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Protein farnesylation negatively regulates brassinosteroid signaling via reducing BES1 stability in *Arabidopsis thaliana*∞

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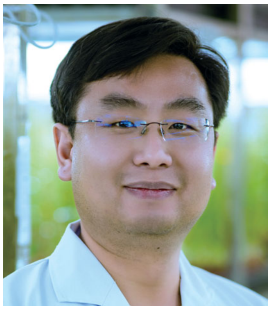

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ABSTRACT

Brassinosteroids (BRs) are a group of steroidal phytohormones, playing critical roles in almost all physiological aspects during the life span of a plant. In *Arabidopsis*, BRs are perceived at the cell surface, triggering a reversible phosphorylation-based signaling cascade that leads to the activation and nuclear accumulation of a family of transcription factors, represented by BES1 and BZR1. Protein farnesylation is a type of post-translational modification, functioning in many important cellular processes. Previous studies demonstrated a role of farnesylation in BR biosynthesis via regulating the endoplasmic reticulum localization of a key bassinolide (BL) biosynthetic enzyme BR6ox2. Whether such a process is also involved in BR signaling is not understood. Here, we demonstrate that protein farnesylation is involved in mediating BR signaling in *Arabidopsis*. A loss-of-function mutant of *ENHANCED RESPONSE TO ABA 1* (*ERA1*), encoding a β subunit of the protein farnesyl transferase holoenzyme, can alter the BL sensitivity of *bak1-4* from a reduced to a hypersensitive level. *era1* can partially rescue the BR defective phenotype of a heterozygous mutant of *bin2-1*, a gain-of-function mutant of *BIN2* which encodes a negative regulator in the BR signaling. Our genetic and biochemical analyses revealed that ERA1 plays a significant role in regulating the protein stability of BES1.

Keywords: *Arabidopsis*, BES1, brassinolide, brassinosteroids, protein farnesylation

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INTRODUCTION

Brassinosteroids (BRs) are an essential group of phytohormones that regulate multiple processes during vegetative and reproductive growth (Mitchell et al., 1970; Grove et al., 1979; Clouse and Sasse, 1998). Within the last few decades, significant efforts have been made to understand BR biosynthetic and signal transduction pathways in many plant species, especially in model plants *Arabidopsis* and rice. Up to date, BR signal transduction pathway is one of the best-characterized hormonal signaling pathways in plants. A series of important regulatory components in BR signaling pathway have been elucidated, from BR perception at the cell surface to gene transcription regulation in the nucleus.

The BR signal transduction pathway starts from the perception of BR by a plasma membrane localized receptor complex containing a major receptor BRASSINOSTEROID-INSENSITIVE 1 (BRI1) or its two paralogs, BRI1-LIKE1 (BRL1) or BRL3, and a major coreceptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) or its redundant SERK family members (Li and Chory, 1997; Li et al., 2002; Nam and Li, 2002; Caño-Delgado et al., 2004; Zhou et al., 2004; Gou et al., 2012). BR binding to its receptor and coreceptor triggers their conformational changes which facilitate their reciprocal phosphorylation (Li, 2010; He et al., 2013). The activated BRI1 can phosphorylate a negative regulator, BRI1 KINASE INHIBITOR 1 (BKI1), resulting in its dissociation from the BRI1 kinase domain (Wang and Chory, 2006). A series of phosphorylation-dephosphorylation processes can then be initiated. The kinase activity of a downstream negative regulator, BRASSINOSTEROID-INSENSITIVE 2 (BIN2), is inhibited by the BR signaling (Li et al., 2001; Li and Nam, 2002), allowing non-phosphorylated forms of a six-member group of downstream transcription factors, represented by BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1 EMS SUPPRESSOR 1 (BES1, also known as BZR2), to be accumulated in the nucleus (Wang et al., 2002; Yin et al., 2002; Zhao et al., 2002). Phosphorylated BZR1 and BES1 can also be activated via a dephosphorylation process by a group of PP2A protein phosphatases, which can positively regulate the BR signaling pathway (Tang et al., 2011). Non-phosphorylated BZR1 and BES1 are able to mediate the expression of thousands of downstream responsive genes (Sun et al., 2010; Yu et al., 2011).

BES1 and BZR1 are the core transcription factors in the BR signaling pathway. Analyses of BES1 and BZR1 target genes led the conclusion that BR signaling can be linked to many biological processes such as protein metabolism, protein trafficking, cell wall biosynthesis, cell signaling, cytoskeleton and chromatin assembling, and so on (Sun et al., 2010; Yu et al., 2011). One of the most important functions of BRs is the promotion of cell elongation (Mitchell et al., 1970), of which the upregulation of cell elongation-related genes by BES1 and BZR1 is an important mechanism (Kim and Wang, 2010). The transcriptional activities of BES1 and BZR1 are mainly regulated by BIN2 and PP2A-type of phosphatases through altering their phosphorylation status. Meanwhile, the stability of BES1 and BZR1 is another key regulatory node in monitoring BR signaling output. Recent studies suggested that the degradation of BES1 through an autophagy- or a proteasome-dependent pathway is mediated by a ubiquitin-binding receptor protein, DOMINANT SUPPRESSOR OF KAR 2 (DSK2), or E3 ubiquitin ligases including MORE AXILLARY GROWTH LOCUS 2 (MAX2) and SINA of *Arabidopsis thaliana* (SINATs), respectively (Wang et al., 2013; Nolan et al., 2017; Yang et al., 2017). However, additional mechanisms involved in the regulation of BES1stability are not well understood.

As a post-translational modification process involved in the addition of a 15-carbon farnesyl isoprenoid to a cysteine residue of the carboxyl terminus of a protein, protein farnesylation has been widely studied in animals. However, studies of farnesylation in regulating plant growth and development are very limited (Running, 2014). It was reported that CYP85A2 (also known as BR6ox2), a cytochrome P450 enzyme that catalyzes the last step in the brassinolide (BL) biosynthesis pathway by converting castasterone (CS) to BL, is farnesylated in *Arabidopsis* (Kim et al., 2005; Northey et al., 2016; Jamshed et al., 2017). The farnesylation of BR6ox2 is required for its endoplasmic reticulum (ER) localization and function. But some BR signaling-related phenotypes seen in a loss-of-function mutant of *ENHANCED RESPONSE TO ABA 1* (*ERA1*), a gene encoding for β subunit of the farnesylation holoenzyme, are not all caused by the loss-of-function of BR6ox2, suggesting farnesylation should have additional roles in regulating the BR signal transduction.

Here we report our discovery that ERA1 is important in modulating BR signaling output in *Arabidopsis* seedlings. Loss-of-function mutants of *ERA1*, *era1-10* and *era1-11*, are hypersensitive to the exogenously applied BL. Additional analyses indicated that ERA1 acts as a downstream component of BIN2 in the BR signaling pathway. Moreover, we discovered that the increased BR signaling output in *era1* seedlings is due to the increased accumulation of BES1. We found that farnesylation not only mediates the expression pattern of *BES1* but also promotes the degradation of BES1. These results indicated that protein farnesylation plays a critical role in modulating BR signal transduction. Our study provides new insights into our better understanding of protein farnesylation in regulating plant growth and development.

RESULTS

ERA1 is an important component in regulating BR signal transduction

As a coreceptor, BAK1 is essential in the perception of BRs and the activation of BRI1 (Gou et al., 2012). A loss-of-function mutant of *BAK1*, *bak1-4* shows reduced elongation and decreased sensitivity to exogenously applied BL (Li et al., 2002). Such a weak *bri1*-like phenotype suggests that *bak1-4* could be a sensitized mutant which can be used to screen for additional BR-related regulatory components by genetic approaches. We generated a large population of T-DNA insertion lines in *bak1-4* and screened for mutants displaying either altered responses to BL or increased defective phenotypes compared to that of the *bak1-4* single mutant. One of these mutants was identified which was initially named *95-5 bak1-4* according to the pool number from which it was originally isolated. Root growth inhibition analysis indicated that *95-5 bak1-4* recovered the lower sensitivity of *bak1-4* to BL compared to that of wild type (WT) (Figure 1). *95-5 bak1-4* was subsequently backcrossed with WT and the *bak1-4* mutation was segregated out. The resulting *95-5* single mutant displayed a number of defective phenotypes including delayed development, heart-shaped and dark green leaves, shortened petioles, delayed flowering time, disordered inflorescence, shortened siliques, and increased number of petals (Figure S1). In addition, *95-5* also showed a hypersensitivity to BL based on the root growth inhibition analysis. Due to DNA rearrangements, we failed to determine the T-DNA insertion site by a thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR) analysis (Liu et al., 1995). Using map-based cloning, we found that the T-DNA responsible for the observed phenotypes was inserted in

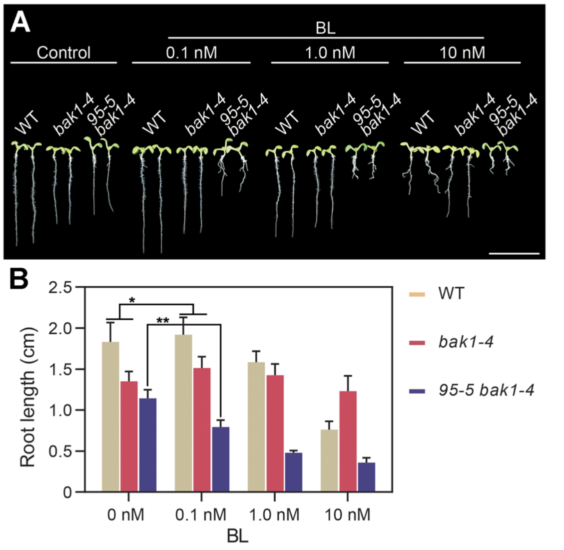

Figure 1. *95-5 bak1-4* double mutant is hypersensitive to bassinolide (BL)

(**A**) Seedlings of the indicated genotypes were grown on the media with various concentrations of BL (0, 0.1, 1.0, and 10 nmol/L) and incubated in a growth chamber with a 16-h light/8-h dark lighting condition at 22°C for 7 d. Scale bar represents 1 cm. (**B**) Measurements of the length of roots from the indicated genotypes. Data are means ± *SD* ($n \geq 50$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$; $^{*}P < 0.05$.

chromosome V, located between the molecular markers MSN9 and K21I16 (Figure S2A). Within this region, there are totally 33 open reading frames. *AT5G40280*, also known as *ERA1*, was proved to be knocked out via an real-time/reverse transcription (RT)-PCR analysis (Figure S2B). We therefore renamed the *95-5* mutant to *era1-10*. Overexpression of *ERA1* in *era1-10* can fully complement its developmental defects and BL-hypersensitive phenotype (Figure 2). To further confirm that loss of *AT5G40280* is responsible for the *era1-10* phenotypes, we also obtained an additional T-DNA insertion allele in *ERA1*, which was named *era1-11* (SALK_116584). RT-PCR analysis indicated that the expression of *ERA1* in both *era1-10* and *era1-11* was undetectable (Figure 3A). Similar to *era1-10*, *era1-11* single mutant was also hypersensitive to BL in a root growth inhibition assay (Figure 3B, C). Previous studies indicated that *era1* has multiple defective phenotypes during vegetative and reproductive stages (Bonetta et al., 2000; Yalovsky et al., 2000; Ziegelhoffer et al., 2000). The phenotypes observed in *era1-10* are consistent with those reported (Figure S1). These results confirmed that loss-of-function of *AT5G40280* is responsible for the BL-hypersensitive phenotypes of *era1-10* and *era1-11*. Because the phenotypes of *era1-10* and *era1-11* are virtually identical, our subsequent analyses were mainly carried out in *era1-11*.

To determine whether ERA1 regulates BR biosynthesis or signal transduction, we treated WT and *era1-11* with a BR biosynthetic inhibitor, brassinazole (BRZ) (Sekimata et al., 2001). We found that 100 nmol/L BRZ application could dramatically inhibit hypocotyl elongation of WT but could only slightly inhibit that of *era1-11*, suggesting an increased resistance of *era1-11* to BRZ (Figure S3). In addition, the expression levels of *DET2*, *CPD* and *DWF4*, three key BR biosynthesis genes, were all down-regulated in *era1-11* seedlings with or without the BL treatment (Figure 3D). These results suggested that the BR signaling is enhanced in *era1-11* and ERA1 likely plays a negative role in regulating the BR signal transduction.

ERA1 acts downstream of BIN2 in the BR signaling pathway

To further investigate the function of ERA1 in the BR signaling pathway, we compared the *era1* seedlings with WT and a BR receptor mutant *bri1-301* for their root growth responses to bikinin and LiCl, two chemical inhibitors of GSK3 kinases (Klein and Melton, 1996; Stambolic et al., 1996; Vert et al., 2005; Xu et al., 2008; De Rybel et al., 2009). Interestingly, unlike WT and *bri1-301* which showed similar root growth inhibition phenotypes, *era1-11* showed a significantly increased sensitivity to 6 µmol/L bikinin and 10 mmol/L LiCl, respectively (Figures 4A, B, S4). Since BIN2, one of the GSK3 kinases, is a target of bikinin and LiCl, we conclude that ERA1 should affect a step downstream of BIN2 in the BR signaling pathway.

To examine the genetic interrelationship between *ERA1* and *BIN2*, we crossed *era1-11* with *bin2-1*, a gain-of-function allele of *BIN2*, exhibiting characteristic BR defective phenotypes. Because a *bin2-1* homozygous line is male sterile and displays a severely dwarfed stature, we used a heterozygous version of *bin2-1*, *bin2-1* (+/–), for further analysis. We obtained an *era1-11 bin2-1* (+/–) double mutant line and compared its phenotypes with the single mutants of *era1-11* and *bin2-1* (+/–), respectively. Surprisingly, root growth inhibition assay showed that, different from *bin2-1* (+/–) which is almost completely insensitive to exogenous BL, *era1-11 bin2-1* (+/–) showed partially restored BR sensitivity. In other words, loss-of-function of *ERA1* in *bin2-1* (+/–) can partially relieve its resistance to BL (Figure 4C, D). In addition, the compacted rosettes observed in *bin2-1* (+/–) can be significantly suppressed in *era1-11 bin2-1* (+/–) (Figure 4E, F). These results further confirmed that ERA1 regulates a step downstream of BIN2.

ERA1 is involved in BES1 degradation

As key transcription factors in the BR signaling pathway, BES1 and its homologs act downstream of BIN2. It was reported that BIN2 interacts with and phosphorylates BES1 to prevent its nuclear accumulation and promote its degradation, consequently inhibiting BES1 target gene expression. We therefore performed immunoblotting analyses to test whether the protein level of BES1 was altered in *era1-11*. In comparison with WT, both non-phosphorylated and phosphorylated BES1 were significantly accumulated in the *era1-11* seedlings (Figure 5A, B). Consistently, 2-h treatment with 10 nmol/L BL resulted in a higher level of accumulation of non-phosphorylated BES1 in *era1-11* compared to that in

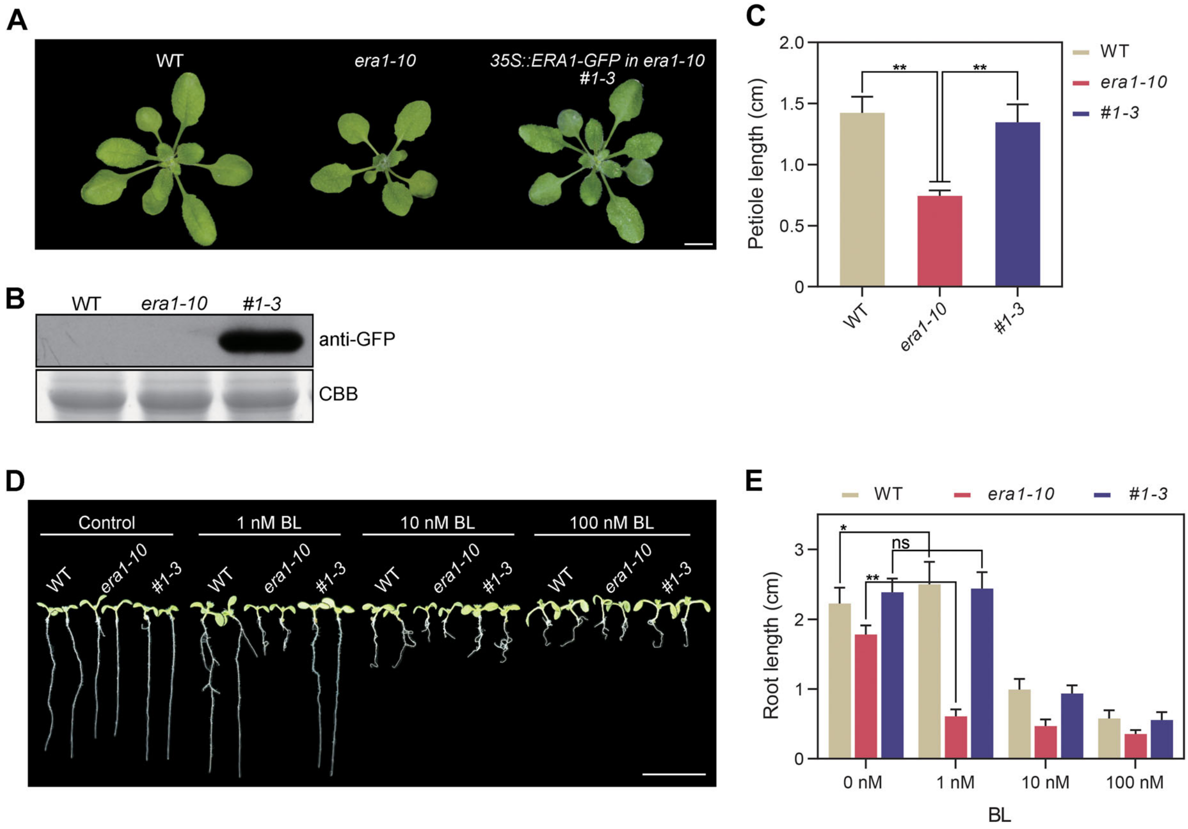

Figure 2. The brassinosteroid (BR)-hypersensitive phenotype of the *era1-10* (*95-5*) mutant can be fully complemented by the overexpression of *ENHANCED RESPONSE TO ABA 1*(*ERA1*)

(**A**) Overexpression of *ERA-GFP* (green fluorescent protein) can restore the rosette defective phenotype of *era1*. Scale bar represents 1 cm. (**B**) Immunoblotting analysis result to show the ERA1-GFP protein levels in 3-week-old wild type, *era1* and *ERA1-GFP* transgenic *Arabidopsis* plants. Coomassie brilliant blue (CBB) staining was used to show equal loadings. (**C**) Measurements of petiole length of the fifth euphylla in the indicated genotypes. Data are means ± *SD* ($n \geq 25$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$. (**D**) Overexpression of *ERA1* in *era1* fully complements its BR-hypersensitive phenotype. Seedlings of the indicated genotypes grown on the media with various concentrations of bassinolide (BL) (0, 1, 10, and 100 nmol/L) in a growth chamber with 16-h light/8-h dark lighting condition at 22°C for 9 d. Scale bar represents 1 cm. (**E**) Measurements of root length upon the treatments as shown in (**D**). Data are means ± *SD* ($n \geq 45$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$; $^{*}P < 0.05$; ns, non-significant.

WT, suggesting an elevated BR signal output in *era1-11*. To further verify the accumulation of BES1 in the *era1-11* seedlings, we generated an *era1-11 pBES1::BES1-YFP* homozygous transgenic line by crossing a single T-DNA inserted homozygous transgenic *pBES1::BES1-YFP* line with *era1-11*. Confocal microscopic analysis showed that BES1-YFP (yellow fluorescent protein) signals were significantly accumulated in the nuclei of the *era1-11* root cells compared to those in WT (Figure 5C, D). These results indeed demonstrated that *era1* mutation leads to the accumulation of BES1 in the nucleus.

To investigate the causes of the BES1 accumulation in the *era1* mutant, we compared the *BES1* transcription levels and BES1 protein degradation speed in *era1-11* and WT. We first compared the mRNA abundance of *BES1* in 7-d-old seedlings of WT and *era1-11* at different time points by quantitative RT-PCR analyses. The seedlings were grown in a growth chamber with 16 h light and 8 h dark (lights were on from 6:00 to 22:00 hours). The expression level of *BES1* is increased but at slightly different rates in WT and *era1-11* after the lights were turned on. At the beginning of the lighting, *BES1* level in *era1-11* is slightly higher than that of WT, but the situation is reversed at the end of the lighting (Figure S5). This observation indicated that ERA1 modulates the expression pattern of *BES1* but does not significantly change its expression level in general. Therefore, the protein stability of BES1 became our main focus. We tested the degradation of recombinant maltose binding protein (MBP)-BES1 using a cell-free degradation system, in which MBP-BES1 protein was incubated with protein extracts from WT and *era1-11* mutant plants, respectively. The degradation rate of MBP-BES1 is reduced in the extracts of the *era1-11* mutant in comparison with that of WT. Supplementation of MG132, an effective proteasome inhibitor, could significantly reduce the BES1 degradation in WT extracts, indicating that the degradation of BES1 is largely proteasome-dependent (Figure 6A, B). To elucidate the mechanism causing the reduced degradation rate of BES1 in *era1-11*, we analyzed ubiquitination level of BES1 in *era1-11* and WT using an anti-ubiquitin antibody. Immunoblotting analysis showed that the

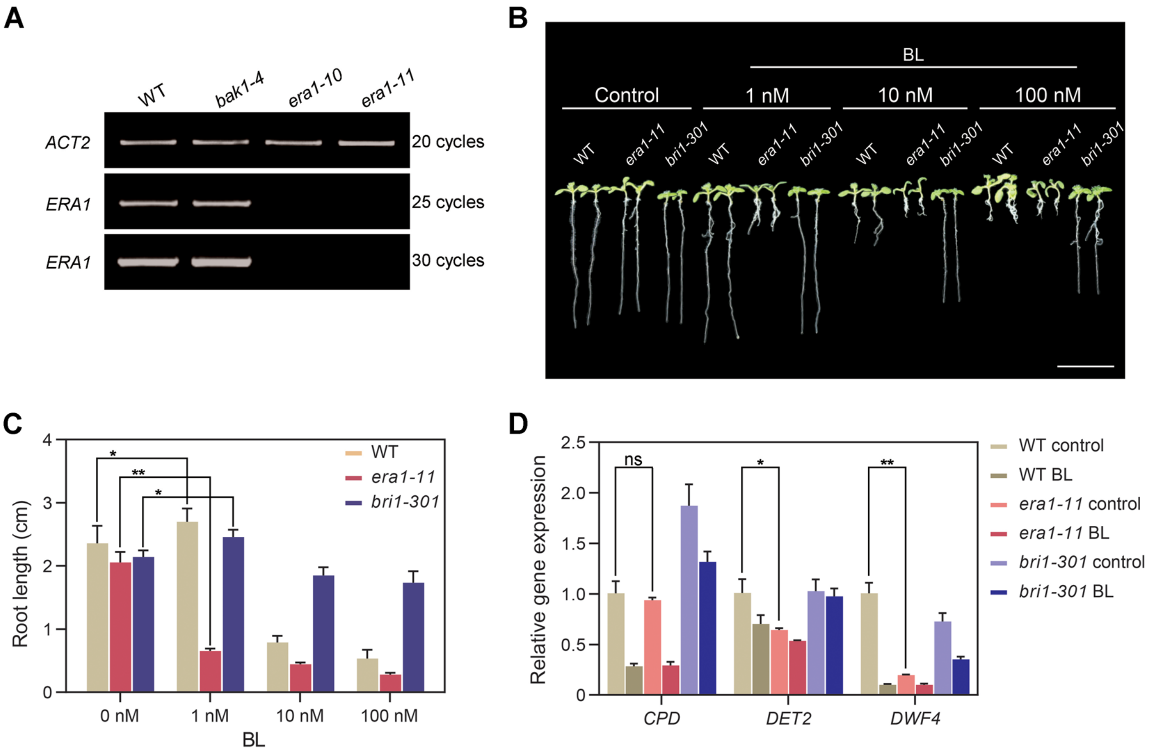

Figure 3. *era1* mutant is hypersensitive to exogenous bassinolide (BL) treatment

(**A**) Real-time/reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis to confirm the genotypes of *era1* mutants in wild type (WT) and in *bak1-4* backgrounds. (**B**) *era1* is more sensitive to BL than WT in a root growth inhibition analysis. Seedlings of the indicated genotypes were grown on the medium with various concentrations of BL (0, 1, 10, 100 nmol/L). The pictures were taken after the seedlings were incubated in a growth chamber with 16-h light/8-h dark photoperiod and 22°C for 7 d. Scale bar represents 1 cm. (**C**) Measurements of root length for seedlings treated with different concentrations of BL, as shown in (**B**). Data are means ± *SD* ($n \geq 50$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$; $^{*}P < 0.05$. (**D**) RT-qPCR analysis results to show the expression levels of a number of brassinosteroids (BR) biosynthetic genes (*CPD*, *DET2*, *DWF4*) in the indicated genotypes. The data are shown as means of three biological repeats ± *SD*. Significance was determined by Student's *t*-test. $^{**}P < 0.01$; $^{*}P < 0.05$; ns, non-significant.

ubiquitination level of BES1 is strongly reduced in *era1-11* (Figure 6C). Taken together, our results indicated that protein farnesylation affects the protein level of BES1 mainly via promoting its ubiquitin-dependent degradation in *Arabidopsis*.

Modulation of BR signal output by ERA1 is BES1-dependent

To further explore the relationships between ERA1 and BES1, we generated a homozygous line of the *era1-11 bes1-1* double mutant by crossing the *era1-11* with *bes1-1*, a partial *bes1* loss-of-function allele in which the *BES1-Long* transcript is completely eliminated whereas the *BES1-Short* transcript is still present (Jiang et al., 2015; Chen et al., 2019). Interestingly, no difference was found in root growth between *bes1-1* and WT, possibly due to gene redundancy. However, the root growth of the *era1-11 bes1-1* double mutant was significantly inhibited compared to that of the *era1-11* single mutant (Figure 7A, B), suggesting that the BES1 level in *era1-11* plays a role in root growth. More importantly, the BR hypersensitivity in the roots of *era1-11* is significantly suppressed by the *bes1-1* mutation (Figure 7A, B). Furthermore, *bes1-1* in *era1-11* can suppress the increased angle between lateral inflorescence branch and main inflorescence axis observed in the *era1-11* single mutant (Figure 7C, D). Previous studies indicated that increased BR signaling leads to increased angle between inflorescence branch and main inflorescence axis in *Arabidopsis* (Gendron et al., 2012). These results indicated that the effect of protein farnesylation on BR signal transduction depends on BES1.

ERA1 affects BR signaling through a BR6ox2-independent pathway in *Arabidopsis* seedlings

It was previously reported that farnesylation of BR6ox2 is essential for its function in BR biosynthesis. It is therefore reasonable to ask whether the BR-hypersensitive phenotype of *era1* identified in this study results from BR6ox2 which is not farnesylated in the mutant. Unlike the significant inhibition of root elongation in *era1* by 0.1 nmol/L BL, the root elongation of *br6ox2* was inhibited dramatically only when the concentration of BL reached to 10 nmol/L (Figure 8A, B). We also checked the expression levels of several BR biosynthetic genes, including *CPD*, *DET2*, and *DWF4*. In contrast to the transcriptional downregulation of *CPD*, *DET2* and *DWF4* in *era1*, a significant upregulation of these three genes was detected in *br6ox2*

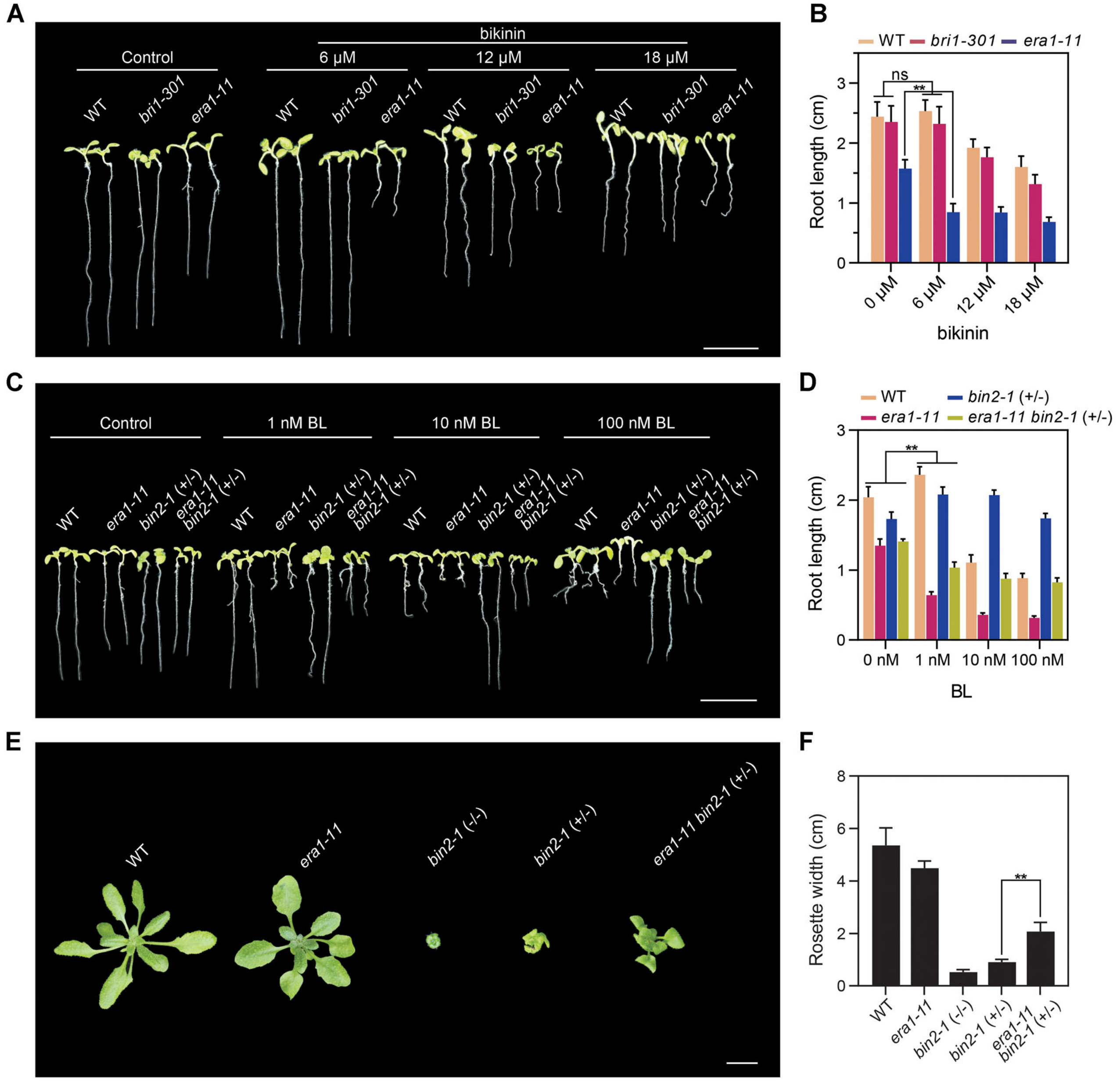

Figure 4. ENHANCED RESPONSE TO ABA 1 (ERA1) acts as a regulatory component downstream of BIN2 in the brassinosteroid (BR) signaling pathway

(**A**) *era1* shows increased sensitivity to bikinin than wild type (WT). Seedlings of the indicated genotypes were grown on the media with various concentrations of bikinin (0, 6, 12, and 18 μmol/L) and incubated in a growth chamber with a 16-h light/8-h dark lighting condition at 22°C for 7 d. Scale bar represents 1 cm. (**B**) Measurements of root length for the indicated genotypes. Data are means ± *SD* ($n \geq 40$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$; ns, non-significant. (**C**) Loss-of-function *ERA1* rescues the hyposensitive phenotype of *bin2-1* (+/–) to bassinolide (**BL**). Seedlings of the indicated genotypes were grown on the media with various concentrations of BL (0, 1, 10, 100 nmol/L) and incubated in a growth chamber with a 16-h light/8-h dark lighting condition at 22°C for 7 d. Scale bar represents 1 cm. (**D**) Measurements of root length for the indicated genotypes. Data are means ± *SD* ($n \geq 45$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$. (**E**) Loss-of-function *ERA1* partially rescues the compact rosette phenotype of *bin2-1* (+/–). Rosette phenotypes of 3-week-old wild type (WT), *era1-11*, *bin2-1* (+/–), and *era1-11 bin2-1* (+/–) double mutant. Scale bar represents 1 cm. (**F**) Measurements of rosette width for the indicated genotypes. Data are means ± *SD* ($n \geq 15$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$.

seedlings (Figure 8E). Furthermore, immunoblotting analysis revealed that non-phosphorylated BES1 was significantly less in *br6ox2* seedlings than in WT (Figure 8C, D). In contrast, both the non-phosphorylated and phosphorylated BES1 were clearly accumulated in *era1-11* seedlings (Figure 8C, D). Taken together, these results indicated that the changes in BR signaling output caused by the loss-of-function of *BR6ox2* are different from those caused by the loss-of-function of *ERA1*, suggesting that BR hypersensitivity of *era1-11* is independent of BR6ox2 in *Arabidopsis* seedlings.

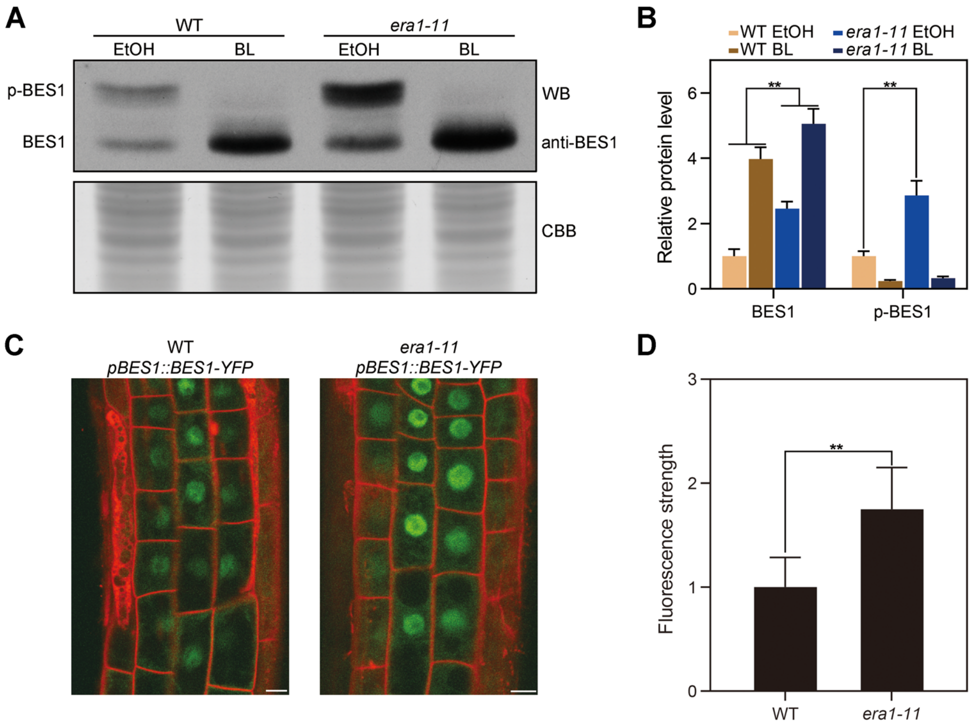

Figure 5. More BRI1 EMS SUPPRESSOR 1 (BES1) is accumulated in *era1* than in wild type (WT)

(**A**) Immunoblotting analysis to show the BES1 protein levels in 7-d-old seedlings of the indicated genetic backgrounds using an anti-BES1 antibody. Seedlings of the indicated genotypes treated with or without 10 nmol/L bassinolide (**BL**) for 2 h were used for total protein extraction. Coomassie brilliant blue (CBB) staining was used to show equal loadings. (**B**) Measurements of relative gray value of the bands in the indicated genotypes and conditions. ImageJ was used in gray value analyses. The data are shown as means of three biological repeats ± *SD*. Significance was determined by Student's *t*-test. $^{**}P < 0.01$. (**C**) BES1 accumulates in the *era1* seedling roots. Four-d-old homozygous transgenic plants carrying a single T-DNA insertion of *pBES1::BES1-YFP* in WT or *era1* background (generated by crossing the *pBES1::BES1-YFP* line with *era1*) were used for confocal microscopic analyses. Root cell membranes were stained with propidium iodide (PI). Scale bars represent 10 μm. (**D**) Measurements of relative fluorescence intensity in the nuclei of the indicated genotypes. ImageJ was used for fluorescence intensity analyses. Data are means ± *SD* ($n \geq 25$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$.

DISCUSSION

Over the past billion years, organisms have developed a range of protein post-translational modifications which have dramatically expanded the functions of proteins. Such evolution has made organisms better adapted to the changing environment, which was also critical to the evolution of higher levels of life, even our human beings (Ambrogelly et al., 2007). One example of such post-translational modifications is protein farnesylation, a type of protein prenylation. This modification makes a significant contribution to the functions of a certain group of proteins that are involved in many biological regulations in eukaryotic cells (Zhang and Casey, 1996; Gelb et al., 2006). Accumulated evidence suggests that disruption of farnesylation is highly associated with various human diseases, especially cancers. More and more researchers therefore are making great effects to elucidate the biochemical mechanisms of farnesylation in regulating protein functions (Berndt et al., 2011). Compared to medical studies, less attention has been paid to protein farnesylation in plants until recently. Significant progress has been made to elucidate how farnesylation is involved in regulating the functions of a number of important proteins in model plant *Arabidopsis*. The biggest advantage of studying the importance of farnesylation in *Arabidopsis* is that we can take advantage of a loss-of-function of *ERA1* mutant, which is totally viable. Many biological roles of farnesylation were revealed via the genetic and biochemical analyses of *era1*. However, the biggest challenge of studying farnesylation is still the identification of target proteins of such modification.

Given the hydrophobicity of the lipids involved in protein farnesylation, it is not surprising that this post-translational modification provides proteins with a hydrophobic C terminus, the consequence of which is to greatly increase the capacity of proteins to interact with cellular membranes. A well-known example is human RAS proteins, including H-RAS, N-RAS and K-RAS, the farnesylation of which is essential to their membrane association and functions (Casey et al., 1989; Hancock et al., 1989; Schafer et al., 1989). The farnesylation of RAS proteins is correlated with many kinds of human cancers. Such modification can therefore be used as a potential anticancer target (Malumbres and Barbacid, 2003). In addition, farnesylation also can affect other characterizations of proteins, including the affinity of protein-protein interactions and protein stability (Wang and Casey,

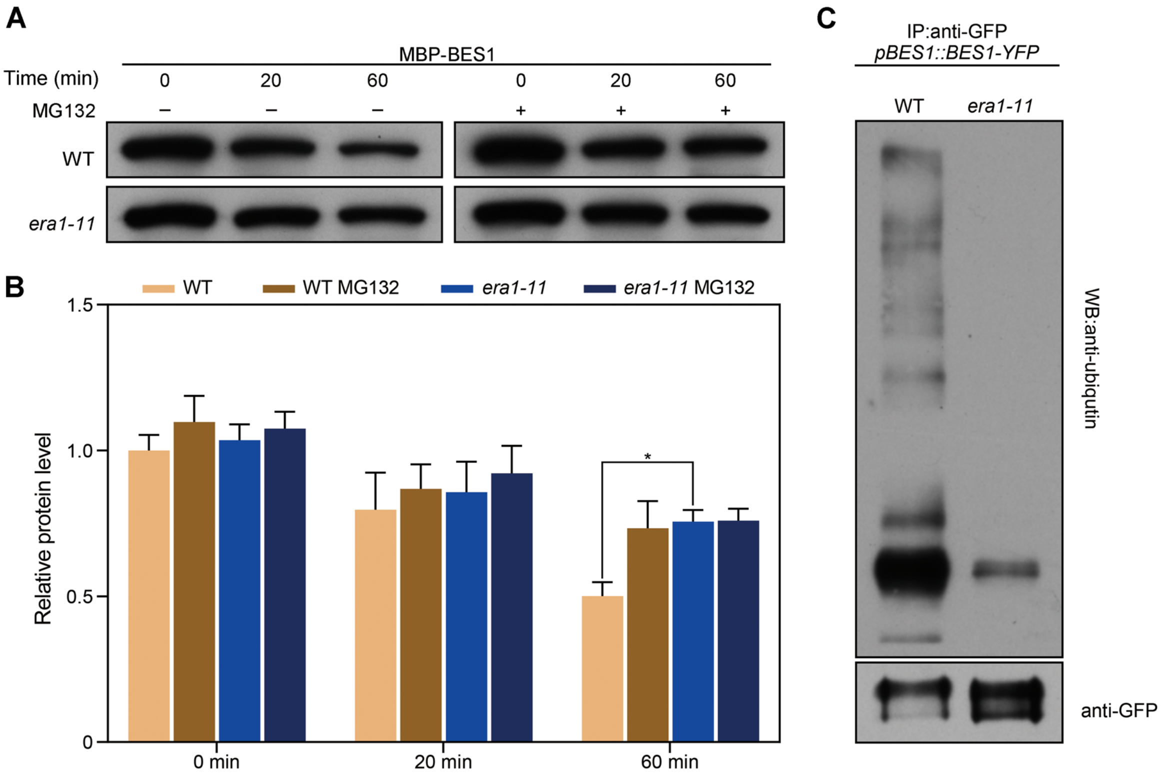

Figure 6. ENHANCED RESPONSE TO ABA 1 (ERA1) promotes the ubiquitin-dependent degradation of BES1
(**A**) Cell-free degradation analysis showed delayed degradation of the recombinant -BRI1 EMS SUPPRESSOR 1 (maltose binding protein [MBP]-BES1) in the *era1* extract compared to that in wild type (WT) extract. The protein level of MBP-BES1 was determined by an immunoblotting analysis using an anti-MBP antibody. (**B**) Measurements of relative gray value of the bands for the indicated genotypes and treatments. ImageJ was used for gray value analyses. The data are shown as means of three biological repeats ± *SD*. Significance was determined by Student's *t*-test. $^*P < 0.05$. (**C**) *In vivo* ubiquitination analysis to show ubiquitination levels of BES1 in WT and in *era1*. The ubiquitination levels of BES1 were determined by an immunoblotting analysis using an anti-ubiquitin antibody.

2016). For example, the stability of human RHO guanosine triphosphatases (GTPases) and a transcriptional activator YAP/TAZ, a yeast SNARE Protein Ykt6, and an *Arabidopsis* ROP2 GTPase is regulated by protein prenylation either directly or indirectly (Pylypenko et al., 2008; Stubbs and Von Zee, 2012; Chai et al., 2016, 2020).

In this report, we provide strong evidence to show the stability of BES1 is regulated by protein farnesylation. We identified a mutant which can alter the BL sensitivity of *bak1-4*, from reduced sensitivity to hypersensitivity relative to WT. Map-based cloning indicated that such a phenotype is associated with the loss-of-function of *ERA1*, a gene essential for farnesylation. Our genetic and biochemical data further proved that the accumulation of BES1 primarily results from the loss-of-function of *ERA1*. The accumulated BES1 is the cause of the BL hypersensitivity of *era1*. In *era1*, the ubiquitination-based BES1 degradation has been greatly suppressed. These observations indicate that ERA1 and protein farnesylation determine BES1 stability (Figure 9). But due to the lack of a conserved CaaX domain at its C terminus, BES1 can unlikely be modified directly by a farnesyl group. It is the ubiquitination of BES1 regulated by farnesylation that consequently affects its stability. The role of farnesylation in regulating the ubiquitination of a plant protein to affect its stability was not reported previously.

Earlier reports showed that farnesylation of BR6ox2 is essential for its normal subcellular localization and function (Northey et al., 2016; Jamshed et al., 2017). In addition, the levels of a number of BRs, including 6-deoxocastasterone (6-deoxo CS), castasterone (CS), and BL are altered in *br6ox2-2* and *era1-2*. BL levels are significantly reduced in the seedlings and inflorescences of *br6ox2-2* and *era1-2*, whereas 6-deoxoCS is accumulated tremendously in the seedlings or inflorescences of *br6ox2-2* compared to that in wild type. However, inconsistently, the accumulation of 6-deoxoCS was not observed in the seedlings or inflorescences of *era1-2*. The level of 6-deoxoCS is actually reduced in the inflorescences of *era1-2* (Northey et al., 2016). These results cannot be explained solely by the loss-of-function of BR6ox2 due to its lack of farnesylation in *era1-2*, suggesting that protein farnesylation may affect BR biosynthesis via an additional manner other than the farnesylation of BR6ox2. Our data demonstrated that protein farnesylation negatively regulates BR signaling output by promoting the ubiquitination-dependent degradation of the key transcription factor BES1. It is known that almost all steps of the BR biosynthesis can

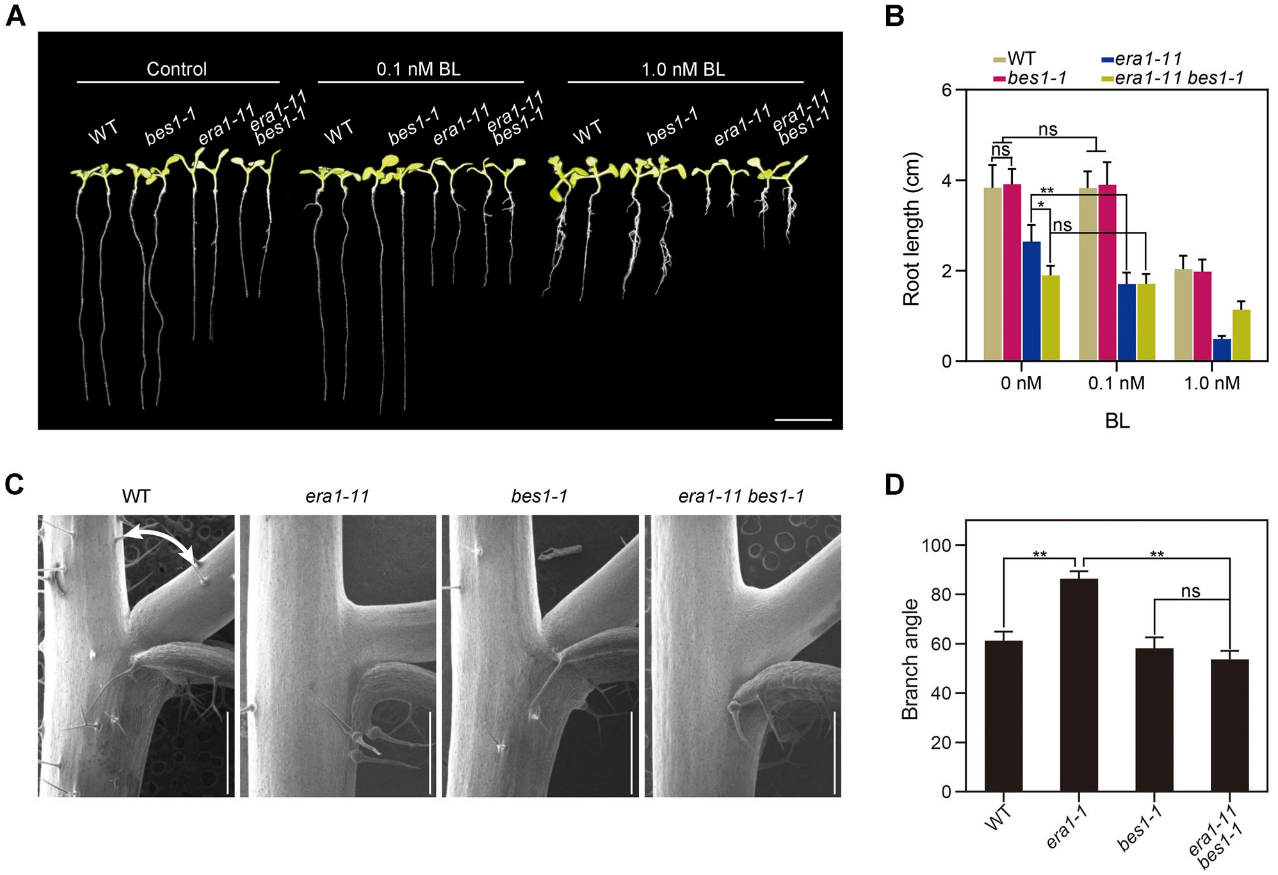

Figure 7. Hypersensitivity of *era1* to brassinosteroids (BR) requires BRI1 EMS SUPPRESSOR 1 (BES1)

(**A**) *bes1-1* partially rescues hypersensitive phenotype of *era1* to BR and significantly reduces root elongation of *era1*. Seedlings of the indicated genotypes were grown on the media with various concentrations of bassinolide (**B**L) (0, 0.1, and 1.0 nmol/L) and incubated in a growth chamber with a 16-h light/8-h dark lighting conditions at 22°C for 10 d. Scale bar represents 1 cm. (**B**) Measurements of the root length of the indicated genotypes. Data shown are means ± *SD* ($n \geq 50$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$; $^{*}P < 0.05$; ns, non-significant. (**C**) *bes1-1* rescues the increased lateral branch angle shown in *era1*. Scanning electron microscopic analyses was used to show the angle of the first lateral branch with the primary inflorescence axis of 7-week-old wild type (WT), *era1-11*, *bes1-1* and *era1-11 bes1-1*. Scale bars represent 1 mm. (**D**) Measurements of the angles between the first lateral branch and the primary inflorescence axis of the indicated genotypes. Data are means ± *SD* ($n = 15$). Significance was determined by Student's *t*-test. $^{**}P < 0.01$; ns, non-significant.

be inhibited by the end products, including CS and BL, via a negative feedback loop, which is important to maintain the homeostasis of the BR signal transduction in plants (He et al., 2005; Tanaka et al., 2005; Sun et al., 2010; Yu et al., 2011; Wei and Li, 2020). Our results indicated that BES1 and p-BES1 are significantly accumulated in the loss-of-function *ERA1* seedlings (Figure 5). Our results also showed that many BR biosynthetic genes in *era1* mutant were dramatically down-regulated (Figure 3D). This negative feedback loop may explain why there is a decreased 6-deoxoCS in *era1* mutant.

Early reports indicated that the non-phosphorylated BES1 is significantly increased in *bes1-D*, a gain-of-function mutant of *BES1*. Consequently, the petioles of *bes1-D* are significantly elongated compared to those of WT (Yin et al., 2002). In *era1*, non-phosphorylated BES1 is also accumulated. But the petioles of *era1* are shorter than those of WT. This inconsistency possibly results from the fact that farnesylation can affect many different processes in addition to its roles in the BR signaling pathway (Zhang and Casey, 1996; Gelb et al., 2006). In other words, the observed phenotype of *era1* is a cumulative effect of many abolished farnesylation related processes. Alternatively, it could also be caused by the tissue-specific expression of an undefined farnesylation substrate which is directly involved in the degradation of BES1.

We have not identified a farnesylated substrate which is responsible for the ubiquitination of BES1. Previous reports suggested that the degradation of BES1 is through a proteasome and an autophagy pathway which are mediated by SINAT E3 ligases and ubiquitin receptor protein DSK2, respectively (Nolan et al., 2017; Yang et al., 2017). However, the aforementioned proteins related to the ubiquitination of BES1 do not contain the conserved CaaX motifs at their C termini that are required for protein farnesylation, suggesting that there should be other proteins involved in the ubiquitination of BES1. Using a bioinformatic approach, we searched for the potential proteins with a CaaX motif that

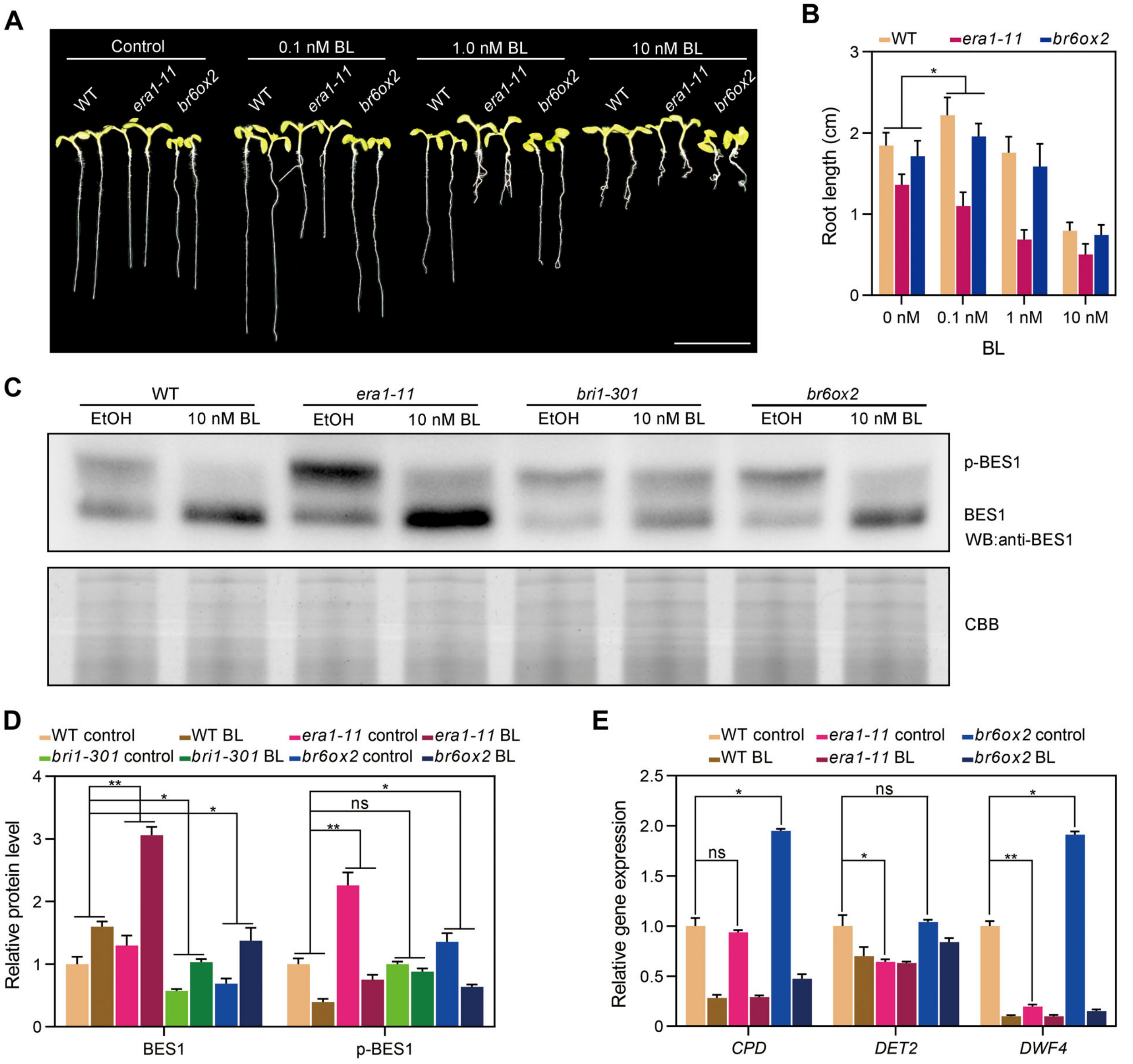

Figure 8. ENHANCED RESPONSE TO ABA 1 (ERA1) regulates brassinosteroids (BR) signaling is independent of BR6ox2

(**A**) *era1* and *br6ox2* showed different responses to exogenous applied bassinolide (BL). Seedlings of the indicated genotypes were grown on the media with various concentrations of BL (0, 0.1, 1, and 10 nmol/L) and incubated in a growth chamber with a 16-h light/8-h dark lighting condition at 22°C for 7 d. Scale bar represents 1 cm. (**B**) Measurements of the root length of the indicated genotypes. Data are means ± *SD* ($n \geq 50$). Significance was determined by Student's *t*-test. $^*P < 0.05$. (**C**) The BRI1 EMS SUPPRESSOR 1 (BES1) protein levels in 7-d-old seedlings of the indicated genotypes treated with or without 10 nmol/L BL for 2 h. Coomassie brilliant blue (CBB) staining was used to show equal loadings. (**D**) Measurements of relative gray values of bands in the indicated genotypes and experimental conditions. ImageJ was used for gray value analyses. The data are shown as means of three biological repeats ± *SD*. Significance was determined by Student's *t*-test. $^{**}P < 0.01$; $^*P < 0.05$; ns, non-significant. (**E**) The expression levels of three brassinosteroid biosynthetic genes in *era1* and *br6ox2* are different. Quantitative real-time/reverse transcription quantitative polymerase chain reaction (qRT-PCR) analysis was used to show the expression levels of the three BR biosynthetic genes (*CPD*, *DET2*, *DWF4*) in the indicated genotypes. The data are shown as means of three biological repeats ± *SD*. Significance was determined by Student's *t*-test. $^{**}P < 0.01$; $^*P < 0.05$; ns, non-significant.

may relate to protein ubiquitination and stability in *Arabidopsis*. UBIQUITIN E2 VARIANT 1A and 1B (UEV1A and UEV1B), MEMBRANE-ANCHORED UBIQUITIN-FOLD PROTEINs PRECURSOR (including MUB1, MUB4, MUB5, MUB6), and four F-box proteins (encoded by *AT5G36820*, *AT5G36730*, *AT4G15475*, *AT2G03580*) were revealed to possess those characteristics, which will be our main research targets in future investigations.

MATERIALS AND METHODS

Plant materials and growth conditions

All the *Arabidopsis thaliana* plants used in this study were in Col-0 background. The plants were grown in a greenhouse with 24 h light at 22°C for general growth and seed harvesting. For seedlings grown on the medium in Petri dishes, the sterilized seeds were grown on 1/2 Murashige and Skoog

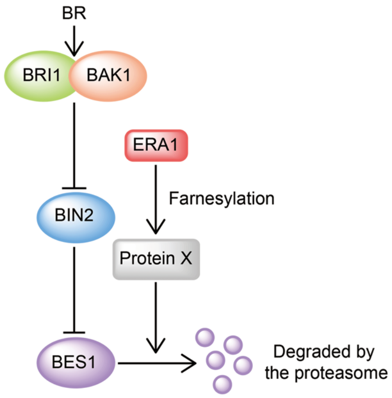

Figure 9. A hypothetical model of ENHANCED RESPONSE TO ABA 1 (ERA1) in mediating brassinosteroids (BR) signal transduction through controlling BRI1 EMS SUPPRESSOR 1 (BES1) stability

BR activates BRASSINOSTEROID-INSENSITIVE 1 (BRI1) BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) receptor-coreceptor complex, triggering sequential phosphorylation of BRI1 and BAK1, which eventually reduces the kinase activity of BRASSINOSTEROID-INSENSITIVE 2 (BIN2). The phosphorylation level of BES1, as the substrate of BIN2, is subsequently reduced, causing non-phosphorylated BES1 to be accumulated in the nucleus to regulate downstream response gene expression. There is an unrevealed protein X which is the substrate of ERA1. Farnesylated protein X can promote the degradation of BES1.

(MS) medium containing 1% sucrose and supplemented with 1% agar. Plates were placed in a 22°C growth chamber with a 16-h light/8-h dark cycle.

T-DNA insertional mutagenesis and BR-related mutant screening

The *bak1-4* plants were transformed with a *pBIB-BASTA* plasmid using a floral dipping method published previously (Clough and Bent, 1998). T2 seeds were harvested together as a pool made from 500 T1 transgenic plants (177 pools in total). About 4,000 T2 seeds of each pool (average about eight T2 seeds from each T1 transgenic line) were grown on the medium containing 100 nmol/L 24-epiBL in a 22°C growth chamber with a 16-h light/8-h dark lighting cycle. Seedlings with altered root growth sensitivity in the presence of 100 nmol/L 24-epiBL compared to *bak1-4* were screened. *95-5 bak1-4* double mutant was identified from #95 pool. The T-DNA position was determined by a map-based cloning strategy.

Generation of constructs and transgenic plants

For complementation, the full-length *ERA1* coding sequence with a start codon was cloned into a Gateway™ Entry vector which was then subcloned into a binary destination vector *pBIB-HYG-35S-GWR-GFP* and *pBIB-HYG-35S-GWR-HA*. For expression analysis, a piece of DNA sequence containing 1.5 kb promoter sequence plus the genomic sequence of *BES1* was cloned into a Gateway™ Entry vector which was then subcloned into a *pBIB-BASTA-GWR-YFP* destination vector. The resulting constructs were transformed into *Arabidopsis* plants by floral dip (Clough and Bent, 1998). *ERA1-GFP* and *ERA1-HA* constructs were transformed to *95-5* mutant. *pBES1::BES1-YFP* construct was transformed to WT. *pBES1::BES1-YFP* in *era1* transgenic lines were generated by crossing *pBES1::BES1-YFP* transgenic lines with an *era1-11* mutant.

Photographing and microscopy

For phenotypic observation, all plants were photographed with a Canon EOS 70D Camera. For lateral branch angle analyses, stem between first node and second node was removed from the plant at the end of its flowering period and carefully set on the sample preparation platform and quickly frozen in liquid nitrogen. The samples were transferred into the chamber of a Hitachi S-3400N scanning electron microscope for image analyses. For expression analyses of BES1-YFP in different genetic backgrounds, the roots of 4-d-old seedlings were stained in 10 μg/mL propyl iodide (PI) aqueous solution for 10 min, and then the roots were mounted in water for observation of YFP and PI signals under a Leica TCS SP8 laser scanning confocal microscope.

RT-PCR and quantitative RT-PCR (qRT-PCR)

RT-PCR was used to determine the expression levels of target genes in mutants. qRT-PCR was carried out to evaluate the expression levels of genes that function in BR biosynthesis. Total RNAs were extracted from rosette leaves (for RT-PCR) and 7-d seedlings treated with or without 10 nmol/L BL (Sigma Aldrich) for 2 h (for qRT-PCR) using an RNAprep pure Plant Kit (TIANGEN DP432). Complementary DNA (cDNA) was generated with a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara). For RT-PCR, genes were amplified from 100 ng total RNA reverse transcripts. For qRT-PCR, genes were amplified from 80 ng total RNA reverse transcripts. All primers used are listed in Supplementary Table S1. SYBR Premix Ex Taq II (Takara) was used in PCR reaction on a StepOnePlus Real-Time PCR System (Applied Biosystems™). All experiments were performed in triplicates.

Immunoblotting analysis

After genotyping, 7-d-old seedlings of different homozygote mutants were treated with or without 10 nmol/L BL for 120 min. Total proteins were extracted with 2 × sodium dodecyl sulfate (SDS) buffer containing 125 mmol/L Tris (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 20 mmol/L dithiothreitol (DTT) and 0.02% (w/v) bromophenol blue. Protein extracts equivalent to 10 mg seedlings were resolved on 8% (for MBP) and 12% (for BES1) SDS polyacrylamide gel electrophoresis \ and transferred onto a nitrocellulose membrane. After blocking with 10% non-fat milk solution, the membranes were incubated with primary antibodies against MBP (1:3,000, ProteinTech, 15089-1-AP) and BES1 (1:5,000, homemade), respectively, and then incubated with the corresponding anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000, Abmart, M21002 for BES1 and MBP). The signals were revealed by a JustGene ECL Plus (CLINX) mixture and were detected by Fuji medical X-ray film.

In vivo ubiquitination analysis

The *pBES1::BES1-YFP* in Col-0 and the *pBES1::BES1-YFP* in *era1* transgenic plants were grown on 1/2 MS medium for 10 d. Plant materials were ground to powder in liquid nitrogen and solubilized with an IP buffer (50 mmol/L Tris-HCl pH7.5, 1 mmol/L ethylenediaminetetraacetic acid, 125 mmol/L NaCl, 0.2% Triton X-100, 5% Glycerol, 1mmol/L phenylmethylsulfonyl fluoride [PMSF], 50 μmol/L MG132, 1 × Protease Inhibitor) (Kim et al., 2009). After centrifugation at 15,000 × *g* for 10 min twice at 4°C, the supernatant was incubated for 3 h with the anti-green fluorescent protein (GFP) agarose beads at 4°C. The beads were then washed five times with an IP buffer, 5 min each time. The eluted samples were analyzed by immunoblots using anti-ubiquitin (Cell Signaling Technology) and anti-GFP (Roche) antibodies.

Cell-free protein degradation assay

The cell-free protein degradation assay was performed as described previously (Wang et al., 2009). Plants were grown at 22℃ in long-day conditions (12 h light/12 h dark cycles) and the 3-week-old leaves were ground to powder in liquid nitrogen. Total proteins were extracted with a cell-free degradation buffer (25 mmol/L Tris-HCl pH 7.5, 10 mmol/L NaCl, 10 mmol/L $MgCl_2$, 4 mmol/L PMSF, 5 mmol/L DTT, and 10 mmol/L adenosine triphosphate) and cell debris was removed by centrifugation at 15,000 × *g* for 10 min at 4℃. Total protein extracts from each of the plant materials were measured the concentration by Bicinchoninic Acid Kit for Protein Determination (Sigma Aldrich) and adjusted to equal concentrations with the degradation buffer. Then, 1,000 ng of recombinant MBP-BES1 proteins were added in 1,000 μL plant extracts (containing 100 mg total proteins) for further reaction. The reaction mixtures were incubated at 30℃ for different periods, and 200 μL reaction mixtures were taken from the tube at each sample and analyzed by immunoblots with MBP antibody.

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AUTHOR CONTRIBUTIONS

J.L. for supervision. Z.F., H.S., and J.L. for conception and design of the work, acquisition of data, analysis and interpretation of the data, and writing of the article; M.L. and Y.M. provided assistance for the experiments. All authors read and approved the contents of the article.

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SUPPORTING INFORMATION

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Figure S1. Plant morphology of *95-5* and wild type (WT) in different growth stages

Mutants exhibit a variety of defective phenotypes at different growth stages, including 4 d after germination (**A**), 3-week-old seedlings and rosette leaves (**B**) and (**C**), 8-week-old plants (**D**); inflorescence (**E**), mature silique (**F**), and flowers (**G**). Scale bars represent 1 cm.

Figure S2. A T-DNA is inserted in the genetic loci of *ENHANCED RESPONSE TO ABA 1 (ERA1)*

(**A**) Map-based cloning analysis. (**B**) Genotypic analysis of *ERA1* in *bak1-4* and in *95-5 bak1-4*. Full-length coding sequence primers are used in polymerase chain reaction amplification.

Figure S3. *era1* showed increased resistance to brassinazole (BRZ) than wild type (WT)

(**A**) Seedlings of the indicated genotypes were grown on the media with or without 100 nM BRZ and incubated in a growth chamber in the dark at 22°C for 5 d. Scale bar represents 1 cm. (**B**) Measurements of the hypocotyl length of the indicated genotypes. Data are means ± *SD* ($n \geq 50$). Significance was determined by Student's *t*-test. $**P < 0.01$; $*P < 0.05$.

Figure S4. *era1* showed increased sensitivity to LiCl than wild type (WT)

(**A**) Seedlings of the indicated genotypes were grown on the media with various concentrations of LiCl (0, 1, 10 mmol/L) and incubated in a growth chamber with a 16-h light/8-h dark lighting condition at 22°C for 7 d. Scale bar represents 1 cm. (**B**) Measurements of the root length of the indicated genotypes. Data are means ± *SD* ($n \geq 40$). Significance was determined by Student's *t*-test. $*P < 0.05$.

Figure S5. The expression level of *BES1* in wild type (WT) and *era1*

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyze the expression level of *BES1* in the indicated genotypes at different times. Seedlings of the indicated genotypes were grown on the media and incubated in a growth chamber with a 16-h light/8-h dark lighting condition at 22°C for 7 d. The light starts at 6:00 and the darkness at 22:00 hours every day. The data are shown as means of three biological repeats ± *SD*. Significance was determined by Student's *t*-test. $*P < 0.05$; ns, non-significant.

Table S1. The primers used in the experiments