins would be important for proper plant growth, development, and survival, especially under adverse conditions.

As a highly conserved process involved in protein degradation in eukaryotes, autophagy is essential for the degradation of unnecessary and dysfunctional cellular components during certain stages of development and under adverse environmental conditions (Qin *et al.*, 2007; Liu and Bassham, 2012). To date, over 30 autophagy-related genes (*ATG*s) associated with the autophagy pathway have been identified (Yoshimoto *et al.*, 2012; Marshall and Vierstra, 2018). In plants, the function of autophagy has been thoroughly studied under stress conditions such as nutrient starvation (Hanaoka *et al.*, 2002; Guiboileau *et al.*, 2013), osmotic stress (Liu *et al.*, 2009; Wang *et al.*, 2015*b*), temperature stress (Zhai *et al.*, 2016), oxidative stress (Xiong *et al.*, 2007; Wang *et al.*, 2015*a*), and the presence of pathogens (Liu *et al.*, 2005; Lai *et al.*, 2011). In response to these stresses, the transcription of several *ATG* genes is up-regulated in conjunction with the autophagosome and autophagic body formation. Down-regulation or mutation of *ATG* genes compromise the response or resistance to these stresses (Thompson *et al.*, 2005; Chung *et al.*, 2010; Zhou *et al.*, 2014*b*; Wang *et al.*, 2015*b*). Furthermore, in addition to the highly conserved core ATG proteins that contribute to autophagosome formation, autophagy receptors also play critical roles in the recognition and sequestration of captured autophagy cargoes by phagophores for degradation. NBR1, a selective autophagy receptor, mediates the selective autophagosomal degradation of ubiquitinated protein aggregates in plant responses to specific abiotic stresses (Svenning *et al.*, 2011; Zhou *et al.*, 2013). Although cold damages a variety of cellular structures and macromolecules, and affects protein conformation and aggregation (Chinnusamy *et al.*, 2007; Sun *et al.*, 2017), the roles of autophagy and the receptor NBR1 in the regulation of protein aggregation under cold stress are poorly understood.

Brassinosteroids (BRs) represent a class of phytohormones that play critical roles in a wide range of growth and developmental processes and responses to various stresses (Vriet *et al.*, 2012). The role of BRs has been established mostly via model plants such as Arabidopsis and rice (Yamamuro *et al.*, 2000; Yin *et al.*, 2002). BRs are perceived by the leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1 (BRI1). BRI1 interacts with BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) and positively regulates BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1), which are essential positive regulators of BR signaling (Wang *et al.*, 2002; Yin *et al.*, 2002; Clouse, 2011). An increase in BR levels leads to dephosphorylation of BZR1, which in turn promotes the binding of dephosphorylated BZR1 (dBZR1) to conserved E-boxes (CANNTG) and/or BRRE elements (CGTGT/CG) in the promoters of target BR-responsive genes (He *et al.*, 2005; Kim and Wang, 2010; Sun *et al.*, 2010; Tang *et al.*, 2011). Numerous research studies have shown that BRs enhance stress resistance and ameliorate cellular damage caused by heavy metals, salt, drought, heat, and oxidative stresses (Bajguz and Hayat, 2009; Xia *et al.*, 2009; Zhou *et al.*, 2014*a*). In addition, emerging evidence has revealed that BRs regulate several signaling pathways and genes in the response to cold stress (Kagale *et al.*, 2007; Kim *et al.*, 2010; Qu *et al.*, 2011; Eremina *et al.*, 2016; Li *et al.*, 2017; Ye *et al.*, 2019). For example, BRs modulate pectin methylesterase (PME) activity and the expression of *AtPME41* in Arabidopsis under chilling conditions (Qu *et al.*, 2011). Moreover, BRASSINOSTEROID-INSENSITIVE2 (BIN2) down-regulates the expression of *C-REPEAT BINDING FACTOR* (*CBF*) via the transcription factors (TFs) CESTA, BZR1, and ICE1 in Arabidopsis in response to cold stress (Eremina *et al.*, 2016; Li *et al.*, 2017; Ye *et al.*, 2019). Recently, we reported that BR-induced cold tolerance is relevant to the alleviation of photoinhibition in conjunction with the reduced accumulation of oxidative proteins, suggesting that BRs participate in the regulation of the degradation of denatured proteins (Xia *et al.*, 2018). Although we recently found that BZR1 mediates BR-induced transcription of *ATG*s and autophagy (Wang *et al.*, 2019*a*), the function and regulation of autophagy and the receptor NBR1 in BR-induced cold tolerance are still unclear.

Here, we show that BRs and their signaling element BZR1 play a vital role in the response to cold by inducing autophagy and NBR1 accumulation in tomato plants. Both cold and BRs led to increased stability of BZR1, which activates the transcription of several *ATG*s and *NBR1* by directly binding to the promoter of these genes, leading to the formation of autophagosomes and autophagic bodies. In addition, the silencing of these *ATG*s or *NBR1* genes resulted in the decreased accumulation of several functional proteins and the increased accumulation of ubiquitinated proteins, and subsequently compromised BR-induced tolerance to cold stress. In summary, these results provide new information indicating that BRs play an extremely important role in preventing the accumulation of cold-induced protein aggregates by inducing NBR1-mediated selective autophagy in tomato plants.

Materials and methods

Plant material

The tomato (*Solanum lycopersicum* L.) cultivar Condine Red was used as the wild type (WT). Its BR-deficient mutant *dwf* (accession LA0571) has a lesion in the BR biosynthetic gene *DWARF* (*DWF*), which encodes the cytochrome P450 CYP85A1. Its BR-overexpressing transgenic plant *DWF*OE was used as described previously (Li *et al.*, 2016).

To obtain *bzr1* mutants, a *BZR1* clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) vector was constructed in accordance with previously reported methods (Pan *et al.*, 2016). The target sequence (GGAAGCCATCATGGAGGGAA) was designed by using the CRISPR-P program (Lei *et al.*, 2014) and then synthesized, annealed, and inserted into the *Bbs*I site of an AtU6-sgRNA-AtUBQ-Cas9 vector. Subsequently, we inserted the

AtU6-sgRNA-AtUBQ-Cas9 cassette into the *Hin*dIII and *Kpn*I sites of a pCAMBIA1301 binary vector.

To generate the tomato *BZR1* overexpression construct, a 981 bp coding DNA sequence was amplified from tomato cDNA with specific primers (see Supplementary Table S1 at *JXB* online). The PCR product was digested with *Asc*I and *Kpn*I and then inserted behind the promoter of CaMV 35S in a pFGC1008-HA plasmid vector. The plasmids (*BZR1*OE-HA and the *BZR1* CRISPR/Cas9 vector) were transformed into *Agrobacterium tumefaciens* strain EHA105, and the transformed *A. tumefaciens* were introduced into Condine Red tomato seeds by previously described methods (Fillatti *et al.*, 1987). The homozygous *bzr1* and *BZR1*OE T_2 progenies were used in the experiments.

Virus-induced gene silencing (VIGS) vectors for silencing *ATG2* or *ATG6* were constructed by using PCR amplification with specific primers (Supplementary Table S2), digested by *Sac*I and *Xho*I, and ligated into the same restriction enzyme cutting sites in a TRV2 vector. To silence the *NBR1a* or *NBR1b* genes, *Eco*RI and *Bam*HI were used to digest the PCR product amplified with specific primers (Supplementary Table S2). The resulting plasmid was electroporated into *A. tumefaciens* strain GV3101, and *A. tumefaciens*-mediated virus inoculation was then conducted in accordance with previously described methods (Ekengren *et al.*, 2003). The plants were maintained at 23 °C and used for experiments 4 weeks after *A. tumefaciens* infection. Leaflets whose transcript levels were approximately 20–40% of those of the control plants (infected with an empty TRV2 vector; hereafter termed TRV plants) were used for the experiment (Supplementary Fig. S1).

Growth conditions and treatments

The tomato seedlings were grown at 23/20 °C (day/night) under a 12 h photoperiod (200 μmol m^{-2} s^{-1} photosynthetic photon flux density) in growth rooms. Five-week-old plants were used for subsequent experiments. To induce cold stress, the plants were transferred to growth chambers (Conviron, Manitoba, Canada) and maintained at 23 °C or 4 °C under 200 μmol m^{-2} s^{-1} photosynthetic photon flux density. To determine the effects of exogenous BRs, the plants were pretreated with 200 nM brassinolide (BL; Sigma-Aldrich, St Louis, MO, USA) 24 h before the cold treatment. BL is a member of the BR family with the highest biological activity. Plants sprayed with a solution consisting of distilled water containing an equal amount of ethanol (in place of BL) served as a control.

Cold tolerance assays

Relative electrolyte leakage (REL), which reflects cell membrane permeability, was measured after exposure to cold for 5 d, by using previously reported methods (Cao *et al.*, 2007). The maximum quantum efficiency of photosystem II (PSII) (Fv/Fm) was examined at 5 d with the Imaging-PAM (IMAG-MAXI; Heinz Walz, Effeltrich, Germany) according to previously described methods (Jin *et al.*, 2014).

Monodansylcadaverine staining

The tomato leaves were stained with monodansylcadaverine (MDC) according to previously reported methods (Wang *et al.*, 2015*b*). In brief, tomato leaves were cut into small pieces and vacuum infiltrated with 100 μM MDC (Sigma-Aldrich, St Louis, MO, USA) until they were completely saturated. Afterwards, the saturated leaves were incubated for 30 min in the dark and then washed twice with phosphate-buffered saline (PBS; Solarbio, Beijing, China). The MDC-stained structures were excited by a 405 nm wavelength and measured at the 400–580 nm detection spectrum via an LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany). MDC-stained autophagic signaling was determined by quantifying the number of autophagic vesicles per 30 000 μm^2 area.

Transmission electron microscopy

To determine the accumulation of autophagic bodies, small pieces (~4 mm × 1 mm) of tomato leaves were excised and immediately fixed with 2.5% glutaraldehyde dissolved in 50 mM PBS buffer (pH 7.2) for 12 h in the dark. Then, the samples were washed with PBS three times and fixed with 1% (v/v) osmium tetroxide for 1.5 h. The leaf specimens were then dehydrated in a graded series of ethanol (30–100%; v/v) and embedded in Epon 812. Sections (70 nm) were prepared by using an ultramicrotome (Leica, Wetzlar, Germany) and collected on Formvar-coated grids. The sections were examined with an H7650 transmission electron microscope (Hitachi, Tokyo, Japan) to identify autophagic bodies.

Yeast one-hybrid assays

Yeast one-hybrid (Y1H) experiments were performed following previously reported methods (Ravindran *et al.*, 2017). The coding DNA sequence of *BZR1* and the promoter sequences of *NBR1a* and *NBR1b* were amplified with specific primers (Supplementary Table S3) and ligated into pGADT7 and pAbAi vectors separately. To obtain the mutant E-box, the sequence CANNTG was mutated to TCNNAA by using the Fast MultiSite Mutagenesis System (TransGen FM201-01; Beijing, China). The primers for vector construction are shown in Supplementary Table S4. Y1H Gold yeast strains were transformed with linearized constructs that contained *NBR1* promoter fragments in pAbAi vectors, after which both empty AD and BZR1-AD vectors were transformed into the transformed Y1H Gold strains. The transformed yeast cells were ultimately selected on SD/Leu- plates that contained 100 ng ml^{-1} aureobasidin A to detect DNA–protein interactions.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were carried out with an EpiQuik™ Plant ChIP Kit (Epigentek, Farmingdale, NY, USA) following the manufacturer's instructions. In brief, leaf samples of 1 g were obtained from BL-treated (200 nM) *35S-BZR1*-HA and WT plants separately after 1 d of cold treatment, after which the input chromatin was extracted from the leaf tissues. Epitope-tagged BZR1 chromatin was immunoprecipitated using an antibody to hemagglutinin (HA) (Pierce, Rockford, IL, USA); goat anti-mouse IgG (Millipore, Darmstadt, Germany) was adopted as the negative control. The ChIP–quantitative PCR (qPCR) results were verified by quantitative real-time–PCR (qRT–PCR) with primers specific for the *NBR1a* and *NBR1b* promoters (Supplementary Table S5) and are reported as the percentage of the input DNA.

Protein extraction and western blot analysis

Tomato leaf tissues were extracted using a buffer containing 5 mM EDTA, 100 mM HEPES (pH 7.5), 10 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 mM EGTA, 10 mM sodium fluoride, 10% glycerol, 50 mM β-glycerophosphate, and 7.5% polyvinylpolypyrrolidone. The extracted proteins were denatured at 95 °C for 10 min, after which they were subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane. For Atg8 detection, the denatured proteins were subjected to SDS-PAGE with a 13.5% gel containing 6 M urea. The membrane was blocked in TBST buffer [150 mM NaCl, 20 mM Tris (pH 7.5), and 0.1% Tween-20] containing 5% skimmed milk for 1 h. After blocking, the membrane was incubated for 1 h in TBST buffer containing 1% bovine serum albumin (BSA; Amresco, Solon, OH, USA) that contained mouse anti-HA monoclonal antibody (Pierce, Rockford, IL, USA), rabbit anti-actin polyclonal antibody (Abcam, Cambridge, UK), mouse anti-ubiquitin monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA), and rabbit anti-autophagy substrate NBR1 (NBR1) polyclonal antibody, rabbit anti-Atg8 polyclonal antibody, rabbit anti-PSII subunit S (PsbS) monoclonal antibody, rabbit anti-violaxanthin deepoxidase (VDE) monoclonal antibody, and rabbit anti-D1 monoclonal antibody (Agrisera, Vännäs, Sweden). The secondary antibodies used were goat anti-rabbit horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology, Danvers, MA, USA) and goat anti-mouse HRP-linked antibody (Millipore, Darmstadt, Germany). The signal on the blot was observed by using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The soluble, insoluble,

and ubiquitinated proteins were examined as described previously (Zhou *et al.*, 2013).

Total RNA extraction and gene expression analyses

Total RNA was extracted from tomato leaves with an RNA extraction kit (Tiangen, Beijing, China) following the manufacturer's instructions. A ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) was adopted to reverse transcribe the RNA to a cDNA template for real-time RT–PCR. qRT–PCR was performed using a LightCycler® 480 II Real-Time PCR detection system (Roche, Basel, Switzerland) with SYBR Green PCR Master Mix (Takara, Dalian, China). The qRT–PCR conditions included pre-denaturation at 95 °C for 3 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 57 °C for 20 s, and extension at 72 °C for 30 s. The tomato *Actin* gene was used as an internal control. The gene-specific primers used are shown in Supplementary Table S6. The relative expression of the genes was analyzed by using previously reported methods (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis of the bioassays was performed using SPSS statistical software (v 19.0, SPSS Inc.; Chicago, IL, USA); ANOVA was used to test for significance. Tukey's test ($P<0.05$) was used to analyze the experimental data.

Accession numbers

Sequence data for the genes studied in this work are available in Sol Genomics Network (https://solgenomics.net/) under the following accession numbers: *Actin* (Sl03g078400), *ATG2* (Sl01g108160), *ATG3* (Sl06g034160), *ATG4* (Sl01g006230), *ATG5* (Sl02g036380), *ATG6* (Sl05g050390), *ATG7* (Sl11g068930), *ATG8a* (Sl07g064680), *ATG8b* (Sl02g080590), *ATG8c* (Sl03g031650), *ATG8e* (Sl08g007400), *ATG8f* (Sl08g078820), *ATG9* (Sl04g008630), *ATG11* (Sl07g005970), *ATG12* (Sl12g049310), *ATG13b* (Sl06g072980), *ATG18a* (Sl08g006010), *BZR1* (Sl04g079980), *DWF* (Sl04g078370), *NBR1a* (Solyc03g112230), *NBR1b* (Solyc06g071770).

Results

BRs are involved in regulating autophagy in response to cold stress

First, we studied whether BR-induced cold tolerance is associated with changes in ubiquitinated protein aggregates by using tomato plants with the same genetic background but differing in their BR biosynthesis ability. These plants were *dwf*, a mutant whose BR biosynthetic gene *DWARF* is defective, *DWF*OE, a transgenic plant that overexpresses *DWARF*, and WT. Under optimal growth conditions, no significant differences in REL, Fv/Fm, D1 protein accumulation, or levels of insoluble protein aggregates were found among the three genotypes (Fig. 1; Supplementary Fig. S2A). After exposure to chilling conditions at 4 °C for 5 d, the *dwf* plants had significantly lower Fv/Fm and higher REL values than the WT plants, while the *DWF*OE plants had higher Fv/Fm and lower REL values than the WT (Fig. 1A, B). In addition, compared with the WT plants, the *dwf* plants had lower D1 accumulation while the *DWF*OE plants had higher D1 accumulation (Supplementary Fig. S2A). After cold stress, the accumulation of insoluble proteins in the *dwf* and *DWF*OE plants was approximately 52% higher and 49% lower, respectively, than that in the WT plants (Fig. 1C). To investigate whether these insoluble proteins were ubiquitinated, the total, soluble, and insoluble proteins were isolated and subjected to SDS-PAGE for analysis of ubiquitination. As shown in Fig. 1D, the accumulation of ubiquitinated protein aggregates in the soluble fraction was similar between the plants that were subjected to cold stress and those that were not. However, the level of ubiquitinated protein aggregates in the insoluble protein fraction in the *dwf* leaves and *DWF*OE leaves was markedly higher and lower, respectively, compared with the level in WT leaves after cold stress. We also examined how cold tolerance and the accumulation of ubiquitinated proteins were altered by exogenous BRs. Applications of BL significantly reduced the REL and increased Fv/Fm and D1 protein levels after cold stress (Supplementary Figs S2B, S3A, B). Moreover, the level of insoluble proteins, and of ubiquitinated proteins in the insoluble protein aggregates, decreased (Supplementary Fig. S3C, D).

To examine the role of BRs in autophagy, MDC staining was performed to detect autophagic vesicles in the cells and transmission electron microscopy (TEM) was used to observe autophagic bodies within vacuoles. The MDC assay revealed no or only weak autophagic signaling in *dwf* and WT leaves but significantly greater signaling in *DWF*OE leaves in the absence of cold stress (Fig. 2A). Cold stress induced autophagic activity by causing the increased formation of autophagic vesicles, which was especially apparent in the *DWF*OE leaves (Fig. 2A). Compared with the number of autophagic vesicles in WT leaves, the number in *dwf* leaves was 45% lower and that in *DWF*OE leaves was 181% higher as determined by MDC staining (Fig. 2B). Moreover, compared with the number of autophagic bodies in WT leaves, the number was 59% lower in *dwf* leaves and 176% higher in *DWF*OE leaves as determined by TEM (Fig. 2C).

Atg8 has been widely used to monitor autophagosomes. To further detect the role of BRs in the initiation of autophagy, we used western blotting with an anti-Atg8 antibody to analyze Atg8–phosphatidylethanolamine (PE) conjugates as a marker for activation of autophagy. The Atg8-PE band was barely detected in WT or *dwf* leaves but was abundant in *DWF*OE leaves under normal conditions (Fig. 2D). Cold stress induced the accumulation of the Atg8-PE band, to a lower abundance in *dwf* leaves and a greater abundance in *DWF*OE leaves compared with WT leaves (Fig. 2D). Consistent with these results, foliar applications of BL significantly induced autophagic activity by increasing the formation of autophagosomes and autophagic bodies in response to cold stress (Supplementary Fig. S4A–D).

We then determined whether BRs induced autophagy by inducing the transcription of *ATG*s and two homologous genes of the selective autophagy receptor NBR1 (*NBR1a* and *NBR1b*) as a response to cold conditions. Full data on transcript levels of the *ATG* and *NBR1* genes are available at Dryad Digital Repository (https://doi.org/10.5061/dryad.h70rxwdds; Chi *et al.*, 2020). Under optimal growth conditions, there were little differences between *dwf* and WT plants in the transcript levels of the 16 *ATG* and 2 *NBR1* genes examined. By contrast, the *DWF*OE plants accumulated more transcripts for most of the studied genes. After cold stress, transcript accumulation was differentially induced for these genes, especially for *ATG2*, *ATG6*, *ATG8a*, *ATG8e*, *ATG8f*, *ATG11*, *ATG12*,

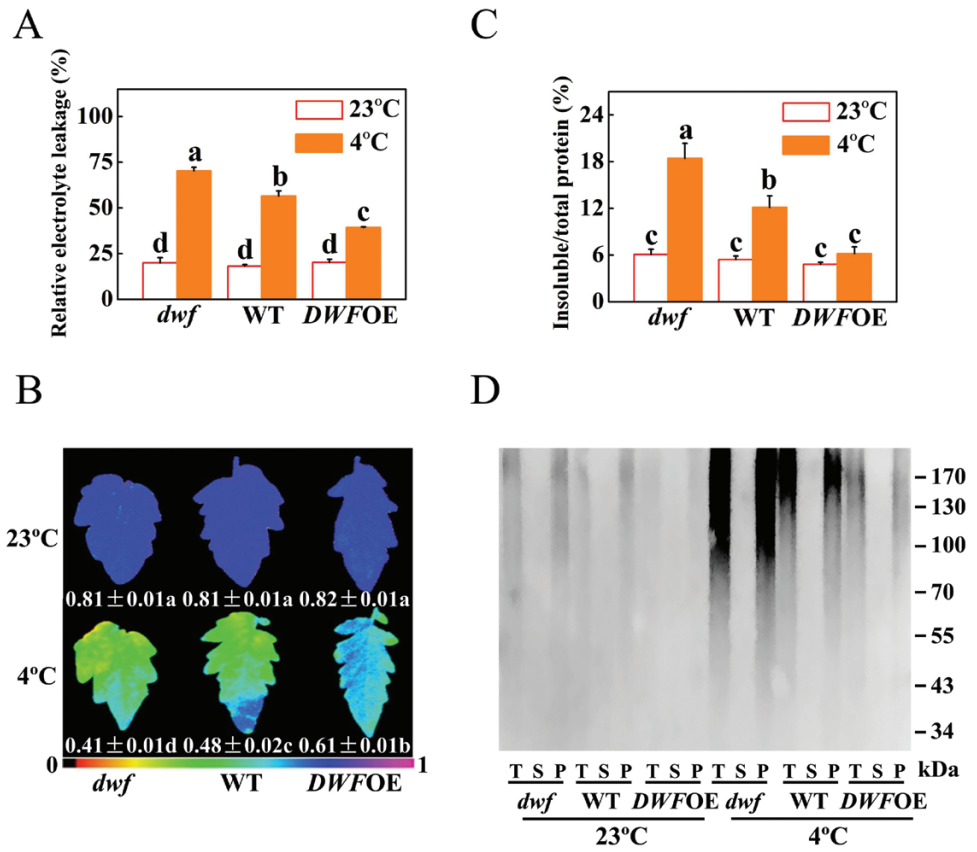

Fig. 1. Influence of brassinosteroids (BRs) on cold tolerance and the accumulation and ubiquitination of insoluble proteins in tomato plants. BR-deficient mutant (*dwf*), wild type (WT; Condine Red), and plants overexpressing the BR biosynthetic *DWARF* gene (*DWF*OE) were transferred to 23 °C or 4 °C for 5 d, after which samples were collected for analysis. (A) Relative electrolyte leakage. (B) Maximum quantum efficiency of photosystem II. The color scale in the image ranges from 0 (black) to 1.0 (purple). (C) Accumulation of insoluble proteins. (D) Ubiquitination of insoluble proteins. Total (T), soluble (S), and insoluble (P) proteins were subjected to SDS-PAGE and detected using an anti-ubiquitin monoclonal antibody. Three independent experiments were performed with similar results. Data are the means ±SD of four biological replicates (A, C) or eight replicates (B). Different letters represent significant differences ($P<0.05$) according to Tukey's test.

ATG13b, *ATG18a*, *NBR1a*, and *NBR1b*. Among these genes, the transcript accumulation of *ATG2*, *ATG6*, *ATG8a*, *ATG8e*, *ATG8f*, *NBR1a*, and *NBR1b* largely depended on the level of BR, while the transcript accumulation of *ATG18a* was almost independent of the level of BR (Fig. 2E). Similarly, the application of exogenous BL up-regulated the transcription of most *ATG* and *NBR1* genes, with the effects on *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* being the most pronounced. Again, the transcript of *ATG18a* was not altered by BL application (Supplementary Fig. S4E). Furthermore, the accumulation of NBR1 protein was induced by cold stress and was especially apparent in *DWF*OE or BL-treated plants (Fig. 2D; Supplementary Fig. S4D).

BZR1 plays a crucial role in BR-induced autophagy

To determine whether BZR1 participates in the BR-based regulation of cold tolerance and autophagy in tomato, we examined the changes in BZR1 protein levels in response to cold stress and BL stimuli using *BZR1*OE plants. Cold stress induced an increased accumulation of phosphorylated BZR1 (pBZR1) and dBZR1. The increase in dBZR1 was more apparent after the application of BL compared with distilled water (Fig. 3A), implying that BZR1 mediates BR-regulated cold tolerance in tomato. To determine how BZR1 regulates cold tolerance, *bzr1*, WT, and *BZR1*OE plants were used. Under optimal growth conditions, Fv/Fm and REL were not related to *BZR1* transcription in the transgenic plants (Supplementary Fig. S5). Compared with the WT plants, the *bzr1* mutants were less tolerant to cold stress, as indicated by the lower Fv/Fm and higher REL values, whereas the *BZR1*OE plants showed increased tolerance, as indicated by the higher Fv/Fm and lower REL values (Fig. 3B, C). However, the application of exogenous BL enhanced cold tolerance only in the WT and *BZR1*OE plants, and not in the *bzr1* plants, suggesting an important role of the BZR1-dependent signaling pathway in BR-induced cold tolerance (Fig. 3B, C).

We then used MDC staining to examine autophagic signaling and performed TEM to observe autophagic bodies in *bzr1*, WT, and *BZR1*OE plants. In the absence of cold stress, little difference was observed among the *bzr1*, WT and *BZR1*OE plants in the number of autophagic vesicles in the leaf samples (Fig. 4A). The application of exogenous BL induced autophagic bodies and increased autophagic signaling; this effect was less significant in *bzr1* plants and more significant in *BZR1*OE plants compared with WT (Fig. 4A). Cold

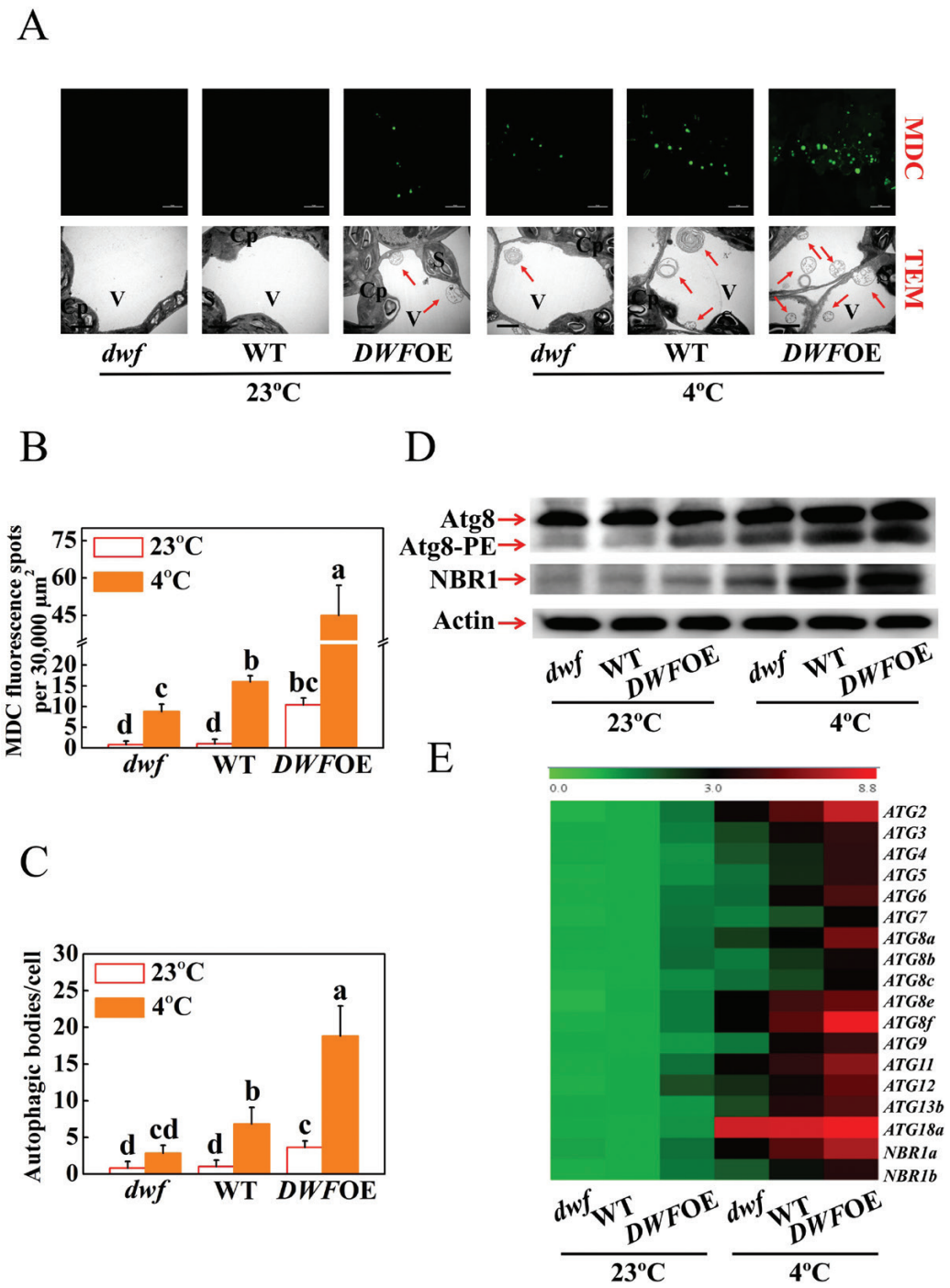

Fig. 2. Brassinosteroid (BR) levels are linked to the induction of autophagy. (A) Monodansylcadaverine (MDC)-stained autophagosome and autophagic bodies (upper panels; bar=25 μm) and transmission electron microscopy (TEM) images of autophagic bodies (lower panels; bar=2 μm). MDC-stained spots are indicated as green signals. Autophagic bodies visualized by TEM are indicated by red arrows. Cp, chloroplast; S, starch; V, vacuole. (B) Number of punctate MDC-stained spots per 30 000 μm^2 area. (C) Number of autophagic bodies in the vacuole. (D) Atg8 and NBR1 protein levels. The non-lipidated and lipidated forms of Atg8 are indicated by Atg8 and Atg8-PE, respectively. Actin was used as the loading control. Three independent experiments were performed with similar results. (E) Transcripts of *ATG* and *NBR1* genes. BR-deficient mutant (*dwf*), wild type (WT; Condine Red), and plants overexpressing the BR biosynthetic *DWARF* gene (*DWF*OE) were transferred to 23 °C or 4 °C, and all leaf samples were collected at 24 h after the imposition of cold stress. In (B) and (C) the reported values are means ±SD calculated by scoring at least 20 randomly chosen fields of view from at least three experiments. For (E), the data represent the means ±SD of four biological replicates. Different letters represent significant differences ($P<0.05$) according to Tukey's test.

stress induced an increase in autophagic signaling and the formation of autophagic bodies in WT and *BZR1*OE plants but not in *bzr1* plants (Fig. 4A). Moreover, applications of BL significantly increased the accumulation of autophagic bodies in WT and *BZR1*OE plants but not in *bzr1* plants (Fig. 4A–C). For example, compared with the number of autophagic bodies identified by TEM in WT leaves, the number in *bzr1* leaves was 61% lower after the application of exogenous BL under cold

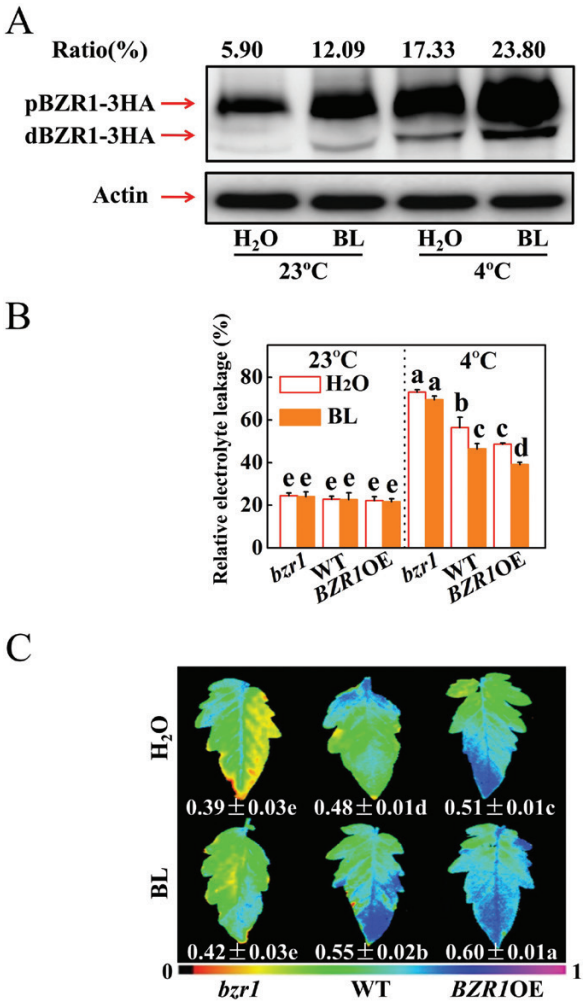

Fig. 3. Influence of BZR1 on cold tolerance in tomato plants. (A) Induction of dephosphorylation of BZR1 in *35S-BZR1*-HA overexpressing (*BZR1*OE) plants by cold stress and brassinolide (BL) application. The phosphorylated and dephosphorylated forms of BZR1 are indicated by pBZR1 and dBZR1, respectively. Total proteins were probed using an anti-HA monoclonal antibody. The ratio reflects the relative abundance of dBZR1 to pBZR1, expressed as a percentage. Actin was used as the loading control. Three independent experiments were performed with similar results. (B) Relative electrolyte leakage. (C) Maximum quantum efficiency of photosystem II (Fv/Fm). The color scale in the image ranges from 0 (black) to 1.0 (purple). *bzr1* mutants, wild type (WT; Condine Red), and *BZR1*OE plants were transferred to 23 °C or 4 °C. BL (200 nM) was applied 24 h before the plants were transferred to 4 °C. REL and Fv/Fm were measured at 5 d after the imposition of cold stress. Data are the mean ±SD of four biological replicates (B) or eight leaf samples (C). Different letters represent significant differences ($P<0.05$) according to Tukey's test.

conditions, while that in *BZR1*OE leaves was 70% higher (Fig. 4C). Furthermore, the abundance of Atg8-PE was not significantly different in any of the plants in the absence of BL, but was greater in WT and *BZR1*OE leaves treated with BL under normal conditions (Fig. 4D). Although cold stress increased the level of Atg8-PE in all the plants, the accumulation of Atg8-PE was lower in the *bzr1* mutant and higher in the *BZR1*OE plants compared with the WT plants (Fig. 4D). Similarly, BL induced the accumulation of NBR1 protein in the WT and *BZR1*OE plants under normal conditions (Fig. 4D). Moreover, under cold stress, the accumulation of NBR1 protein was lower in the *bzr1* mutant and higher in the *BZR1*OE plants compared with the WT plants (Fig. 4D).

Consistent with the changes in the accumulation of autophagic bodies, the transcription levels of the *ATG* and *NBR1* genes showed no differences among the *bzr1*, WT, and *BZR1*OE plants without exogenous BL at 23 °C (Fig. 4E). BL application promoted the transcription of a subset of *ATG* and *NBR1* genes (*ATG2*, *ATG6*, *NBR1a*, and *NBR1b*) by 51–75% in the WT plants and by 89–117% in the *BZR1*OE plants, but had little effect on the transcription of these genes in *bzr1* plants. Similarly, cold stress induced 1.53- to 3.32-fold and 2.29- to 4.36-fold increases in the number of transcripts of the *ATG* and *NBR1* genes in WT and *BZR1*OE plants, respectively, but had little effect in *bzr1* plants. Notably, with the exception of *ATG18a*, these genes were up-regulated after BL application (Fig. 4E).

BZR1 functions as a transcription factor for NBR1a *and* NBR1b

As a TF, dBZR1 can bind to a subset of gene promoters that contain CANNTG and/or CGTGT/CG motifs. Recently, we reported that BZR1 can bind to the promoters of *ATG2* and *ATG6* genes and regulate their transcription (Wang *et al.*, 2019*a*). To investigate whether BZR1 further regulates the selective autophagy receptor *NBR1* genes, the promoter region (~1.5 kb) located upstream of the predicted transcriptional start sites of *NBR1a* and *NBR1b* was inspected. The *NBR1a* promoter contains five E-box sequences (CANNTG) and one BRRE element (CGTGT/CG), and the *NBR1b* promoter contains three E-box sequences (Fig. 5A). To examine whether BZR1 could directly regulate the transcription of *NBR1a* and *NBR1b* *in vitro*, a Y1H assay was performed. As shown in Fig. 5B, the yeast cells that contained the bait vector harboring the *NBR1a* and *NBR1b* promoter regions grew on selective medium when transformed with BZR1-AD; however, when transformed with the empty pGADT7 vector, the yeast cells did not grow on the same selective medium. In addition, the yeast cells that contained the bait vector harboring the mutated E-box regions of the *NBR1a* and *NBR1b* promoters could not grow on the selective medium when transformed with BZR1-AD and empty pGADT7 (Supplementary Fig. S6). This indicates that BZR1 could directly bind to the E-box regions of the *NBR1a* and *NBR1b* promoters *in vitro*.

We also carried out ChIP–qPCR assays to verify whether BZR1 proteins could bind to the promoters of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* *in vivo* under cold stress. As shown in Fig. 5C, the promoter sequences of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* were strikingly enriched via an anti-HA antibody immunoprecipitating 3HA-tagged BZR1 protein in the *BZR1*OE plants compared with the WT plants after cold treatment for 24 h, whereas no difference in the efficiency of the pull-down of these promoter sequences was found using the IgG control antibody. Thus, BZR1 may directly regulate the transcription of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* by binding to their promoters. Taken together, our results demonstrate that, in response to cold and BR, BZR1 up-regulates the

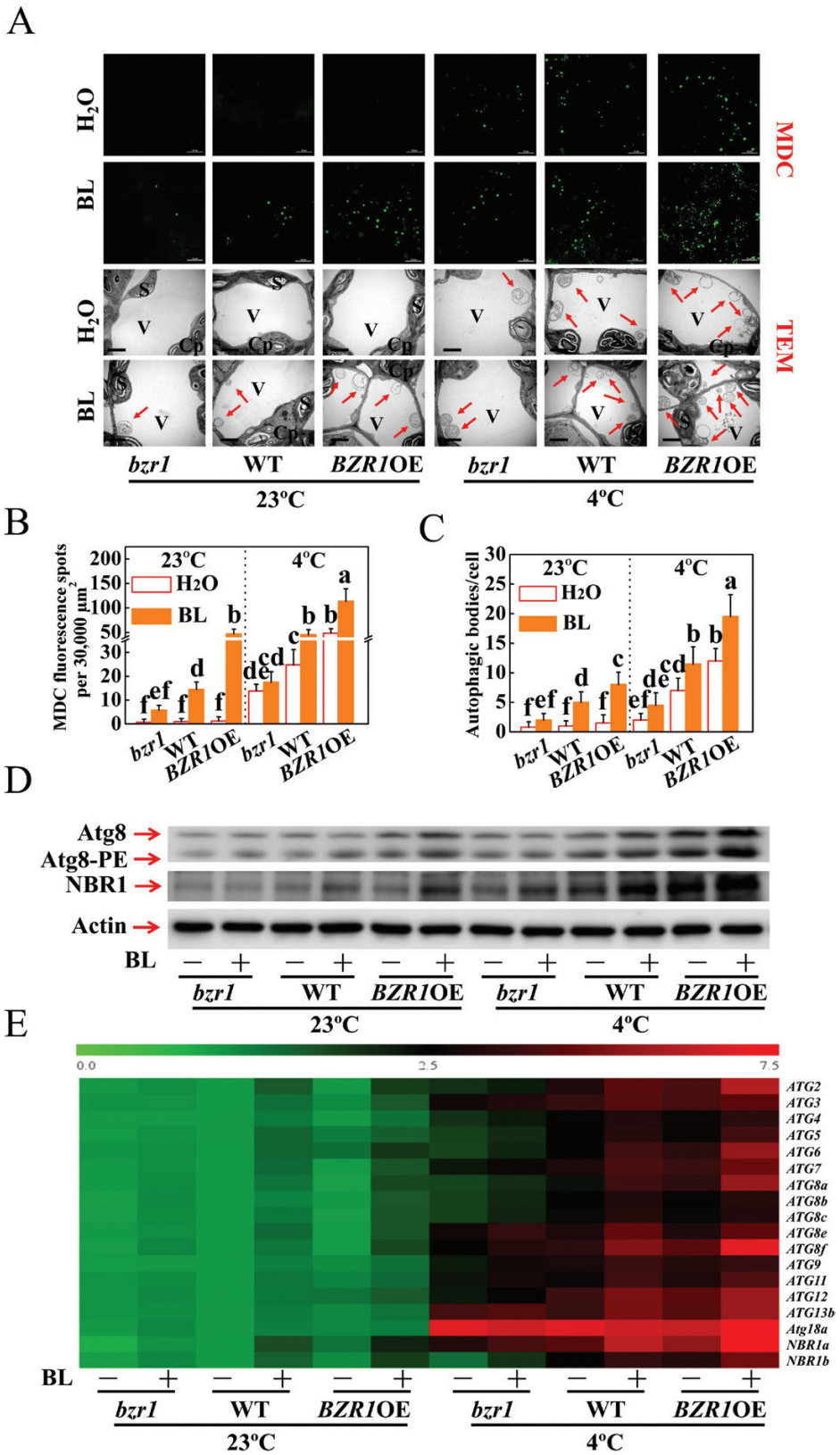

Fig. 4. BZR1 plays a role in the induction of autophagy. (A) Monodansylcadaverine (MDC)-stained autophagosome and autophagic bodies (upper panels; bar=25 μm) and transmission electron microscopy (TEM) images of autophagic bodies (lower panels; bar=2 μm). MDC-stained spots are indicated as green signals. Autophagic bodies visualized by TEM are indicated by red arrows. BL, brassinolide; Cp, chloroplast; S, starch; V, vacuole. (B) Number of punctate MDC-stained spots per 30 000 μm^2 area. (C) Number of autophagic bodies in the vacuole. (D) Atg8 and NBR1 protein levels. The non-lipidated and lipidated forms of Atg8 are indicated by Atg8 and Atg8-PE, respectively. Actin was used as the loading control. Three independent experiments were performed with similar results. (E) Transcripts of *ATG* and *NBR1* genes. *bzr1* mutants, wild type (WT; Condine Red), and *BZR1*-overexpressing plants (*BZR1*OE) were transferred to 23 °C or 4 °C. BL (200 nM) was applied 24 h before the plants were transferred to 4 °C. All leaf tissues were collected at 24 h after the imposition of cold stress. In (B) and (C) the reported values are means ±SD calculated by scoring at least 20 randomly chosen fields of view from at least three experiments. For (E), the data are the means ±SD of four biological replicates. Different letters represent significant differences ($P<0.05$) according to Tukey's test.

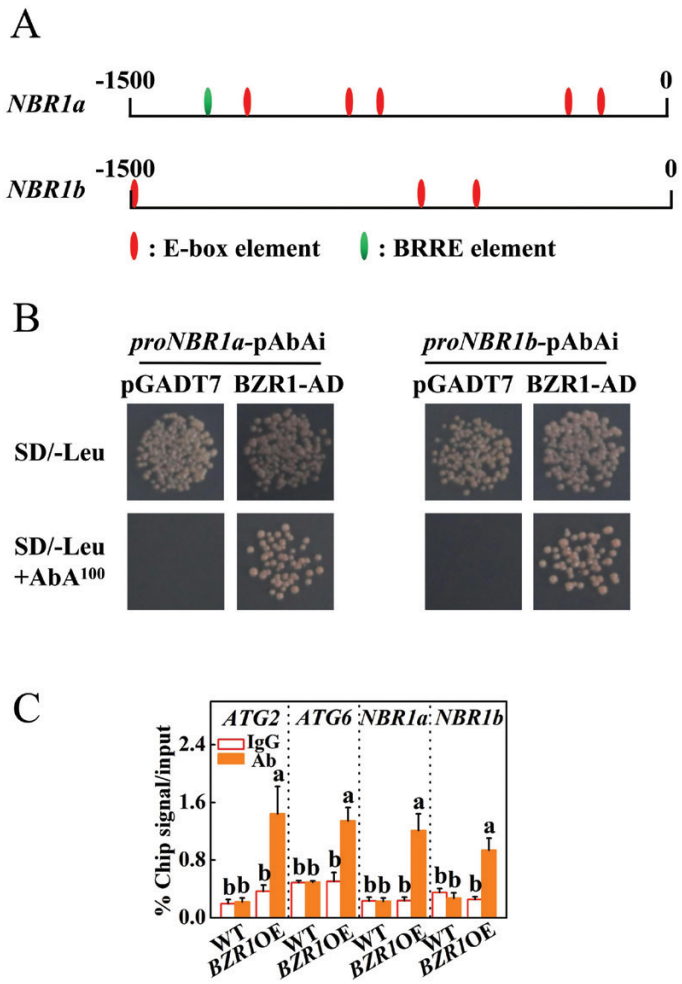

Fig. 5. BZR1 binds to the *ATG* and *NBR1* promoters *in vitro* and *in vivo*. (A) E-box and BRRE elements in the promoters of tomato *NBR1a* and *NBR1b*. The numbering is from the predicted transcriptional start sites. (B) Yeast one-hybrid (Y1H) experiment showing the binding of BZR1-AD to the *NBR1a* and *NBR1b* promoters. (C) Direct binding of BZR1 to the promoters of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* by ChIP–qPCR in *35S-BZR1*-HA overexpressing (*BZR1*OE) plants. Brassinolide (200 nM) was applied 24 h before the plants were transferred to cold (4 °C). All leaf tissues were collected at 24 h after the imposition of cold. Different letters represent significant differences ($P<0.05$) according to Tukey's test; the comparison was done separately for the promoter of each gene.

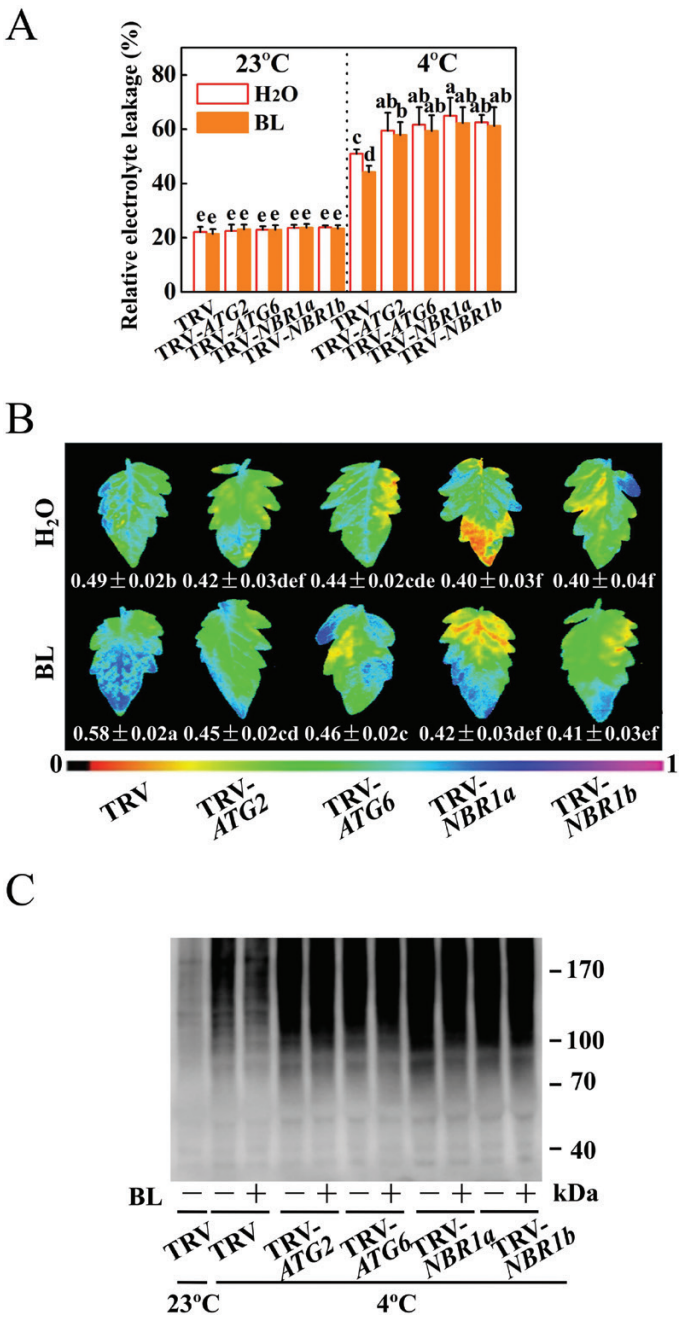

Fig. 6. Cold tolerance is influenced by *ATG*s and *NBR1*s in tomato plants. (A) Relative electrolyte leakage. (B) Maximum quantum efficiency of photosystem II (Fv/Fm). The color scale in the image ranges from 0 (black) to 1.0 (purple). (C) Accumulation of ubiquitination for total proteins. Three independent experiments were performed with similar results. Brassinolide (BL; 200 nM) was applied 24 h before the plants were transferred to cold (4 °C). REL, Fv/Fm, and ubiquitinated proteins were all measured at 5 d after the imposition of cold stress. Data represent the means ±SD of four biological replicates in (A) and eight leaf samples in (B). Different letters represent significant differences ($P<0.05$) according to Tukey's test.

transcription of autophagy-related genes (*ATG2* and *ATG6*) and selective autophagy receptors (*NBR1a* and *NBR1b*).

ATG2, ATG6, NBR1a, *and* NBR1b *play critical roles in the cold response*

To further study the roles of BR-regulated *ATG* and *NBR1* genes in response to cold,VIGS was used to suppress the transcript levels of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* in tomato plants. Under optimal growth conditions, Fv/Fm, REL, and ubiquitinated proteins were not altered in *ATG2*-, *ATG6*-, *NBR1a*-, or *NBR1b*-silenced plants (Supplementary Fig. S7). Under chilling stress, however, in contrast to the control plants infected with the empty vector (TRV), plants whose *ATG* or *NBR1* genes were silenced showed a decrease in Fv/Fm and an increase in both REL and ubiquitinated protein accumulation (Fig. 6A–C). While the application of BL attenuated the cold-induced decrease in Fv/Fm, increase in REL, and accumulation of ubiquitinated proteins in TRV plants, it had no effect on the chilling-induced changes in Fv/Fm, REL, and the accumulation of ubiquitinated proteins in *ATG2*-, *ATG6*-, *NBR1a*- or *NBR1b*-silenced plants (Fig. 6A–C).

Next, we determined how these genes are involved in regulating the induction of autophagy. While cold and BL treatment induced autophagic activity and the formation of autophagic bodies in TRV plants, silencing of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* significantly attenuated both cold-induced and BL-induced autophagic activity and autophagic body formation (Fig. 7A–C; Supplementary Fig. S8). Similarly, the abundance of Atg8-PE increased after cold stress, and this response was more pronounced after BL treatment in TRV plants. However, the silencing of *ATG2*, *ATG6*, *NBR1a*, or *NBR1b* compromised the cold- and BL-induced accumulation of Atg8-PE (Fig. 7D). Furthermore, the level of NBR1 also increased in response to cold stress, and this response was promoted by the application of exogenous BL in TRV plants. Interestingly, this response was promoted by the BL treatment in *ATG2*- or *ATG6*-silenced plants more strongly than in TRV plants (Fig. 7D). These results indicate that the breakdown of the autophagic pathway inhibited the degradation of NBR1 and its conjugated proteins in *ATG2*- or *ATG6*-silenced plants under cold stress. Moreover, the accumulation of NBR1 protein was decreased in the *NBR1a*- or *NBR1b*-silenced plants (Fig. 7D).

Autophagy is involved in the regulation of functional protein homeostasis

Because a deficiency in autophagy reduces protein degradation and the generation of amino acids (Barros *et al.*, 2017), we analyzed the accumulation of D1, PsbS, and VDE proteins, which are involved in photoprotection, in *ATG*- or *NBR1*-silenced plants in response to cold and BL treatment. Under optimal growth conditions, silencing of *ATG2*, *ATG6*, *NBR1a*, or *NBR1b* did not influence the accumulation of PsbS, D1, or VDE (Supplementary Fig. S9). However, BL treatment differentially increased the accumulation of PsbS, VDE, and D1 in TRV plants under optimal growth conditions (Supplementary Fig. S9). Cold stress reduced the accumulation of D1 protein, but increased the accumulation of PsbS and VDE in TRV plants (Fig. 8). However, less accumulation of D1, PsbS, and VDE was observed in *ATG2*-, *ATG6*-, *NBR1a*-, or *NBR1b*-silenced plants than in TRV plants (Fig. 8). The application of BL induced a clear increase in the accumulation of all these proteins in TRV plants under cold stress; however, this was not apparent in the *ATG2*-, *ATG6*-, *NBR1a*-, or *NBR1b*-silenced plants (Fig. 8). These results indicate that autophagy regulates the homeostasis of the functional proteins involved in photoprotection.

Discussion

Cold-induced autophagy is subject to BR regulation

Cold stress leads to a metabolic imbalance that is involved in oxidative stress and a high risk of protein misfolding, causing the formation of dysfunctional and non-functional proteins; the protein control machinery can recognize and ubiquitinate the stress-generated misfolded proteins to target them for degradation. Consistent with our earlier observations that *dwf* plants had a relatively high accumulation of oxidative proteins while *DWF*OE plants had a relatively low accumulation of these proteins (Xia *et al.*, 2018), here we demonstrated that cold stress induced a relatively high accumulation of insoluble proteins in conjunction with ubiquitination in *dwf* plants, and a relatively low accumulation of insoluble proteins in conjunction with ubiquitination in *DWF*OE plants. We previously reported that damaged and denatured cellular proteins were ubiquitinated in response to stress conditions in plants (Zhou *et al.*, 2013). Therefore, the relatively high level of ubiquitinated protein aggregates in the *dwf* plants is attributable to the reduced capacity for degrading ubiquitinated proteins in these plants, suggesting that BR levels are important for degrading ubiquitinated protein aggregates.

Ubiquitinated proteins are degraded via the autophagic pathway and the ubiquitin–proteasome system (UPS) pathway. However, the UPS pathway is restricted in its ability to degrade aggregated proteins, which are not small enough to pass through the narrow proteasome entrance channel (Bence *et al.*, 2001). Autophagy represents a major mechanism in plants for degrading macromolecular ubiquitinated protein aggregates as a response to stresses (Zhou *et al.*, 2014*c*; Marshall and Vierstra, 2018). Many studies have identified the role of autophagy in various stress conditions, such as heat, salinity, drought, nutrient starvation, and endoplasmic reticulum stress (Doelling *et al.*, 2002; Liu *et al.*, 2009; Liu and Bassham, 2013; Zhou *et al.*, 2013; Wang *et al.*, 2016). The data from the present study provide evidence for the involvement of autophagy in the response to cold stress. Consistent with the role of autophagic degradation, cold stress induced the transcription of most of the examined *ATG* and *NBR1* genes, with *ATG2*, *ATG6*, *NBR1a*, *NBR1b*, and *ATG18a* showing a more significant induction than the other *ATG* genes. Interestingly, the number of transcripts of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* was closely related to the capacity for BR biosynthesis, while the number of transcripts of *ATG18a* did not differ among the *dwf*, WT, and *DWF*OE plants after cold stress (Fig. 2E). Consistent with these changes in transcription of the *ATG* and *NBR1* genes relative to that in the WT plants, autophagic activity was lower, in conjunction with decreased autophagic body formation and NBR1 accumulation, in the *dwf* plants, but higher, with greater autophagic body formation and NBR1 accumulation, in the *DWF*OE plants (Fig. 2A–D). In short, these results suggest that BRs are important not only for preventing the functional proteins from being damaged but also for degrading aggregated or damaged proteins. To our knowledge, this study is one of only a few that have demonstrated a role for phytohormones such as BRs in the regulation of autophagy and its receptor NBR1. BRs have been identified that play an important role in the plant response to cold stress (Eremina *et al.*, 2016; Li *et al.*, 2017). For instance, BRs induce the dephosphorylation and SUMOylation of CESTA, and regulate it in both CBF-independent and CBF-dependent pathways to alter the expression of the *COR* gene to improve the freezing resistance of plants (Eremina *et al.*, 2016). BZR1, a crucial TF in BR signaling cascades (Belkhadir and Jaillais, 2015), participates in the regulation of numerous genes under a variety of conditions, including cold stress (Sun *et al.*, 2010; Li *et al.*, 2017; Yin

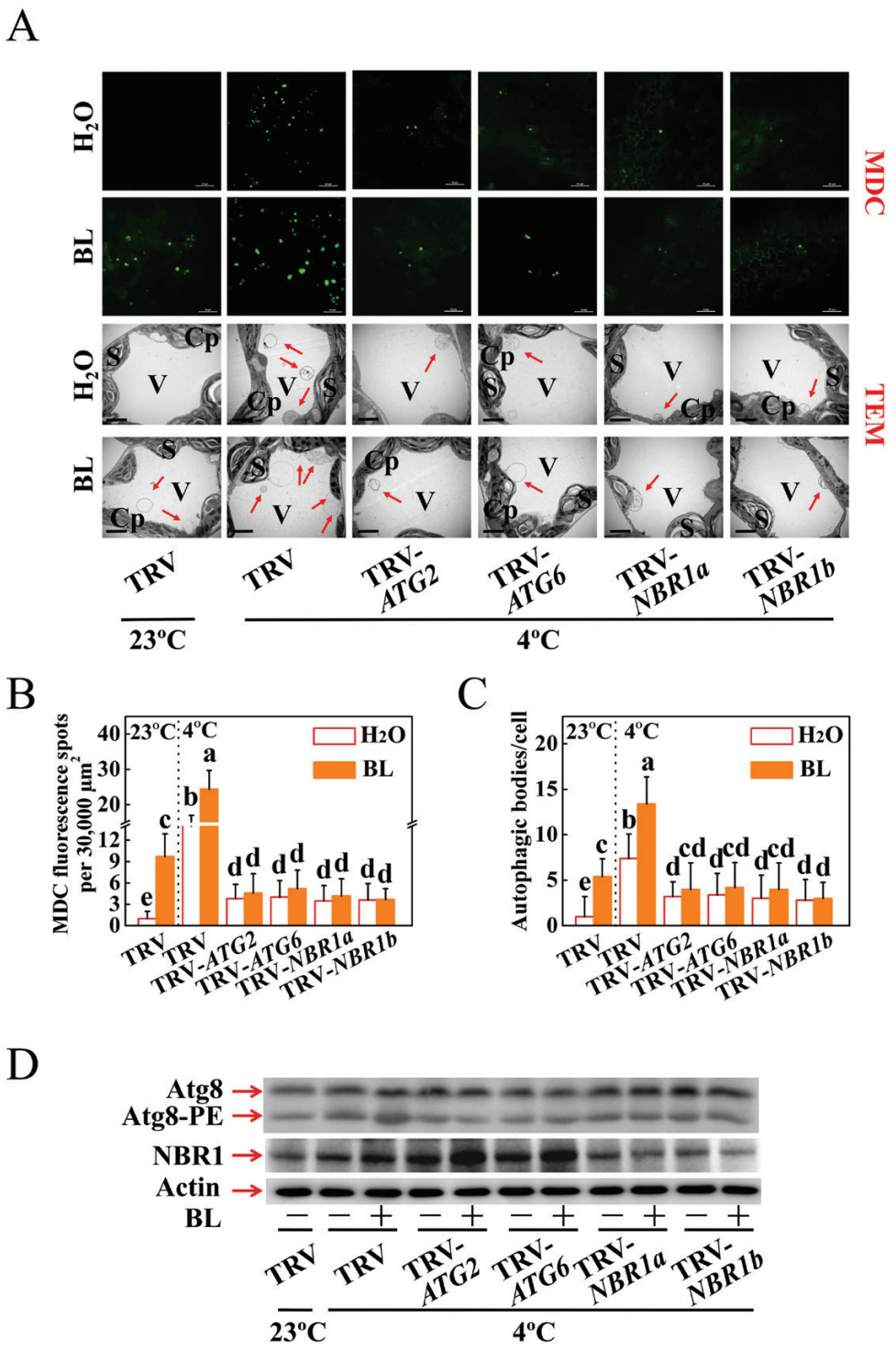

Fig. 7. *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* are important in cold- and BR-induced autophagy. (A) Monodansylcadaverine (MDC)-stained autophagosome and autophagic bodies (upper panels; bar=25 μm) and transmission electron microscopy (TEM) images of autophagic bodies (lower panels; bar=2 μm). MDC-stained spots are indicated as green signals. Autophagic bodies visualized by TEM are indicated by red arrows. BL, brassinolide; Cp, chloroplast; S, starch; V, vacuole. (B) Number of punctate MDC-stained spots per 30 000 μm^2 area. (C) Number of autophagic bodies in the vacuole. (D) Atg8 and NBR1 protein levels. The non-lipidated and lipidated forms of Atg8 are indicated by Atg8 and Atg8-PE, respectively. Actin was used as the loading control. Three independent experiments were performed with similar results. BL (200 nM) was applied 24 h before the plants were transferred to cold (4 °C). All leaf samples were collected 24 h after the imposition of cold stress. In (B) and (C) the reported values are means ±SD calculated by scoring at least 20 randomly chosen fields of view from at least three experiments. Different letters represent significant differences (P<0.05) according to Tukey's test.

et al., 2018; Wang *et al*., 2019*a*). Cold induced the increased accumulation of dephosphorylated BZR1 (active form), and activated BZR1 directly up-regulates the expression of *CBF* genes to modulate cold signaling (Li *et al*., 2017). In addition, several CBF-independent *COR* genes that are regulated by BZR1 also play crucial roles in promoting freezing tolerance

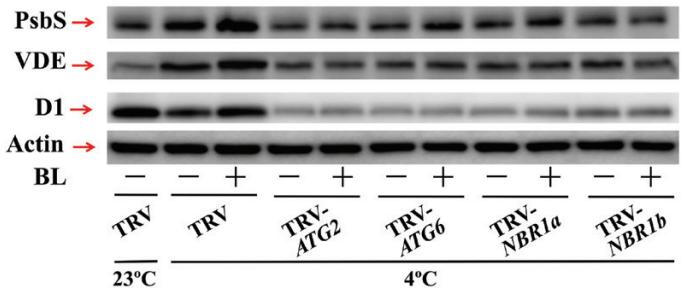

Fig. 8. Accumulation of the photosystem II subunit S (PsbS), violaxanthin deepoxidase (VDE), and D1 proteins in *ATG2*-, *ATG6*-, *NBR1a*-, or *NBR1b*-silenced plants. Brassinolide (200 nM) was applied 24 h before the plants were subjected to cold (4 °C), and leaf tissues were collected at 24 h after the imposition of cold stress. Actin was used as the loading control. Three independent experiments were performed with similar results.

in plants (Li *et al.*, 2017). Hence, BZR1 can regulate CBF-dependent and CBF-independent transcriptional regulation of cold stress responses in plants. Here, we found that BZR1 positively regulates cold tolerance by activating autophagy in tomato. The *bzr1* mutants exhibited decreased tolerance to cold, whereas, compared with the WT plants, the *BZR1*OE plants exhibited increased tolerance, as indicated by changes in Fv/Fm and REL (Fig. 3B, C). Importantly, the *bzr1* and *BZR1*OE plants had lower and higher autophagic activity, respectively, in conjunction with decreases and increases in the initiation of autophagy, which indicated that BZR1 is involved in regulating autophagy as a response to cold. Thus, BZR1 regulates plant cold tolerance through multiple pathways.

The transcripts of numerous *ATG* genes and autophagy receptors respond to changes in the conditions of the growth environment (Zhou *et al.*, 2014*b*; Chen *et al.*, 2015; Wang *et al.*, 2015*b*; Zhai *et al.*, 2016). However, the transcriptional regulation of autophagy and its receptors is largely unknown. Based on the Y1H screen, 225 TFs from 35 families were found to bind to the promoters of four *ATG8* genes (Wang *et al.*, 2019*b*), indicating that numerous TFs potentially regulate the autophagy genes. Recently, we reported that BZR1 can bind to the promoters of *ATG2* and *ATG6* and regulate the expression of these genes under nitrogen starvation (Wang *et al.*, 2019*a*). However, little is known about whether TFs participate in the transcriptional regulation of the autophagy receptor *NBR1* genes under various conditions. Here, we found that the transcripts of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* were not only BR responsive but also BZR1 dependent. These findings are in sharp contrast with findings regarding *ATG18a*, which was responsive to cold but not BR levels (Figs 2E, 4E; Supplementary Fig. S4E). BZR1 is present in two forms, pBZR1 and dBZR1. As currently understood, increased BR levels lead to the accumulation of dBZR1 in the nucleus. The increased accumulation of dBZR1 increases its binding to conserved E-boxes and/or BRRE elements in the promoters of target genes, leading to the transcriptional response to BR (He *et al.*, 2005; Kim and Wang, 2010; Sun *et al.*, 2010). Our results demonstrated that BZR1 can bind to the promoters of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* and regulate the expression of these genes under cold stress, suggesting that BZR1 acts as a TF for these genes in response to chilling. BZR1 has also been reported to be degraded by autophagy to balance growth and the stress response in Arabidopsis (Zhang *et al.*, 2016; Nolan *et al.*, 2017). Thus, BZR1 and autophagy can regulate each other. We previously reported that, in tomato, HsfA1a activated the expression of *ATG10* and *ATG18f* to induce autophagy, which enhanced drought tolerance by degrading ubiquitinated protein aggregates (Wang *et al.*, 2015*b*). Therefore, plants use different TFs to activate *ATG* genes and autophagy receptors in response to different developmental and environmental stimuli.

Notably, differences among *dwf*, WT, and *DWF*OE plants or among *bzr1*, WT, and *BZR1*OE plants in terms of autophagic activity and the abundance of NBR1 protein, as well as in the number of transcripts of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b*, were observed only after cold treatment or exogenous BL. Therefore, we measured the changes in BZR1 protein levels in response to cold stress and BL application. The accumulation of both pBZR1 and dBZR1 increased after cold treatment, and this increase was more pronounced in plants treated with exogenous BL (Fig. 3A). Thus, similar to the BL treatment, cold stress increased the stability of both pBZR1 and dBZR1 (Fig. 3A). Increased accumulation of dBZR1 would increase its binding to conserved E-boxes in the promoters of target genes, leading to the BR response. Taken together, these findings strongly confirm that cold-induced autophagy is attributable to the increased stability of dBZR1, which leads to increased transcriptional activation of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b*, and the subsequent formation of autophagosomes and autophagic bodies and increased abundance of NBR1 protein in tomato.

ATGs *and* NBR1 *are involved in the BR-induced cold response*

Studies have shown that the transcription of the *ATG* and *NBR1* genes is differentially up-regulated in response to different stressors (Zhou *et al.*, 2013; Chen *et al.*, 2015; Wang *et al.*, 2015*a*; Zhai *et al.*, 2016). For example, *ATG10* and *ATG18f* were induced by drought; *ATG18a* was induced by pathogen attack, salt, and drought stresses; and *NBR1*s were induced by heat stress (Liu *et al.*, 2009; Lai *et al.*, 2011; Zhou *et al.*, 2013, 2014*b*; Wang *et al.*, 2015*b*). Genetic experiments demonstrated that the induction of the *ATG* and *NBR1* genes is sufficient for defense against a variety of stresses, while the silencing and mutation of these genes resulted in poorer tolerance or a compromised response to the stresses (Thompson *et al.*, 2005; Chung *et al.*, 2010; Zhou *et al.*, 2014*b*; Minina *et al.*, 2018). For example, overexpression of *ATG18a* improved stress tolerance by altering antioxidant activity and anthocyanin accumulation via increased autophagy in apple (Sun *et al.*, 2018*a*, *b*), while RNAi-*AtATG18a* plants were highly sensitive to salinity and drought stresses (Liu *et al.*, 2009). The evidence here revealed that the transcript levels of two *ATG* genes and two *NBR1* genes (*ATG2*, *ATG6*, *NBR1a*, and *NBR1b*) were highly regulated by the levels of BR and BZR1 (Figs 2E, 4E; Supplementary Fig. S4E). Furthermore, transcript suppression by VIGS confirmed that suppression of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* differentially increased the plant sensitivity to

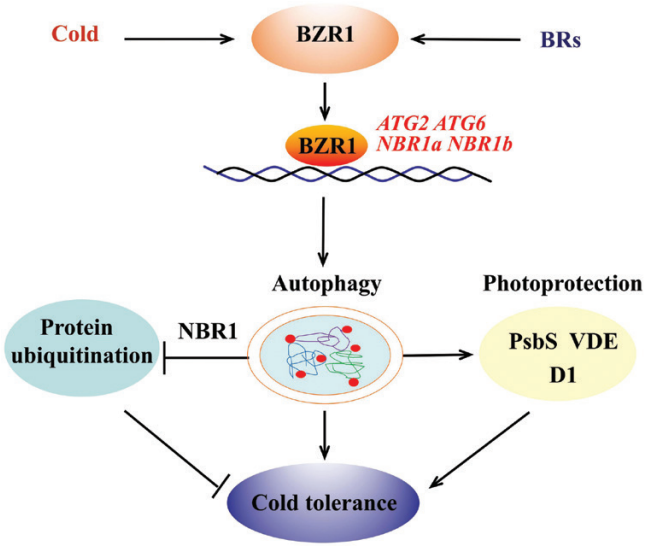

Fig. 9. A proposed model for the induction of cold tolerance by BZR1 through the activation of autophagy in tomato. Both cold and brassinosteroids (BRs) can induce the stability of BRASSINAZOLE RESISTANT 1 (BZR1), which activates the transcription of the autophagy genes *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* by directly binding to their promoters, subsequently enhancing autophagy. The increase in autophagy promotes photoprotection via greater accumulation of functional proteins (PsbS, VDE, and D1) and increases the degradation of stress-damaged insoluble ubiquitinated protein aggregates via the selective autophagy receptor NBR1. Arrows denote positive regulation; bar ends denote negative regulation.

cold and partially attenuated BR-induced cold tolerance and autophagic activity via the decreased formation of autophagic bodies (Fig. 6, 7). On the basis of these results, we argue that *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* are not only critical for the response to cold stress but also crucial for BR-induced cold tolerance. However, we cannot exclude a potential role for other *ATG* genes because we analyzed the transcripts of only 16 of the identified *ATG* genes in the tomato genome. Notably, we found that *ATG18a* was highly responsive to cold but not to BR or BZR1. Moreover, additional experiments are needed to determine whether the induction of this gene is important for the cold response (Figs 2E, 4E; Supplementary Fig. S4E).

BR-induced stress tolerance is frequently related to the alleviation of photoinhibition of PSII (Xia *et al.*, 2009). Our results also demonstrated that not only BRs but also *ATG*s are important for the alleviation of photoinhibition of PSII. In general, the degree of photoinhibition strongly depends on the capacity for photoprotection, which involves various processes, such as chloroplast avoidance movement, the dissipation of light energy as thermal energy, the reactive oxygen species scavenging machinery, cyclic electron flow, and the photorespiratory process (Takahashi and Badger, 2011). By examining the changes in the PsbS, VDE, and D1 proteins involved in photoprotection, we found that the accumulation of these proteins was induced by BRs and that silencing of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* compromised their accumulation (Fig. 8; Supplementary Fig. S9). These results suggest that the homeostasis of many functional proteins could be controlled by BR signaling and might occur in an autophagy-dependent manner in plants. Given the role of autophagy in protein aggregate degradation, recycling, and amino acid release (Barros *et al.*, 2017), an investigation of the maintenance of homeostasis of these functional proteins via BRs and autophagy in plants is warranted.

In summary, the comprehensive genetic and molecular analyses presented here provide new perspectives on the role of BRs in the regulation of autophagy in response to cold stress in plants. We demonstrated that cold and BRs induced the stability of BZR1, which up-regulates the expression of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* by directly binding to their promoters, thereby resulting in increased autophagy and increased levels of NBR1 protein. The increase in autophagy and the selective autophagy receptor NBR1 enhanced photoprotection via greater accumulation of functional proteins (PsbS, VDE, and D1) and promoted the degradation of stress-damaged ubiquitinated protein aggregates, thereby leading to increased tolerance to cold (Fig. 9). To our knowledge, this is the first report that explains the mechanism underlying the induction of autophagy and the receptor NBR1 by BRs under cold stress through a BZR1-dependent process.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Relative mRNA abundance of *ATG2*, *ATG6*, *NBR1a*, and *NBR1b* in *ATG2*-, *ATG6*-, *NBR1a*-, or *NBR1b*-silenced WT plants.

Fig. S2. Accumulation of D1 protein as influenced by BR levels in tomato plants.

Fig. S3. Cold tolerance, accumulation, and ubiquitination of insoluble proteins as influenced by exogenous BRs in tomato plants.

Fig. S4. Induction of autophagy by foliar applications of brassinolide in tomato plants.

Fig. S5. Values of relative electrolyte leakage and maximum quantum efficiency of photosystem II of *bzr1* mutants, WT, and *BZR1*OE plants under optimal conditions.

Fig. S6. BZR1 binds to the *NBR1* promoters *in vitro*.

Fig. S7. Functions of brassinolide in *ATG2*-, *ATG6*-, *NBR1a*-, or *NBR1b*-silenced plants under optimal conditions.

Fig. S8. *ATG2*, *ATG6*, *NBR1a*, or *NBR1b* are important in BR-induced autophagy under optimal conditions.

Fig. S9. Effects of brassinolide on the accumulation of PSII subunit S, violaxanthin deepoxidase, and D1 protein in *ATG2*-, *ATG6*-, *NBR1a*-, or *NBR1b*-silenced plants under optimal conditions.

Fig. S10. The loading control for ubiquitination of insoluble proteins.

Table S1. Primers used for *BZR1* overexpressing vector construction.

Table S2. Primers used for VIGS vectors construction.

Table S3. Primers used for yeast one-hybrid assays.

Table S4. Primers used for construction of *NBR1a* and *NBR1b* promoter mutant vectors.

Table S5. Primers used for ChIP–qPCR assays.

Table S6. Primers used for qRT–PCR assays.

Data availability

The original data of transcripts of the *ATG* and *NBR1* genes in Fig. 2E, Fig. 4E, and Supplementary Fig. S4E are available at Dryad Digital Repository. https://doi.org/10.5061/dryad.h70rxwdds

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Competing interests

The authors disclose no potential conflicts of interest.

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