Article

Developmental Cell

SINAT E3 Ligases Control the Light-Mediated Stability of the Brassinosteroid-Activated Transcription Factor BES1 in *Arabidopsis*

Highlights

- SINAT E3 ligases mediate ubiquitination and degradation of dephosphorylated BES1
- SINATs negatively regulate BR signaling through BES1
- Light promotes SINAT protein accumulation to regulate BES1 level

Authors

Mengran Yang, Chengxiang Li, Zhenying Cai, ..., Qi Xie, Guiliang Tang, Xuelu Wang

Correspondence

xlwang@mail.hzau.edu.cn

In Brief

The function of BES1 largely depends on its phosphorylation status and protein stability. Yang et al. show that SINAT E3 ligases specifically mediate degradation of dephosphorylated BES1, and light can stabilize SINAT proteins to regulate BES1 level. Therefore, the SINATs-BES1 module links light and BR signaling to regulate plant development.

Yang et al., 2017, Developmental Cell *41*, 47–58
April 10, 2017
http://dx.doi.org/10.1016/j.devcel.2017.03.014

CellPress

Developmental Cell
Article

SINAT E3 Ligases Control the Light-Mediated Stability of the Brassinosteroid-Activated Transcription Factor BES1 in *Arabidopsis*

Mengran Yang,[1,2] Chengxiang Li,[1] Zhenying Cai,[1] Yinmeng Hu,[2] Trevor Nolan,[3] Feifei Yu,[4] Yanhai Yin,[3] Qi Xie,[4] Guiliang Tang,[5] and Xuelu Wang[2,6,*]

[1]State Key Laboratory of Genetic Engineering, Department of Genetics, School of Life Sciences, Fudan University, Shanghai 200438, China
[2]Center of Integrative Biology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[3]Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA
[4]State Key Laboratory of Plant Genomics, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[5]Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931, USA
[6]Lead Contact
*Correspondence: xlwang@mail.hzau.edu.cn
http://dx.doi.org/10.1016/j.devcel.2017.03.014

SUMMARY

The plant hormones brassinosteroids (BRs) participate in light-mediated regulation of plant growth, although the underlying mechanisms are far from being fully understood. In addition, the function of the core transcription factor in the BR signaling pathway, BRI1-EMS-SUPPRESSOR 1 (BES1), largely depends on its phosphorylation status and its protein stability, but the regulation of BES1 is not well understood. Here, we report that SINA of *Arabidopsis thaliana* (SINATs) specifically interact with dephosphorylated BES1 and mediate its ubiquitination and degradation. Our genetic data demonstrated that SINATs inhibit BR signaling in a BES1-dependent manner. Interestingly, we found that the protein levels of SINATs were decreased in the dark and increased in the light, which changed BES1 protein levels accordingly. Thus, our study not only uncovered a new mechanism of BES1 degradation but also provides significant insights into how light conditionally regulates plant growth through controlling accumulation of different forms of BES1.

INTRODUCTION

As sessile organisms, plants must integrate various ambient environmental stimuli and endogenous hormonal cues to optimize their growth and development. Brassinosteroids (BRs) are growth-promoting plant hormones that respond to light and control development (Chory et al., 1991; Li et al., 1996; Yang et al., 2011). As seeds germinate under the ground, seedlings undergo a process of hypocotyl elongation called skotomorphogenesis, also known as etiolation. Upon the perception of light, hypocotyl elongation is inhibited, the cotyledons open and expand, and proplastids develop into mature chloroplasts, a process known as photomorphogenesis or de-etiolation (Wei and Deng, 1996). BRs are believed to be involved in light-mediated plant growth regulation as both BR-deficient and BR-insensitive mutant seedlings show constitutive photomorphogenesis phenotypes (Li et al., 1996; Szekeres et al., 1996). However, the underlying molecular mechanisms of BR signaling in response to light are not well understood.

BRs are perceived by a plasma membrane receptor kinase, BRASSINOSTEROID INSENSITIVE 1 (BRI1), which initiates a signaling cascade to activate the downstream transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1) (Li and Nam, 2002; Li and Chory, 1997; Wang et al., 2011; Wang and Chory, 2006; Yang et al., 2011). BES1 and BZR1 play central roles in BR-regulated gene expression and plant development. The *bes1-D* and *bzr1-D* mutations, both caused by a substitution from proline to leucine in the PEST domains of BES1 and BZR1, respectively, have greatly increased stability and lead to constitutive BR responses in plants (Wang et al., 2002; Yin et al., 2002), suggesting that the regulation of the protein stability of BES1 and BZR1 is important in BR signaling and many developmental processes. A recent study revealed that MORE AXILLARY GROWTH LOCUS 2 (MAX2), an F-box protein, regulates shoot branching in *Arabidopsis* by mediating tissue-specific degradation of both phosphorylated and dephosphorylated BES1 in the strigolactone signaling pathway (Wang et al., 2013). However, an E3 ligase that ubiquitously controls BES1 and BZR1 stability in plants has not been identified.

Several recent studies suggested that BES1 and BZR1 are involved in BR-regulated photomorphogenesis. First, PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), a key downstream transcription factor in light signaling, directly interacts with BZR1 to regulate gene expression and promote seedling etiolation interdependently with BZR1 in *Arabidopsis* (Oh et al., 2012). A subsequent study reported that CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), an E3 ligase, mediates the dark-dependent degradation of the phosphorylated (inactive) form of BZR1 (Kim et al., 2014). However, both phosphorylated and dephosphorylated BES1 and BZR1 are regulated by light conditions, and the dephosphorylated form is more important in regulating plant development (Vert and Chory, 2006).

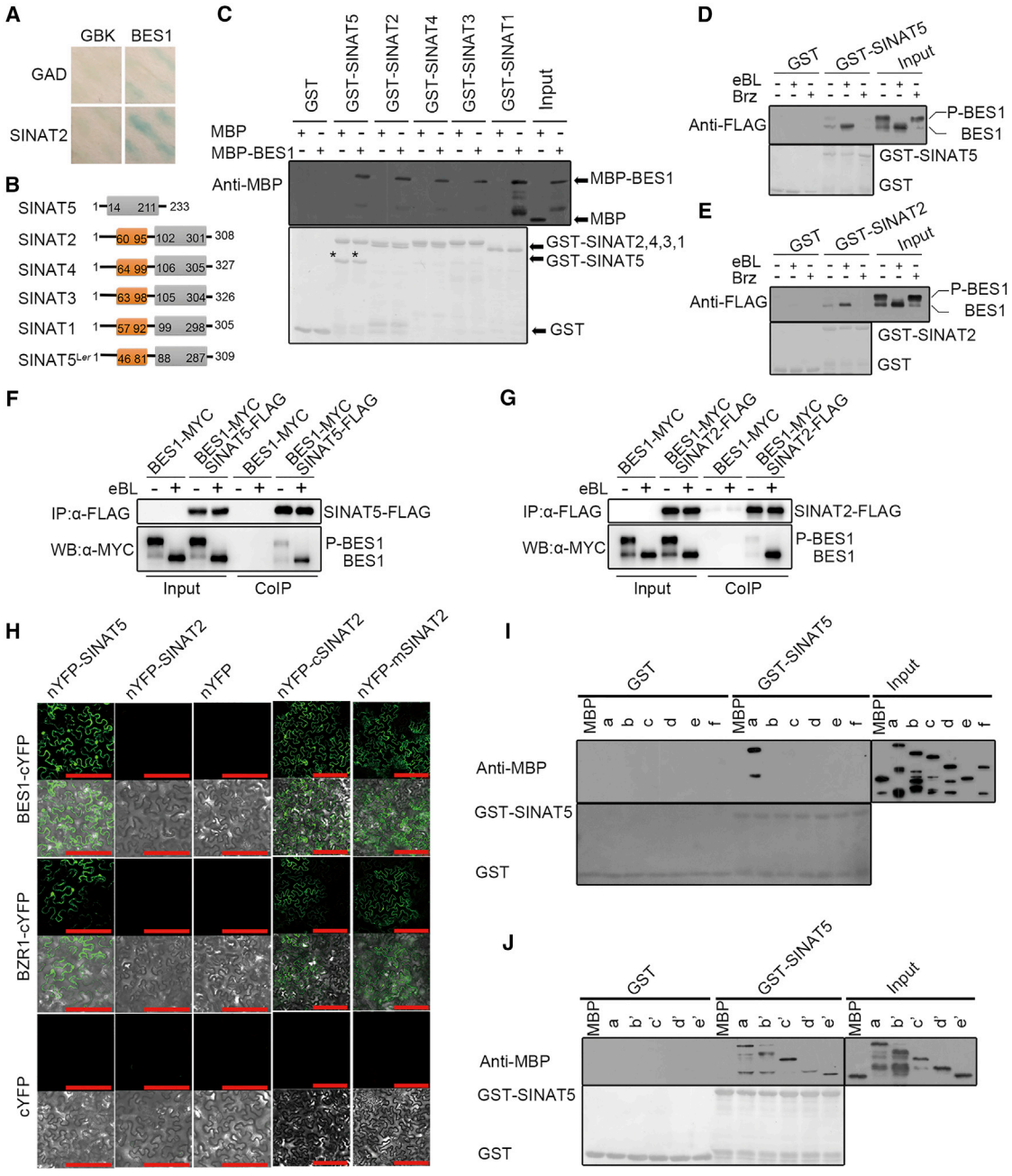


Figure 1. SINATs Interact with BES1 and BZR1 In Vitro and In Vivo

(A) Identification of SINAT2 interacting with BES1 in a yeast-two-hybrid assay.

(B) Domain architectures of SINATs predicted by SMART (http://smart.embl-heidelberg.de). Orange boxes indicate the RING domain; gray boxes indicate the SINA domain; numbers indicate the amino acid positions of each domain.

(C) SINATs interacted with BES1 in the in vitro pull-down assay. GST and GST-SINAT fusion proteins were used to pull down interacting proteins. MBP or MBP fusion proteins were detected by western blotting with anti-MBP antibodies. GST and GST-SINATs stained with Ponceau S (bottom panel) were used to show equal loading. Asterisks indicate GST-SINAT5.

(D and E) SINAT5 and SINAT2 mainly interacted with dephosphorylated BES1 in the semi-in vivo pull-down assays. GST, GST-SINAT5, and GST-SINAT2 fusion proteins purified from *E. coli* were used to pull down interacting proteins. The pulled-down BES1-FLAG from the total protein extracts of the *BES1-FLAG* overexpression line was detected by western blotting with anti-FLAG antibodies. GST, GST-SINAT5, and GST-SINAT2 stained with Ponceau S (bottom panel) were used to show equal loading.

(F and G) SINAT5 and SINAT2 mainly interacted with the dephosphorylated BES1 in the co-immunoprecipitation (IP) assays. Plants expressing both the *35S:SINAT-FLAG* and the *35S:BES1-MYC* were used. Plants solely expressing the *35S:BES1-MYC* were used as a negative control. The SINAT-FLAG was immunoprecipitated with anti-FLAG agarose, and the co-immunoprecipitated BES1-MYC was detected by western blotting (WB) with anti-MYC antibodies.

(legend continued on next page)

Therefore, other E3 ligases that regulate protein stability of BES1 and BZR1 might exist.

In this study, we identified a family of RING domain containing proteins, SINA of *Arabidopsis thaliana* (SINATs), which interact with BES1 and BZR1. We showed that SINATs function as E3 ubiquitin ligases and mediate BES1 ubiquitination and degradation. Genetic analysis indicated that SINATs play an important role in controlling BR-regulated plant development in a BES1-dependent manner in the light. Interestingly, we found that light can regulate the levels of SINATs to control dephosphorylated BES1 level. Thus, we not only uncovered a novel mechanism of BES1 degradation, but also provided a framework to illustrate how BES1 stability can be differentially regulated via different E3 ligases under diverse environmental conditions.

RESULTS

SINATs in the RING Finger Protein Family Interact with BES1 and BZR1

From a yeast-two-hybrid screen intended for BES1 interactors (Yin et al., 2005), we identified a RING finger E3 ligase, SINA of *A. thaliana* 2 (SINAT2) encoded by *At3g58040*. We further confirmed this interaction using full-length BES1 and SINAT2 in yeast (Figure 1A). SINAT2 is homologous to a RING finger protein SINA (Seven in absentia) from *Drosophila* (Carthew and Rubin, 1990). In addition to *SINAT2*, four other *SINA* genes were identified in *A. thaliana*: *SINAT5* (*At5g53360*), *SINAT4* (*At4g27880*), *SINAT3* (*At3g61790*), and *SINAT1* (*At2g41980*) (Wang et al., 2008). SINAT2, SINAT4, SINAT3, and SINAT1 proteins possess a RING domain and a SINA domain, while SINAT5 from the ecotype Columbia (Col-0) lacks the RING domain, but the allele from Landsberg (*Ler*) ecotype contains it (Figure 1B). A previous study found that SINAT5^{Ler} acts as a RING-type E3 ubiquitin ligase regulating lateral root development by mediating the degradation of NAC1 (Xie et al., 2002).

We then tested the interaction of BES1 with the five SINATs via in vitro pull-down assays and found that they directly interacted with BES1 (Figure 1C). Using semi-in vivo pull-down assays and in vivo co-immunoprecipitation assays, we found that the five SINATs mainly interacted with dephosphorylated BES1 (Figures 1D–1G and S1A–S1F). However, when we tested the interaction of the full-length SINATs with BES1 and its homolog BZR1 by bimolecular fluorescence complementation (BiFC) assays, an interaction was only observed between SINAT5 and BES1 or BZR1 (Figure 1H, columns 1–3 and Figure S1H, columns 1–3). We then speculated that BES1 and BZR1 might be degraded by co-expressed SINAT2, -4, -3, or -1, because they may serve as E3 ubiquitin ligases. To test this hypothesis, we separately fused the N-terminal RING domain (nSINAT) and the C-terminal SINA domain (cSINAT) of the SINATs to the YFP N terminus (nYFP) and conducted BiFC assays. We observed that the fragments containing only the SINA domain (cSINAT) strongly interacted with BES1 and BZR1, but did not observe an interaction between nSINATs and BES1 or BZR1 (Figures 1H and S1H). In addition, we also constructed a RING domain disrupted mutant of SINAT2 (C63S), in which the second conserved Cys in the RING domain was mutated into Ser (Figure S1G; Xie et al., 2002), and found that the C63S SINAT2 mutant (mSINAT2) interacts with BES1 and BZR1 in the BiFC assays (Figure 1H, column 5).

We further tested the interaction of the other four BES1 homologs (BEH1, BEH2, BEH3, and BEH4) with SINAT5, a representative member of the SINAT family, by BiFC assays. We found that BEH2 and BEH3 interacted with SINAT5 (Figure S1I). Interestingly, the PEST-domain mutated form of BES1, bes1-D (Yin et al., 2002), also interacted with SINAT5 (Figure S1I), suggesting that the PEST domain of BES1 did not mediate its interaction with SINATs. Full-length and truncated MBP-BES1 proteins were used in glutathione S-transferase (GST) pull-down assays to map the regions of BES1 that mediate its interaction with SINATs (Figure S1J). We found that the fragments lacking the BES1 N-terminal DNA-binding domain could not be pulled down by GST-SINAT5 (Figure 1I), but the fragments containing the N-terminal domain could interact with GST-SINAT5 (Figure 1J), suggesting that the N-terminal DNA-binding domain of BES1 is required and sufficient for its interaction with SINAT5.

SINATs Function as E3 Ub-Ligases to Mediate Ubiquitination and Degradation of BES1

The aforementioned interactions indicated that BES1 and BZR1 may be substrates of SINATs, which may function as E3 ubiquitin ligases. The self-ubiquitination of SINAT2, -4, -3, and -1, and SINAT5^{Ler}, all of which contain both RING and SINA domains, indicated that these proteins have E3 ligase activity; in contrast, SINAT5, which lacks the RING domain, is inactive (Figures 2A and S2A). To determine whether BES1 is a substrate of SINATs, we conducted an in vitro ubiquitination assay and found that in the presence of ubiquitin, E1, and E2, the SINAT2, -4, -3, and -1, and SINAT5^{Ler}, could polyubiquitinate BES1, but SINAT5 was not able to mediate ubiquitination of BES1 (Figures 2B and S2B). In addition, bes1-D could also be ubiquitinated by SINATs, but at a relatively lower level compared with the wild-type BES1 (Figures 2B and S2B). In addition, we conducted an in vivo ubiquitination assay using the *BES1-MYC/SINATs-RNAi* and the *BES1-MYC/SINAT-overexpression* transgenic plants treated with MG132, a 26S proteasome inhibitor. The results indicated that the ubiquitination levels of BES1-MYC in the *SINAT2*, *-4*, *-3*, and *-1 overexpression* lines were much higher than that in the *SINATs-RNAi* line (Figure 2C).

Next, we examined the degradation of recombinant MBP-BES1 using cell-free degradation assays, in which MBP-BES1 protein was incubated with protein extracts from wild-type plants (Col-0) or extracts from the *SINATs-RNAi* line or the *SINAT*

(H) SINAT5, the C-terminal region (SINA domain) of SINAT2, and the C63S SINAT2 mutant (mSINAT2) interacted with BES1 and BZR1 in the BiFC assays. Scale bars, 200 μm.

(I and J) The N terminus of BES1 is required and sufficient for its interaction with SINAT5. The truncated BES1 without the N-terminal region could not interact with SINAT5 in an in vitro pull-down assay (I). All of the truncated forms of BES1 containing the N terminus interacted with SINAT5 in vitro (J). GST and GST-SINAT5 fusion proteins were used to pull down the full-length or truncated MBP-BES1 proteins. MBP and MBP-BES1 proteins were detected by western blotting with anti-MBP antibodies. GST and GST-SINAT5 stained with Ponceau S (lower panels) were used for equal loading.

See Figure S1 for additional information.

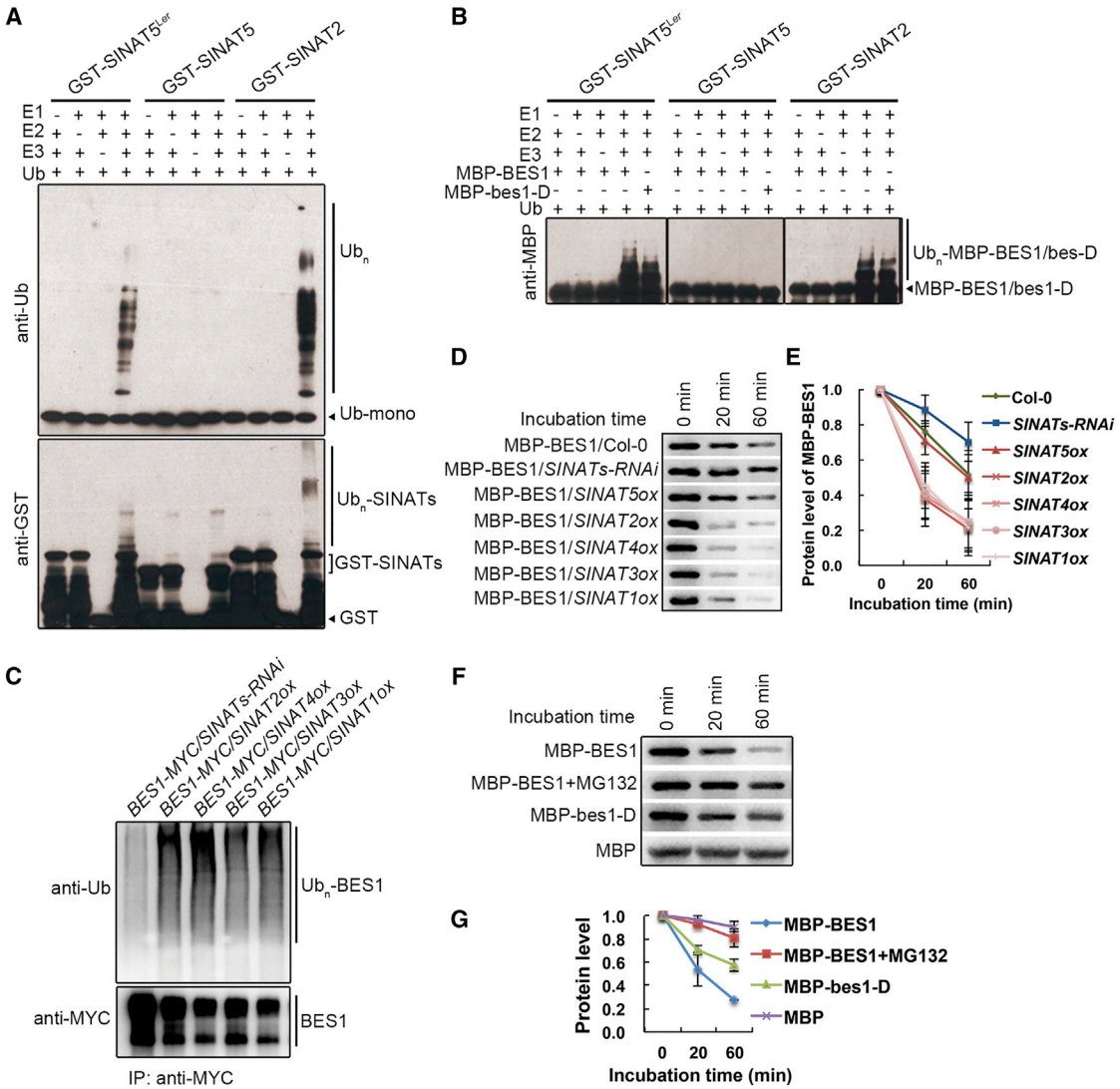


Figure 2. SINATs Function as E3 Ub-Ligases to Mediate BES1 Ubiquitination and Degradation

(A) Detection of self-ubiquitination of GST-fused SINAT5^{Ler}, SINAT5, and SINAT2 in vitro. Ubiquitin-attached protein bands were detected by western blotting with anti-Ub antibodies (upper gels) and anti-GST antibodies (lower gels).

(B) Detection of the ubiquitination of MBP-fused BES1 and bes1-D by SINAT5^{Ler}, SINAT5, and SINAT2 in vitro. The full-length MBP-BES1 and MBP-bes1-D fusion proteins were used as substrates for the in vitro ubiquitination assays. Anti-MBP antibodies were used in western blotting for detecting substrates.

(C) Ubiquitination assay of BES1-MYC in the *RNAi* line and in the *overexpression* lines of *SINATs* in vivo. The BES1-MYC and BES1-MYC-Ub proteins were immunoprecipitated with anti-c-MYC agarose and were detected by western blotting with anti-Ub antibodies (upper gels) and anti-MYC antibodies (lower gels).

(D) In vitro cell-free degradation assays showed the delayed or accelerated degradation of the recombinant MBP-BES1 proteins in the *RNAi* line or in the overexpression lines of *SINATs*, respectively. The protein level of MBP-BES1 was determined by western blotting with anti-MBP antibodies.

(E) Quantitative results for western blotting in (D). The initial protein levels of MBP-BES1 were defined as "1." Data are means ± SD (n = 3).

(F) Cell-free degradation assays showed the proteasome-dependent degradation of MBP-BES1 and delayed degradation of MBP-bes1-D in the overexpression lines of *SINAT2*. MBP was used as a substrate for the negative control. The protein levels were detected by western blotting with anti-MBP antibodies.

(G) Quantitative results for western blotting in (F). The initial protein levels were defined as "1." Data are means ± SD (n = 3).

See Figure S2 for additional information.

overexpression lines, which are described below. The results indicated that the degradation rate of BES1 was reduced in the *SINATs-RNAi* line and increased in the *SINAT2*, *-4*, *-3*, and *-1* overexpression lines (Figures 2D, 2E, and S2C). The degradation rate of BES1 in the *SINAT5* overexpression line was similar to that in the wild-type. Addition of MG132 significantly reduced BES1 degradation, indicating that its degradation is largely proteasome dependent under this condition (Figures 2F, 2G, and S2D–S2F). Moreover, the ubiquitinated BES1 could be detected in the semi-in vivo assays especially when it was incubated with protein extracts from the *SINAT2-overexpression* line treated with MG132 (Figure S2G). Although bes1-D could also be degraded in the *SINAT2*, *-4*, *-3*, and *-1* overexpression lines, its degradation rate was much slower than that of the wild-type

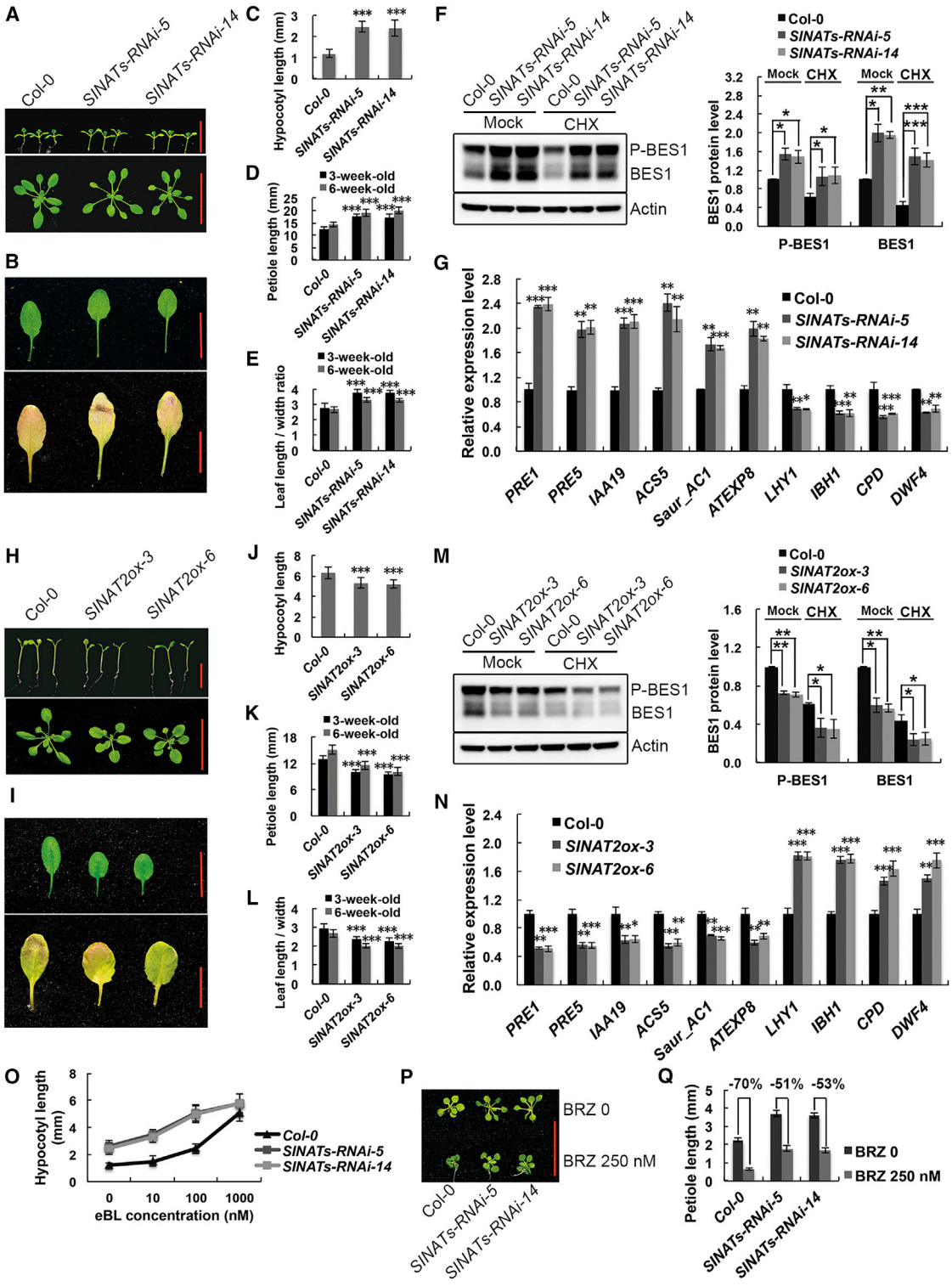


Figure 3. SINATs Act as Negative Regulators of BR Signaling

(A) Phenotypes of the *SINATs-RNAi* seedlings grown on 1/2 MS medium for 9 days (upper) and in soil for 3 weeks (lower). Scales bars, 0.5 cm (upper) and 4 cm (lower).

(B) The fifth leaves of 3-week-old plants (upper) and 6-week-old plants (lower). Scales bars, 2 cm.

(C) Hypocotyl length in upper panel of (A).

(D and E) Petiole length (D) and the ratio of leaf length/width (E) of the fifth leaves shown in (B).

(legend continued on next page)

BES1 (Figures 2F, 2G, and S2D–S2F). Taken together, our results indicated that SINATs function as E3 ligases to regulate BES1 ubiquitination and degradation.

SINATs Are Ubiquitously Expressed in *Arabidopsis*

To elucidate the biological functions of *SINATs*, we further analyzed their expression patterns. qRT-PCR analysis showed that all five *SINATs* were expressed in 9-day-old seedlings, roots, rosette leaves, cauline leaves, stems, flowers, and siliques (Figure S3A). The expression of *SINAT5* and *SINAT2* in roots was much higher than that in seedlings and other tissues. In reporter assays using promoter:GUS (β-glucuronidase) fusions to detect the spatial expression of the *SINATs*, the expression of GUS was only detected when driven by the *SINAT2*, *SINAT4*, and *SINAT1* promoters. Consistent with the qRT-PCR results, GUS was found to be ubiquitously expressed in seedlings and all tissues tested (Figure S3B), which was similar to the expression pattern of *BES1* (Yin et al., 2002).

We next explored the subcellular localization of SINATs; to this end, GFP-SINATs were transiently expressed in tobacco (*Nicotiana benthamiana*) pavement cells. We found that all five of the SINATs localized in both the nucleus and cytoplasm (Figure S3C). In addition, we constructed the transgenic plants expressing the *35S*:*GFP-SINAT2* and found that the GPF-SINAT2 was localized in both nucleus and cytoplasm (Figure S3D). The GFP-tagged SINAT2 is functional in *Arabidopsis*, as the transgenic lines had significantly shorter hypocotyls than the wild-type plants (Figure S3E). The subcellular localization of SINATs was very similar to that of BES1 (Yin et al., 2002) and consistent with our BiFC results.

Knockdown of *SINATs* Enhances BR Signaling and Overexpression of *SINATs* Inhibits BR Signaling

To study the functions of the *SINAT* family genes in plants, we constructed RNAi transgenic plants to knock down *SINATs* expression. The expression levels of all five *SINATs* were reduced in the *SINATs-RNAi* lines (Figure S4A), and the *SINATs-RNAi* lines exhibited a typical BR-enhanced phenotype (Figures 3A, 3B, and S4B), including longer hypocotyls (Figure 3C), longer petioles (Figure 3D), and increased ratio of leaf length to width (Figure 3E). To investigate whether the phenotypes of the *SINATs-RNAi* lines were related to BES1 accumulation, we performed western blotting and found that the endogenous BES1 protein was present at significantly higher levels in *SINATs-RNAi* lines (Figures 3F and S4C). Furthermore, the BES1 level was still higher in the *SINATs-RNAi* lines after treatment with the protein synthesis inhibitor cycloheximide (CHX), indicating that this phenomenon was due to the reduced BES1 degradation rather than the elevated protein synthesis (Figure 3F). In addition, we tested the expression of a set of BES1 target genes, and found that the expression levels of the BR-inducible *PRE1*, *PRE5*, *IAA19*, *ACS5*, *SAUR_AC1*, and *ATEXP8* genes were upregulated while the expression levels of the BR-repressed *LHY1*, *IBH1*, *CPD*, and *DWF4* genes were downregulated in the *SINATs-RNAi* lines compared with the wild-type (Figure 3G). These results suggested that knockdown of *SINATs* can enhance BR signaling.

We also constructed transgenic overexpression lines for each *SINAT* driven by the 35S promoter. The *SINAT5* overexpression lines showed no distinctive phenotypic changes (Figures S4G and S4H), although the expression level of *SINAT5* was higher than that in the wild-type (Figure S4I). The level of BES1 and the expression levels of *CPD* and *DWF4* were also similar between the *SINAT5* overexpression line and the wild-type plants (Figures S4J and S4K). In contrast, the *SINAT2* overexpression lines had significantly shorter hypocotyls, shorter leaf petioles, and decreased ratio of leaf length to width compared with the wild-type plants (Figures 3H–3L and S4E). The expression level of *SINAT2* is shown in Figure S4D. The endogenous BES1 level was lower in the *SINAT2* overexpression lines than that in the wild-type in both the CHX-treated and untreated plants (Figures 3M and S4F), which is consistent with the idea that SINATs mediate BES1 degradation. Furthermore, the BR-inducible genes were downregulated while the BR-repressed genes were upregulated in the *SINAT2* overexpression lines compared with the wild-type (Figure 3N). Moreover, the *SINAT4*, *-3*, and *-1* overexpression lines had phenotypes similar to that of the *SINAT2* overexpression line (Figures S4L–S4Z). These results indicate that overexpression of *SINAT2*, *-4*, *-3*, and *-1* can inhibit BR signaling.

In addition, we tested the sensitivity of the *SINATs-RNAi* lines to BRs in hypocotyl elongation and found that the *SINATs-RNAi* lines exhibited BR hypersensitivity when grown on medium

(F) Endogenous BES1 levels in the wild-type and *SINATs-RNAi* lines. Ten-day-old seedlings treated with mock or CHX (1 mM) for 2 hr were used. Left panel: BES1 was detected with anti-BES1 antibodies, and ACTIN was used for equal loading. Right panel: quantification of BES1 levels in the left panel. The phosphorylated and dephosphorylated BES1 levels in mock-treated Col-0 were defined as "1."

(G) Expression levels of BES1 target genes in the *SINATs-RNAi* seedlings. The expression levels in Col-0 were defined as "1."

(H) Phenotypes of the *SINAT2* overexpression lines grown on 1/2 MS medium under dim light (25 μmol m^{-2} s^{-1}) for 9 days (upper) and in soil for 3 weeks (lower). Scales bars, 0.5 cm (upper) and 4 cm (lower).

(I) The fifth leaves of 3-week-old plants (upper) and 6-week-old plants (lower). Scales bars, 2 cm.

(J) Hypocotyl length in upper panel of (H).

(K and L) Petiole length (K) and ratio of leaf length/width (L) of the fifth leaves shown in (I).

(M) Endogenous BES1 levels in the wild-type and *SINAT2* overexpression lines. Ten-day-old seedlings treated with mock or CHX (1 mM) for 2 hr were used. Left panel: BES1 was detected with anti-BES1 antibodies, and ACTIN was used for equal loading. Right panel: quantification of BES1 levels in left panel. The phosphorylated and dephosphorylated BES1 levels in mock-treated Col-0 were defined as "1."

(N) Expression levels of BES1 target genes in the *SINAT2 overexpression* lines. The expression levels in Col-0 were defined as "1."

(O) eBL sensitivity assays in hypocotyl length. Seedlings were grown on 1/2 MS medium with various concentrations of eBL for 9 days.

(P) Fifteen-day-old seedlings grown on 1/2 MS medium with or without 250 nM BRZ. Scale bar, 2 cm.

(Q) Petiole length of the third leaves shown in (P).

Data are means ± SD. n = 3 in (F), (G), (M), and (N); n > 20 in (C), (J), and (O); n > 15 in (D), (E), (K), (L), and (Q). *p < 0.05, **p < 0.01, ***p < 0.001. See Figures S3 and S4 for additional information.

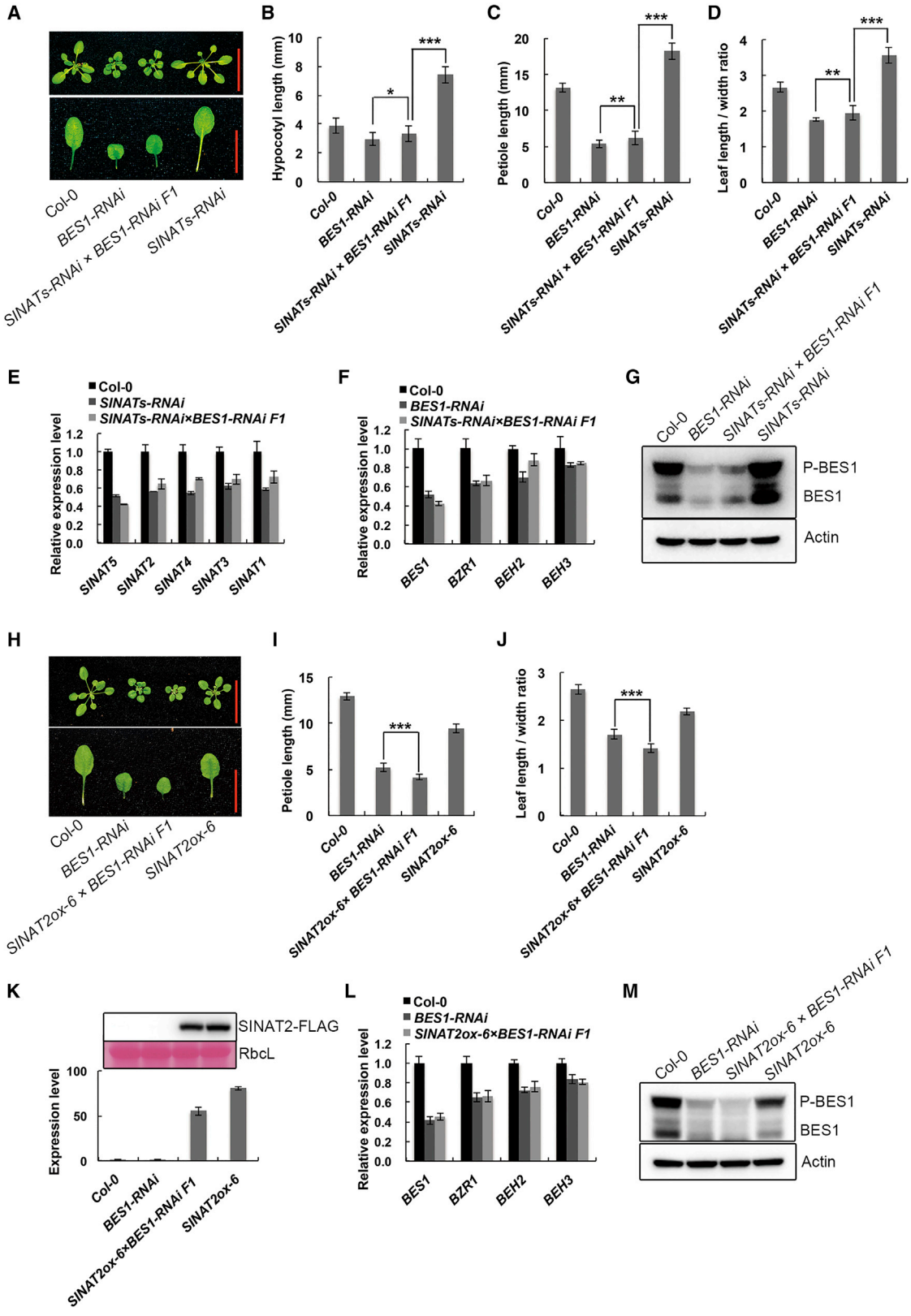


Figure 4. SINATs Act through BES1 to Regulate Plant Growth

(A) Rosettes and the fifth leaves of the wild-type, *BES1-RNAi*, *SINATs-RNAi*, and *SINATs-RNAi* × *BES1-RNAi* F1 plants grown under long-day conditions (16 hr light/8 hr dark cycles) for 3 weeks. Scale bars, 4 cm (upper) and 2 cm (lower).

(legend continued on next page)

containing various concentrations of epibrassinolide (eBL) (Figure 3O). We also tested the sensitivity of *SINATs-RNAi* to brassinazole (BRZ), a BR biosynthesis inhibitor. The results indicated that the *SINATs-RNAi* lines were hyposensitive to BRZ in petiole elongation (~51% inhibition) compared with the wild-type (~70% inhibition; Figures 3P and 3Q). Taken together, these results demonstrated that *SINAT2*, *-4*, *-3*, and *-1* negatively regulate BR signaling.

SINATs Function through BES1 to Regulate Plant Growth

To verify whether the biological functions of SINATs require BES1, we crossed the *SINATs-RNAi* transgenic line with the *BES1-RNAi* transgenic plants (Yin et al., 2005). In the F1 plants, the expression of the *SINATs* and *BES1* family genes were repressed to a level similar to that in each parent (Figures 4E and 4F), and the BES1 protein level was also greatly reduced in *BES1-RNAi* and the F1 plants (Figure 4G). Phenotypic analysis showed that the *BES1-RNAi* counteracted the BR-enhanced phenotypes caused by *SINATs-RNAi*, as quantified by hypocotyl length, petiole length, and the ratio of leaf length to width (Figures 4A–4D). Moreover, in the F1 plants, BES1 levels were higher than in the *BES1-RNAi* transgenic lines (Figure 4G), which is consistent with the degradation of BES1 by SINATs. However, the levels of BES1 in the F1 plants were still very low compared with the wild-type, which should not significantly affect the phenotypes resulting from *BES1-RNAi*.

Furthermore, we crossed the *SINAT2-overexpression* line with the *BES1-RNAi* line. In the F1 plants, the expression of *SINAT2* and *BES1* family genes was comparable with each parent (Figures 4K and 4L). We found that *SINAT2-ox* could further enhance the phenotypes of the *BES1-RNAi* line (Figures 4H–4J). We further detected the BES1 protein level, and found that *SINAT2-ox* caused additional BES1 decrease in the F1 plants compared with the *BES1-RNAi* line (Figure 4M). Taking these data together, we concluded that *BES1* is genetically epistatic to *SINATs*, and the function of the SINATs depends on BES1.

SINATs Protein Levels Were Enhanced by Light

Although SINATs play negative roles in BR signaling, we found that eBL treatment did not affect the expression of *SINATs* or their protein levels (Figures 5A, 5B, and S5A–S5H), which significantly induced BES1 dephosphorylation (Figures 5C and S5I–S5L). Unlike the phenotypes under light, the hypocotyl length of the *SINATs-RNAi* lines grown in the dark was almost indistinguishable from that of the wild-type seedlings (Figures 5D and 5E). The protein level of BES1 was also similar in the dark-grown *SINATs-RNAi* lines and in the wild-type plants (Figure 5F). In addition, *SINATs-RNAi* lines displayed long, slender, obliquely upward-pointing petioles under light (Figures 5G, 3A, 3B, and S4B), suggesting that SINATs may function primarily in the light. We grew the *35S*:*SINAT-FLAG* transgenic lines in continuous light (L) for 5 days and then transferred them to the dark (D) for 2, 4, 6, 8, or 12 hr. The results showed that the protein levels of most of the SINATs, except for SINAT5, dramatically decreased with increasing time in the dark (Figures 5H and S5M–S5P). Furthermore, when the dark-treated plants were returned to light (L-D-L), SINAT levels increased (right side of Figure 5H and right side of Figures S5M–S5P). The transcript levels of the *SINATs* were not dramatically affected by dark treatment (Figures S5Q–S5U), suggesting that light affects SINAT levels through a post-transcriptional mechanism. We then tested whether the regulation of SINATs by light depends on light quality, and found that both red and blue light could increase the levels of SINAT2, -4, -3, and -1 while far-red light had no such effects (Figures S6B–S6E). In contrast, SINAT5 protein level was insensitive to dark treatment or any kind of light treatment (Figure S6A). In addition, we found that the SINAT dynamics in *cop1-4* mutant (Mcnellis et al., 1994) were similar to that of the wild-type (Figures S6F and S6G). Therefore, the levels of SINATs regulated by light were red light and blue light specific and were independent of the COP1-mediated pathway.

COP1 mediates the degradation of phosphorylated BZR1 (pBZR1) in darkness (Kim et al., 2014), and SINATs mainly interact with dephosphorylated BES1, so we hypothesized that SINATs may promote the degradation of dephosphorylated BES1 in the light. To test this hypothesis, we first tested the BES1 protein level in the same samples from the *35S*:*SINAT2-FLAG* transgenic line and found that the dephosphorylated BES1 was accumulated after the plants were transferred to the dark and was reduced when the dark-treated plants were returned to light (Figure 5H), which is consistent with the reduced

(B) Hypocotyl length of the wild-type, *BES1-RNAi*, *SINATs-RNAi*, and *SINATs-RNAi* × *BES1-RNAi* F1 seedlings grown on 1/2 MS medium under dim light (25 μmol m^{-2} s^{-1}) for 8 days. The data are means ± SD ($n > 20$). *$p < 0.05$, ***$p < 0.001$.

(C and D) Petiole length (C) and ratio of leaf length/width (D) of the fifth leaves shown in lower panel of (A). Data are means ± SD ($n > 10$). **$p < 0.01$, ***$p < 0.001$.

(E) Relative expression levels of *SINATs* in the wild-type, *SINATs-RNAi*, and *SINATs-RNAi* × *BES1-RNAi* F1 lines. The expression level of each *SINAT* in Col-0 was defined as "1." Data are means ± SD ($n = 3$).

(F) Relative expression levels of *BES1* and its homologs in the wild-type, *BES1-RNAi*, and *SINATs-RNAi* × *BES1-RNAi* F1 lines. The expression level of *BES1* and its homologs in Col-0 was defined as "1." Data are means ± SD ($n = 3$).

(G) The protein levels of endogenous BES1 in the wild-type, *BES1-RNAi*, *SINATs-RNAi*, and *SINATs-RNAi* × *BES1-RNAi* F1 seedlings. BES1 was detected by western blotting with anti-BES1 antibodies. Endogenous ACTIN detected with anti-ACTIN antibodies was used for equal loading.

(H) Rosettes and the fifth leaves of the wild-type, *BES1-RNAi*, *SINAT2ox-6*, and *SINAT2ox-6* × *BES1-RNAi* F1 plants grown under long-day condition (16 hr light/8 hr dark cycles) for 3 weeks. Scale bars, 4 cm (upper) and 2 cm (lower).

(I and J) Petiole length (I) and ratio of leaf length/width (J) of the fifth leaves shown in lower panel of (H). Data are means ± SD ($n > 10$). ***$p < 0.001$.

(K) Expression level of *SINAT2* in the wild-type, *BES1-RNAi*, *SINAT2ox-6*, and *SINAT2ox-6* × *BES1-RNAi* F1 plants. The expression level of *SINAT2* in Col-0 was defined as "1." Data are means ± SD ($n = 3$). SINAT2-FLAG was detected with anti-FLAG antibodies. The Rubisco large subunit (RbcL) stained with Ponceau S was used for equal loading.

(L) Relative expression levels of *BES1* and its homologs in the wild-type, *BES1-RNAi*, and *SINAT2ox-6* × *BES1-RNAi* F1 lines. The expression level of *BES1* and its homologs in Col-0 was defined as "1." Data are means ± SD ($n = 3$).

(M) The protein levels of endogenous BES1 in the wild-type, *BES1-RNAi*, *SINAT2ox-6*, and *SINAT2ox-6* × *BES1-RNAi* F1 seedlings. BES1 was detected by western blotting with anti-BES1 antibodies. Endogenous ACTIN detected with anti-ACTIN antibodies was used for equal loading.

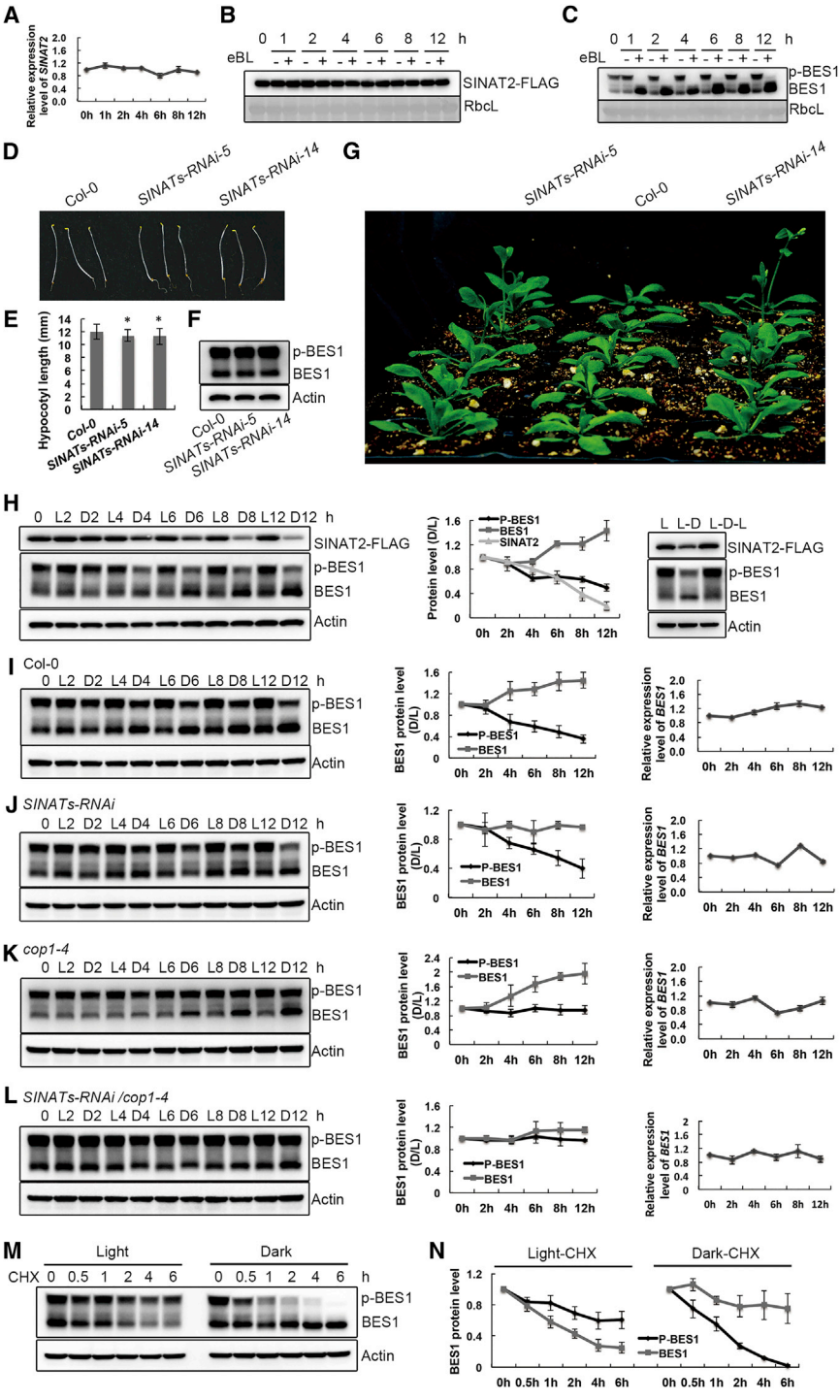


Figure 5. Light, Not BRs, Controls the Protein Levels of SINATs to Regulate BES1 Accumulation

(A) Transcript levels of *SINAT2* in Col-0 treated with 5 μM eBL. The data are normalized to the mock treatment at each time point.

(B) SINAT2 levels with or without eBL treatment. The *35S:SIANT2-FLAG* seedlings were treated with 5 μM eBL (+) or mock solvent (−) for the indicated time. The RbcL stained with Ponceau S was used for equal loading.

(C) BES1 phosphorylation status detected with anti-BES1 antibodies in samples used in (B). The RbcL was used for equal loading.

(D) Phenotype of the *SINATs-RNAi* seedlings grown in the dark for 4 days.

(E) Hypocotyl length in (D).

(F) Endogenous BES1 detected with anti-BES1 antibodies in the dark-grown wild-type and *SINATs-RNAi* lines. ACTIN was used for equal loading.

(G) Phenotypes of the 4-week-old *SINATs-RNAi* plants.

(H) SINAT2 and BES1 levels under different light conditions. Left panel: the *35S:SIANT2-FLAG* seedlings were grown under continuous light for 5 days and either remained in the light (L) or were transferred to the dark (D) for the indicated time period. Middle panel: SINAT2 and BES1 protein ratio of dark/light in the left panel. Protein levels were normalized to that in the light-grown seedlings at each time point. Right panel: L, continuous light; L-D, the continuous light-grown seedlings were transferred to the dark for 6 hr; L-D-L, the dark-treated seedlings were then transferred back to the light for another 6 hr. The SINAT2-FLAG was detected with anti-FLAG antibodies. BES1 was detected with anti-BES1 antibodies in the same samples. ACTIN was used for equal loading.

(I–L) Endogenous BES1 levels in the wild-type (I), the *SINATs-RNAi* line (J), the *cop1-4* mutant (K), and the *SINATs-RNAi/cop1-4* line (L) under different light conditions. The experimental conditions were the same as in the left panel of (H). Left panels: BES1 was detected with anti-BES1 antibodies. ACTIN was used for equal loading. Middle panels: BES1 protein ratio of dark/light in the left panels. Levels of both phosphorylated and dephosphorylated BES1 were normalized to that in light-grown seedlings at each time point. Right panels: transcript levels of *BES1* in the seedlings used in the left panels. The data are normalized to that in light-grown seedlings at each time point.

(M) Endogenous BES1 levels after CHX treatment in the light or transferred to the dark. The wild-type seedlings were grown under continuous light for 14 days and treated with 1 mM CHX, and either remained in the light or were transferred to the dark for the indicated time period. BES1 was detected with anti-BES1 antibodies. ACTIN was used for equal loading.

(N) Quantification of BES1 levels in (M). Phosphorylated and dephosphorylated BES1 levels at 0 hr were defined as "1."

Data are means ± SD. n = 3 in (A), (H–L), and (N); n > 20 in (E). *p < 0.05.

See Figures S5 and S6 for additional information.

levels of SINATs in the dark. Furthermore, we used the wild-type, the *SINATs-RNAi* line, the *cop1-4* mutant (Mcnellis et al., 1994), and the *SINATs-RNAi/cop1-4* line to determine the levels of BES1 under different light conditions. Plants were grown on half-strength Murashige and Skoog (1/2 MS) medium containing 1% sucrose to exclude the effects of starvation. When the wild-type (Col-0) plants were transferred to the dark, the level of the dephosphorylated BES1 was increased while the level of

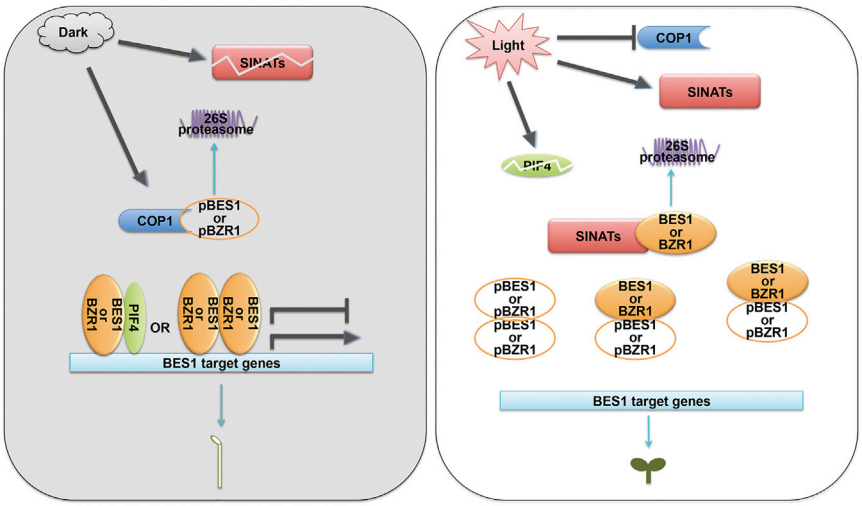


Figure 6. A Proposed Model to Illustrate How Different Light Conditions Control Plant Growth and Development via Differentially Regulating pBES1 and pBZR1 and Dephosphorylated BES1 and BZR1 Stability

In the dark, SINATs are degraded in a self-dependent manner to enhance the dephosphorylated BES1/BZR1 level, and pBES1/pBZR1 are degraded through the COP1 pathway leading to a high ratio of BES1/BZR1 to pBES1/pBZR1. The active BES1-BES1 dimers and BZR1-BZR1 dimers and BES1- or BZR1-PIF4 dimers regulate downstream gene expression to promote hypocotyl elongation. In the light, COP1 is inactive and SINATs accumulate to mediate the degradation of dephosphorylated BES1 and BZR1. A low level of the dephosphorylated BES1/BZR1 and low ratio of BES1/BZR1 to pBES1/pBZR1 as well as labile PIF4 lead to reduced hypocotyl elongation.

phosphorylated BES1 was decreased (Figure 5I), leading to an increased ratio of dephosphorylated/phosphorylated BES1. However, after the plants were transferred to the dark, in the *SIANTs-RNAi* line we only observed the phosphorylated BES1 to be decreased while the dephosphorylated BES1 was almost unchanged, and in the *cop1-4* mutant only the dephosphorylated BES1 was dramatically accumulated but the phosphorylated BES1 was almost unchanged (Figures 5J and 5K). Furthermore, both phosphorylated and dephosphorylated BES1 showed no significant change in the *SINATs-RNAi/cop1-4* line with dark treatment (Figure 5L). The changes in BES1 protein level were not caused by an altered *BES1* expression level (right side of Figures 5I–5L). These results suggest that light regulates the protein level of SINATs to control BES1 accumulation.

To directly measure the BES1 degradation without the disturbance of de novo protein synthesis, we treated the wild-type plants with the protein synthesis inhibitor cycloheximide (CHX) to estimate the half-life of BES1 protein under light or in the dark. In the light, the dephosphorylated BES1 was remarkably decreased after CHX treatment (Figures 5M and 5N), which was likely caused by the SINAT activity. In contrast, the dephosphorylated BES1 was stable in the dark, which was consistent with the low SINAT level. The phosphorylated BES1 was decreased dramatically with CHX treatment in the dark (Figures 5M and 5N), which was likely mediated by COP1. These results suggest that SINATs specifically degrade the dephosphorylated BES1 in the light.

DISCUSSION

In this study, we identified the RING-type E3 ligases, SINA of *A. thaliana* (SINATs), which can ubiquitously and directly regulate the stability of BES1 in the light. First, SINATs strongly interact with dephosphorylated BES1 and BZR1 both in vitro and in vivo. Second, SINAT2, -4, -3, and -1 possess E3 ubiquitin ligase activity and can mediate BES1 ubiquitination and degradation. Third, the *SINATs-RNAi* lines showed BR-enhanced phenotypes and higher BES1 levels in the light, while *SINAT2*, *-4*, *-3*, and *-1* overexpression lines showed the opposite phenotypes. Furthermore, knockdown of *BES1* and its homologs reduced the enhanced BR signaling in the *SINATs-RNAi* lines, and overexpression of *SINAT2* further enhanced the phenotypes of the *BES1-RNAi* line. Finally, the levels of SINATs were decreased in the dark and increased in the light. SINAT-mediated BES1 degradation appears different from MAX2-mediated BES1 degradation (Wang et al., 2013), since SINATs broadly regulate the dephosphorylated BES1 degradation.

SINATs bind to the N-terminal region of BES1 rather than to the PEST domain, to regulate degradation of dephosphorylated BES1; this provides an explanation as to why the N-terminal region is involved in BES1 degradation, as deletion of this region stabilizes the protein (Yin et al., 2005). The PEST peptide motif can target proteins for destruction (Rechsteiner and Rogers, 1996; Rogers et al., 1986). Many important regulators in animals, plants, and yeast are degraded by the 26S or 20S proteasomes based on their PEST motifs, including IκBa (Van Antwerp and Verma, 1996), phytochrome (Rechsteiner, 1990), and mIκBNS (Park et al., 2014), while other domains may function to trigger protein degradation in response to specific signals (Van Antwerp and Verma, 1996). We found that SINATs interact with the PEST-domain mutated form of BES1, bes1-D (Figure S1I), further confirming that the PEST domain is not essential for BES1 degradation by SINATs. However, the ubiquitination level and degradation rate of bes1-D were lower than that of the wild-type BES1 (Figures 2B and 2F), suggesting that the PEST domain mutation may affect the entry of bes1-D into the 26S proteasome. In addition, SINATs mediated BES1 degradation with a preference for the dephosphorylated BES1, which could provide a more direct and efficient way to attenuate BR signaling, as dephosphorylated BES1 is the active form and is more important in regulating BR signaling and plant development (Vert and Chory, 2006). This result differs from that predicted by an early hypothesis that the degradation of BES1 and BZR1 was dependent on their phosphorylation (He et al., 2002; Yin et al., 2002). The levels of both phosphorylated and dephosphorylated BES1 changed in the *SINATs-RNAi* transgenic lines and in the *SINAT2*, *-4*, *-3*, and *-1* overexpression lines (Figures 3F, 3M, S4O, S4T, and S4Y), suggesting a dynamic exchange between phosphorylated and dephosphorylated BES1 in plants.

Our results revealed that SINATs and BES1 link light and BR signaling to regulate photomorphogenesis. Although SINATs negatively regulate BR signaling, the expression level of *SINATs* and the stability of SINATs proteins were not regulated by BRs (Figures 5A, 5B, and S5A–S5H). Interestingly, dark treatment reduced the levels of SINATs (Figures 5H and S5N–S5P), indicating that SINATs are regulated by light signals to control BES1 degradation and BR signaling. As was previously reported, autodegradation of E3 ligases induced by specific signals is a general regulatory mechanism existing in both animals and plants (Chen et al., 2015; David et al., 2006; Yu et al., 2015). Our results indicated that light promotes the accumulation of SINATs, with the exception of SINAT5 (Figures 5H and S5M–S5P), which is catalytically inactive, suggesting that darkness may induce the autodegradation of SINAT2, -4, -3, and -1. In addition, the protein level of BES1 and BZR1 as well as the ratio of the dephosphorylated to the phosphorylated BES1 and BZR1 is critical for determining the rate of plant growth. Our CHX treatment assays further demonstrated that BES1 has a relatively short half-life (Figures 5M and 5N), suggesting that the regulation of BES1 protein stability is very important for its function. Furthermore, we found that the phosphorylated BES1 and the dephosphorylated BES1 have different half-lives under different light conditions, as the degradation of the phosphorylated BES1 is very fast in the dark, while the dephosphorylated BES1 is more labile in the light (Figures 5M and 5N). SINATs promote the degradation of dephosphorylated BES1 and BZR1 in the light, while COP1 degrades phosphorylated BES1 and BZR1 (pBES1 and pBZR1) in the dark. Such mechanisms may function to accurately and sensitively promote the transition between skotomorphogenesis and photomorphogenesis when seedlings emerge from the soil or are under low-light conditions during growth.

The SINATs-BES1 module not only regulates plant development but also may participate in stress responses. A co-submitted study suggests that SINAT-mediated BES1 degradation is involved in starvation stress (Nolan et al., 2017 [this issue of *Stem Cell Reports*]), demonstrating that starvation induced the transcription of *SINATs* and that the *SINATs-RNAi* line was hypersensitive to starvation. During plant development, the light condition is likely closely related to starvation status. Under short-term darkness (several hours), the protein levels of SINATs were reduced to promote BR signaling and hypocotyl elongation, while long-term darkness (several days) could lead to starvation, which will cause the enhanced transcription of *SINATs* and reduced BES1 to repress growth, saving energy and thus promoting survival. Therefore, both transcriptional and post-transcriptional regulation of *SINATs* by light and/or starvation conditions may be key for balancing plant growth and survival.

Taken together, we propose a model to explain how BES1 and BZR1 are degraded under different light conditions to regulate plant growth (Figure 6). When light-grown plants were transferred to the dark (such as day-night transition or covered by obstacles), the protein levels of SINATs are reduced and COP1 becomes activated (Kim et al., 2014), leading to a high level of dephosphorylated BES1/BZR1 and a high ratio of dephosphorylated to phosphorylated BES1/BZR1. Dephosphorylated BES1/BZR1 (the dominant form in the dark) can form BES1-BES1 and BZR1-BZR1 dimers as well as BES1- or BZR1-PIF4 heterodimers (Oh et al., 2012) to regulate downstream gene expression and promote hypocotyl elongation. However, when seedlings are exposed to the light (such as emerging seedlings from soil), the levels of SINATs are increased to mediate degradation of dephosphorylated BES1/BZR1 and COP1 becomes inactive, reducing the ratio of BES1/BZR1 to pBES1/pBZR1. PIF4 is labile in the light (Lorrain et al., 2008), and the absence of active BES1-BES1, BZR1-BZR1, and BES1- or BZR1-PIF4 dimers leads to inhibition of hypocotyl elongation (Figure 6). The mechanisms of light-mediated regulation of SINATs will require further investigation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Arabidopsis
- METHOD DETAILS
  - Construction of Transgenic Lines
  - Phenotypic Analysis
  - Yeast-Two-Hybrid Assays
  - *In Vitro* Pull-Down Assay
  - *Semi-In Vivo* Pull-Down Assay
  - Co-immunoprecipitation Assay
  - BiFC Assay and Subcellular Localization Analysis
  - *In Vitro* Ubiquitination Assay
  - *In Vivo* Ubiquitination Assay
  - Cell-Free Protein Degradation Assay
  - Semi-*In Vivo* Ubiquitination Assay
  - Gene Expression Analysis by Quantitative RT-PCR
  - GUS Staining Assay
  - Immunoblotting Analysis of SINAT-FLAG and BES1
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2017.03.014.

AUTHOR CONTRIBUTIONS

X.W. and M.Y. conceived the project. M.Y. conducted most of the experiments with the assistance of C.L., Z.C., and Y.H. T.N. and Y.Y. conducted the yeast-two-hybrid assays. The in vitro ubiquitination assays were conducted in Q.X.'s laboratory with the help of F.Y. G.T. provided the RNAi plasmid. X.W., M.Y., and C.L. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Hongquan Yang (Fudan University) for providing the *cop1-4* mutant seeds. This work was supported by grant 2015CB910200 of the National Key Basic Research Foundation of China, grants 31430046, 91535104, and 31271300 (to X.W.) of the National Natural Science Foundation of China, and initiative grants 2662015PY020 and 2014RC002 of Huazhong Agricultural University (to X.W.).

Received: June 17, 2016
Revised: January 11, 2017
Accepted: March 14, 2017
Published: April 10, 2017

REFERENCES

Cai, Z., Liu, J., Wang, H., Yang, C., Chen, Y., Li, Y., Pan, S., Dong, R., Tang, G., Barajas-Lopez Jde, D., et al. (2014). GSK3-like kinases positively modulate abscisic acid signaling through phosphorylating subgroup III SnRK2s in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *111*, 9651–9656.

Carthew, R.W., and Rubin, G.M. (1990). *Seven in absentia*, a gene required for specification of R7 cell fate in the *Drosophila* eye. Cell *63*, 561–577.

Chen, S., Lory, N., Stauber, J., and Hoecker, U. (2015). Photoreceptor specificity in the light-Induced and COP1-mediated rapid degradation of the repressor of photomorphogenesis SPA2 in *Arabidopsis*. PLoS Genet. *11*, e1005516.

Cheng, Y., Zhu, W., Chen, Y., Ito, S., Asami, T., and Wang, X. (2014). Brassinosteroids control root epidermal cell fate via direct regulation of a MYB-bHLH-WD40 complex by GSK3-like kinases. Elife. http://dx.doi.org/10.7554/eLife.02525.

Chory, J., Nagpal, P., and Peto, C.A. (1991). Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in *Arabidopsis*. Plant Cell *3*, 445–459.

David, D., Harumi, S., Angie, M., Tanay, D., Michael, E., Karen, O.R., Somasekar, S., and Dixit, V.M. (2006). ATM engages autodegradation of the E3 ubiquitin ligase COP1 after DNA damage. Science *313*, 1122–1126.

He, J.X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *99*, 10185–10190.

Ji, L., Liu, X., Yan, J., Wang, W., Yumul, R.E., Kim, Y.J., Dinh, T.T., Liu, J., Cui, X., Zheng, B., et al. (2011). *ARGONAUTE10* and *ARGONAUTE1* regulate the termination of floral stem cells through two microRNAs in *Arabidopsis*. PLoS Genet. *7*, e1001358.

Jiang, J., Zhang, C., and Wang, X. (2015). A recently evolved isoform of the transcription factor BES1 promotes brassinosteroid signaling and development in *Arabidopsis thaliana*. Plant Cell *27*, 361–374.

Kim, B., Jeong, Y.J., Corvalan, C., Fujioka, S., Cho, S., Park, T., and Choe, S. (2014). Darkness and *gulliver2/phyB* mutation decrease the abundance of phosphorylated BZR1 to activate brassinosteroid signaling in *Arabidopsis*. Plant J. *77*, 737–747.

Li, J., and Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell *90*, 929–938.

Li, J., and Nam, K.H. (2002). Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. Science *295*, 1299–1301.

Li, J., Nagpal, P., Vitart, V., Mcmorris, T.C., and Chory, J. (1996). A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science *272*, 398–401.

Lorrain, S., Allen, T., Duek, P.D., Whitelam, G.C., and Fankhauser, C. (2008). Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. Plant J. *53*, 312–323.

Mcnellis, T.W., von Arnim, A.G., Araki, T., Komeda, Y., Miséra, S., and Deng, X.W. (1994). Genetic and molecular analysis of an allelic series of *cop1* mutants suggests functional roles for the multiple protein domains. Plant Cell *6*, 487–500.

Oh, E., Zhu, J.Y., and Wang, Z.Y. (2012). Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. Nat. Cell. Biol. *14*, 802–809.

Park, K.C., Jeong, J., and Kim, K.I. (2014). Regulation of mIκBNS stability through PEST-mediated degradation by proteasome. Biochem. Biophys. Res. Commun. *443*, 1291–1295.

Rechsteiner, M. (1990). PEST sequences are signals for rapid intracellular proteolysis. Semin. Cell Biol. *1*, 433–440.

Rechsteiner, M., and Rogers, S.W. (1996). PEST sequences and regulation by proteolysis. Trends Biochem. Sci. *21*, 267–271.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science *234*, 364–368.

Szekeres, M., Németh, K., Koncz-Kálmán, Z., Mathur, J., Kauschmann, A., Altmann, T., Rédei, G.P., Nagy, F., Schell, J., and Koncz, C. (1996). Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell *85*, 171–182.

Tang, G., Yan, J., Gu, Y., Qiao, M., Fan, R., Mao, Y., and Tang, X. (2012). Construction of short tandem target mimic (STTM) to block the functions of plant and animal microRNAs. Methods *58*, 118–125.

Teotia, S., Singh, D., Tang, X., and Tang, G. (2016). Essential RNA-based technologies and their applications in plant functional genomics. Trends Biotechnol. *34*, 106–123.

Van Antwerp, D.J., and Verma, I.M. (1996). Signal-induced degradation of IκBα: association with NF-κB and the PEST sequence in IκBα are not required. Mol. Cell. Biol. *16*, 6037–6045.

Vert, G., and Chory, J. (2006). Downstream nuclear events in brassinosteroid signalling. Nature *441*, 96–100.

Wang, X., and Chory, J. (2006). Brassinosteroids regulate dissociation of BKI1, a negative regulator of BRI1 signaling, from the plasma membrane. Science *313*, 1118–1122.

Wang, Z.Y., Nakano, T., Gendron, J., He, J., Meng, C., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., and Asami, T. (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev. Cell *2*, 505–513, 509.

Wang, M., Jin, Y., Fu, J., Zhu, Y., Zheng, J., Hu, J., and Wang, G. (2008). Genome-wide analysis of SINA family in plants and their phylogenetic relationships. DNA Seq. *19*, 206–216.

Wang, H., Yang, C., Zhang, C., Wang, N., Lu, D., Wang, J., Zhang, S., Wang, Z.X., Ma, H., and Wang, X. (2011). Dual role of BKI1 and 14-3-3 s in brassinosteroid signaling to link receptor with transcription factors. Dev. Cell *21*, 825–834.

Wang, Y., Sun, S., Zhu, W., Jia, K., Yang, H., and Wang, X. (2013). Strigolactone/MAX2-induced degradation of brassinosteroid transcriptional effector BES1 regulates shoot branching. Dev. Cell *27*, 681–688.

Wei, N., and Deng, X.W. (1996). The role of the *COP/DET/FUS* genes in light control of *Arabidopsis* seedling development. Plant Physiol. *112*, 871–878.

Xie, Q., Guo, H.S., Dallman, G., Fang, S.Y., Weissman, A.M., and Chua, N.H. (2002). SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. Nature *419*, 167–170.

Yang, C.J., Zhang, C., Lu, Y.N., Jin, J.Q., and Wang, X.L. (2011). The mechanisms of brassinosteroids' action: from signal transduction to plant development. Mol. Plant *4*, 588–600.

Yin, Y., Wang, Z.-Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., and Chory, J. (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell *109*, 181–191.

Yin, Y., Vafeados, D., Tao, Y., Yoshida, S., Asami, T., and Chory, J. (2005). A new class of transcription factors mediates brassinosteroid-regulated gene expression in *Arabidopsis*. Cell *120*, 249–259.

Yu, H., Zhang, Y., Moss, B.L., Bargmann, B.O., Wang, R., Prigge, M., Nemhauser, J.L., and Estelle, M. (2015). Untethering the TIR1 auxin receptor from the SCF complex increases its stability and inhibits auxin response. Nat. Plants *1*, http://dx.doi.org/10.1038/nplants.2014.30.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-MBP	The Wang Laboratory	N/A
Mouse monoclonal anti-FLAG	Abmart	Cat. #M20008
Rabbit polyclonal anti-MYC	Sigma-Aldrich	Cat. #PLA0001
Mouse monoclonal anti-MYC	Abmart	Cat. #M20002
Mouse monoclonal anti-Ub	The Xie Laboratory	N/A
Mouse monoclonal anti-Ub	SANTA CRUZ BIOTECHNOLOGY	Cat. #Ub (P4D1): sc-8017; RRID:AB_628423
Mouse monoclonal anti-GST	ENZYBIO	BE2013
Rabbit polyclonal anti-BES1	The Yin Laboratory	N/A
Mouse monoclonal anti-ACTIN	Abmart	Cat. #M20009
anti-FLAG M2 Affinity Gel	Sigma-Aldrich	Cat. #A2220-5ML; RRID:AB_10063035
anti-c-MYC agarose gel	Sigma-Aldrich	Cat. #A7470-1ML; RRID:AB_10109522
Chemicals, Peptides, and Recombinant Proteins		
MG132	Sigma-Aldrich	Cat. #C2211-5MG; CAS: 133407-82-6
Cycloheximide (CHX)	Sigma-Aldrich	Cat. #01810-1G; CAS: 66-81-9
Protease inhibitor cocktail	Sigma-Aldrich	Cat. #P9599-5ML
Experimental Models: Organisms/Strains		
Arabidopsis: 35S::BES1-FLAG	The Wang Laboratory	N/A
Arabidopsis: 35S::SINAT1-FLAG	This paper	N/A
Arabidopsis: 35S::SINAT2-FLAG	This paper	N/A
Arabidopsis: 35S::SINAT3-FLAG	This paper	N/A
Arabidopsis: 35S::SINAT4-FLAG	This paper	N/A
Arabidopsis: 35S::SINAT5-FLAG	This paper	N/A
Arabidopsis: 35S::BES1-MYC	This paper	N/A
Arabidopsis: pSINAT2::GUS	This paper	N/A
Arabidopsis: pSINAT4::GUS	This paper	N/A
Arabidopsis: pSINAT1::GUS	This paper	N/A
Arabidopsis: 35S::GFP-SINAT2	This paper	N/A
Arabidopsis: SINATs-RNAi	This paper	N/A
Arabidopsis: BES1-RNAi	Yin et al., 2005	N/A
Arabidopsis: *cop1-4*	The Yang Laboratory, Mcnellis et al., 1994	N/A
Oligonucleotides		
Primers for quantitative RT-PCR	See Table S1	N/A
Recombinant DNA		
pGBKT7-BES1	This paper	N/A
pGADT7-SINAT2	This paper	N/A
pGEX-4T-1-SINAT	This paper	N/A
pMAL-c2x-BES1 (Full length of wild type fusion, truncated fusion and point-mutated fusion)	This paper	N/A
PET28a-wheat E1	The Xie Laboratory	N/A
PET28a- human E2 UbcH5B	The Xie Laboratory	N/A
PET28a-Ub	The Xie Laboratory	N/A
pXY104-BES1/BZR1/BEH1, 2, 3, 4-cYFP	The Wang Laboratory, Wang et al., 2013	N/A
pXY104-bes1-D-cYFP	This paper	N/A
pXY106-nYFP-SINAT/nSINAT/cSINAT/mSINAT	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pCAMBIA2300-BES1-MYC	This paper	N/A
pCAMBIA1306-SINAT-FLAG	This paper	N/A
pCAMBIA1300-221-GFP-SINAT	This paper	N/A
pFGC5941-SINATs-RNAi	This paper	N/A
pCAMBIA1300-221-pSINAT2,4,1-GUS	This paper	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Xuelu Wang (xlwang@mail.hzau.edu.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis

The Columbia (Col-0) ecotype, as well as the *35S:BES1-FLAG* line, the *35S:BES1-MYC* line, the *BES1-RNAi* line, the *SINATs-RNAi* lines, the *35S:SINAT-FLAG* lines, the *pSINAT2-GUS* line, the *pSINAT4-GUS* line, the *pSINAT1-GUS* line, the *35S:GFP-SINAT2* lines, and the *cop1-4* mutant were used in this study. All of the transgenic lines and mutants are in the Col-0 ecotype. Seeds were germinated on 1/2 MS medium or directly in soil. Plants were grown at 23°C under long day conditions (16 h light/8 h dark cycles), unless otherwise stated. Generation of transgenic lines and phenotypic analysis were described in the Method Details.

METHOD DETAILS

Construction of Transgenic Lines

To create the knockdown lines of *SINATs*, a "two-hit" artificial miRNA (amiRNAs) strategy was used as described (Ji et al., 2011; Tang et al., 2012; Teotia et al., 2016). Briefly, two artificial microRNAs (amiRNAs: amiR1 and amiR2) were constructed using a 2× 35S promoter and a 35S terminator in pOT2-Poly-Cis vector, resulting in pOT2-*SINATs*-amiR1-amiR2. The pOT2-*SINATs*-amiR1-amiR2 was then sub-cloned into the binary vector pFGC5941-PacI for plant transformation and production of transgenic plants. For overexpression of *SINATs*, the CDS of each *SINAT* was cloned into the binary vector pCAMBIA1306 fused with a FLAG-tag at the C-terminus. The constructs were then transformed into Col-0 via the Agrobacterium strain GV3101.

Phenotypic Analysis

For hypocotyl length analysis, plants were grown at 23°C under long-day conditions (16 h light/8 h dark cycles) with a photon fluence rate of 120 μmol m^{-2} s^{-1} for *SINATs-RNAi* lines and 25 μmol m^{-2} s^{-1} for *SINAT* overexpression lines. Hypocotyl length was measured with ImageJ software. For petiole length and leaf length/width ratio analysis, plants were grown for three weeks or six weeks, and the fifth leaves were harvested and photographed for further analysis. The petiole length, leaf length, and leaf width were measured with ImageJ software. Measurements of the leaf length and leaf width were taken at the longest and widest point of each leaf.

Yeast-Two-Hybrid Assays

The yeast-two-hybrid analysis was performed with the Yeast Matchmaker 3 Two-hybrid System (Clontech). The yeast-two-hybrid screen was done as previously described (Yin et al., 2005). To confirm the interaction, full-length *BES1* was cloned into pGBKT7 using *Eco*RI and *Sma*I sites and *SINAT2* was cloned into pGADT7 using *Eco*RI and *Bam*HI sites. The resulting plasmids were transformed into Y187 yeast and selected on -Leu -Trp plates. Positive colonies were used for LacZ filter assays using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma). Reactions were allowed to proceed for 3-4 h before image acquisition.

In Vitro Pull-Down Assay

The CDS (coding sequence) of each *SINAT* was cloned into pGEX-4T-1 to obtain GST-SINAT recombinant proteins. For MBP-BES1 and truncated MBP-BES1 recombinant proteins, the corresponding CDS was cloned into pMAL-c2x. GST fusion proteins and MBP fusion proteins were purified using glutathione beads (Genscript) and amylose resin (NEB), respectively. Glutathione beads containing GST or GST-SINATs were incubated with MBP, MBP-BES1, or truncated MBP-BES1 proteins in 1×PBS buffer at 4°C for 1 h. The beads were washed 8-10 times with wash buffer (1× PBS, 0.1% Triton X-100) and boiled with 1×SDS loading buffer, separated by SDS-PAGE, and immunoblotted with anti-MBP antibodies (produced in our lab by rabbits immunized with full-length MBP protein).

Semi-In Vivo Pull-Down Assay

The semi-*in vivo* pull-down assays were performed as described previously (Cai et al., 2014). *35S:BES1-FLAG* transgenic plants were grown on 1/2 MS medium with or without 1 μM BRZ for 15 d and seedlings were treated with 1 μM eBL for 1 h. Plant materials were

ground to powder in liquid nitrogen and solubilized with 2× protein extraction buffer (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol, and protease inhibitor). The extracts were centrifuged twice at 12,000 rpm for 10 min and the resulting supernatants were collected and incubated with either GST or GST-SINATs pre-incubated GST beads at 4°C for 1 h. The beads were washed 8-10 times with wash buffer and boiled with 1×SDS loading buffer, separated by SDS-PAGE, and immunoblotted with anti-FLAG antibodies (Abmart).

Co-immunoprecipitation Assay

Transgenic plants were grown on 1/2 MS medium for 15 d, and half of the seedlings were treated with 1 μM eBL for 1 h. Plant materials were ground to fine powder in liquid nitrogen and solubilized with 2× protein extraction buffer (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol, and protease inhibitor). The extracts were centrifuged twice at 12,000 rpm for 10 min and the resulting supernatants were collected and incubated with prewashed anti-FLAG M2 agarose gel (Sigma) at 4°C for 1.5 h. The agarose gel was washed 3 times with the 1× extraction buffer and boiled with 1×SDS loading buffer, separated by SDS-PAGE, and immunoblotted with anti-FLAG antibodies (Abmart) and anti-MYC antibodies (Sigma).

BiFC Assay and Subcellular Localization Analysis

For the bimolecular fluorescence complementation (BiFC) assays, each full-length *SINAT*, *SINAT-N*, or *SINAT-C* was fused with N-terminal YFP, and *BES1*, *BES1* homologs or *bes1-D* were fused with C-terminal YFP. For the observation of subcellular localization, the *SINAT* fragments were inserted into a binary vector pCAMBIA1300-221 fused with GFP at the N-terminus. Agrobacterium strain GV3101 was transformed with the above vector or control vector and then injected into young leaves of *Nicotiana benthamiana*. Plants were grown in the dark for 1 d and then transferred to long-day conditions (16 h light/8 h dark) for 2 d. Fluorescence signals in pavement cells were observed with confocal microscopy (Zeiss for Figures 1H column 1-3, S1H column 1-3, and S1I, Leica SP8 for Figures 1H column 4 and 5, S1H column 4-11, and S3C). For the observation of subcellular localization in *Arabidopsis*, the transgenic line expressing the *35S*:*GFP-SINAT2* were grown on 1/2 MS medium for 9 d and fluorescence signals in root tip cells were observed with confocal microscopy (Leica SP8).

In Vitro Ubiquitination Assay

The in vitro ubiquitination assays were carried out as described (Xie et al., 2002). For the in vitro self-ubiquitination assay, approximately 200 ng of each GST-SINAT fusion protein was mixed with 50 ng of wheat E1, 50 ng of human E2 UbcH5B, and 2 mg of his-ubiquitin. The reactions were done in ubiquitination buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM ATP, 5 mM $MgCl_2$, and 2 mM DTT. After incubating at 30°C for 1.5 h with agitation in an Eppendorf Thermomixer, samples were heated to 95°C in 5×SDS loading buffer and were analyzed by SDS-PAGE and immunoblotted with anti-GST antibodies (ENZYBIO) and anti-Ub antibodies (The Xie Laboratory). To confirm that SINATs mediate BES1 ubiquitination, 50 ng of MBP-BES1 fusion protein was incubated together with the ubiquitination mixture at 30°C for 1.5 h. The mixture was then analyzed by SDS-PAGE and immunoblotted with anti-MBP antibodies.

In Vivo Ubiquitination Assay

The *35S*:*BES1-MYC*/ *SINATs-RNAi* and the *35S*:*BES1-MYC*/ *SINAT-ox* transgenic plants were grown on 1/2 MS medium for 15 days and were treated with 50 μM MG132 for 1 h. Plant materials were ground to powder in liquid nitrogen and solubilized with 2× protein extraction buffer (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol, 50 μM MG132, and protease inhibitor). The extracts were centrifuged twice at 12,000 rpm for 10 min and the resulting supernatants were collected and incubated with prewashed anti-c-MYC agarose gel (Sigma) at 4°C for 1.5 h. The agarose gel was washed 3 times with the 1× extraction buffer and boiled with 1×SDS loading buffer, separated by SDS-PAGE, and immunoblotted with anti-MYC antibodies (Abmart) and anti-Ub (SANTA CRUZ BIOTECHNOLOGY) antibodies.

Cell-Free Protein Degradation Assay

The cell-free protein degradation assays were performed as described (Wang et al., 2013). Plants were grown at 23°C in long-day conditions (16 h light/8 h dark cycles) and were ground to powder in liquid nitrogen. Total proteins were extracted with degradation buffer (25 mM Tris-HCl, [pH 7.5], 10 mM NaCl, 10 mM $MgCl_2$, 4 mM PMSF, 5 mM DTT, and 10 mM ATP) and cell debris was removed by centrifugation at 12,000 rpm for 10 min. Total protein extracts from each of the plant material were adjusted to equal concentrations with the degradation buffer. Then, 100 ng of recombinant MBP-BES1, MBP-bes1-D, or MBP proteins were added in 100 μL plant extracts (containing 500 mg total proteins) for individual assays. The reaction mixtures were incubated at 30°C for the indicated time. Reactions were blocked by adding 5×SDS loading buffer and samples were used for the immunoblotting analysis with anti-MBP antibodies. The band intensity was quantified using ImageJ software.

Semi-*In Vivo* Ubiquitination Assay

The recombinant MBP-BES1 protein bound to amylose resin (NEB) was incubated with equal amounts of protein extracts of *SINATs-RNAi* and *SINAT2ox* plants in degradation buffer with or without 50 μM MG132. After incubated at 30 °C for the 2 hours, The resin was washed 3 times with the 1× extraction buffer and boiled with 1×SDS loading buffer, separated by SDS-PAGE, and immunoblotted with anti-MBP antibodies and anti-Ub (SANTA CRUZ BIOTECHNOLOGY) antibodies.

Gene Expression Analysis by Quantitative RT-PCR

Total RNAs were extracted using the Tiangen RNApre Plant Kit (Tiangen) and 2 μg of total RNA was used for reverse transcription (M-MLV reverse trascriptase, TaKaRa). For qRT-PCR, cDNAs were combined with SYBR master mix (Invitrogen/Bio-Rad). Primers were designed by Beacon design 8.0 software. A U-box gene (*At5g15400*) was used to normalize the data. qRT-PCR was performed in triplicate with an Eppendorf Thermocycler (Eppendorf) or a Bio-Rad C1000 Thermocycler (Bio-Rad). The data were collected and analyzed with Eppendorf or Bio-Rad real-time PCR detection systems and software. Primers for quantitative RT-PCR are listed in Table S1.

GUS Staining Assay

Gus staining assays were performed as described (Cheng et al., 2014; Jiang et al., 2015). Transgenic plants containing the *pSINAT2*:*GUS*, *pSINAT4*:*GUS*, and *pSINAT1*:*GUS* reporters were grown on 1/2 MS plates for 4 d or 15 d. Different tissues and organs from adult plants grown on soil were used. The samples were soaked in 90% acetone for 30 min for fixation and then stained in X-Gluc solution (100 mM NaH_2PO_4, pH 7.2, 2 mM K-ferrocyanide, 2 mM X-Gluc, and 0.1% Triton X-100) at 37°C in the dark for 6 to 12 h. Samples were then rinsed sequentially with 75% and 100% ethanol to remove the chlorophyll. Digital images were taken with a stereomicroscope (Zeiss, Discovery V20).

Immunoblotting Analysis of SINAT-FLAG and BES1

For extraction of total proteins, seedlings were grown on 1/2 MS medium at 23°C, ground to a fine powder in liquid nitrogen, and boiled at 95°C for 5 min in 5% SDS. After centrifugation at 12,000 rpm for 10 min, the supernatant containing total proteins was mixed with 5× SDS loading buffer and then separated on 12% SDS-PAGE gels. Since BES1, SINATs-FLAG, and ACTIN have similar molecular weights, we separated our analyses onto two separate blots. One membrane was probed with anti-BES1 or anti-FLAG antibodies and the other with anti-ACTIN (Abmart) antibodies for equal loading.

QUANTIFICATION AND STATISTICAL ANALYSIS

The band intensity of western blotting, as well as hypocotyl length, petiole length, leaf length, and leaf width were measured with ImageJ software. The data for qRT-PCR were collected with Eppendorf or Bio-Rad real-time PCR detection systems. These data were assumed to follow normal distributions and were subjected to two-tailed Student's t test. Statistical tests were performed in Microsoft Excel 2011. Statistical parameters including the exact value of n, the precision measures (mean ± SD) and statistical significance can be found in the Figure Legends. In Figure Legends, n means number of plants for phenotypic analysis, or numbers of biological replications for western blotting, or numbers of technical replicates for qRT-PCR. In Figures, asterisks denote statistical significance test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) as compared to wild-type controls, unless otherwise specified by lines connecting the compared pieces of data.

Developmental Cell, Volume *41*

Supplemental Information

SINAT E3 Ligases Control the Light-Mediated Stability of the Brassinosteroid-Activated Transcription Factor BES1 in *Arabidopsis*

Mengran Yang, Chengxiang Li, Zhenying Cai, Yinmeng Hu, Trevor Nolan, Feifei Yu, Yanhai Yin, Qi Xie, Guiliang Tang, and Xuelu Wang

Supplemental Inventory

Supplemental Figures

Figure S1, related to Figure 1. SINATs interact with BES1 and its homologs.

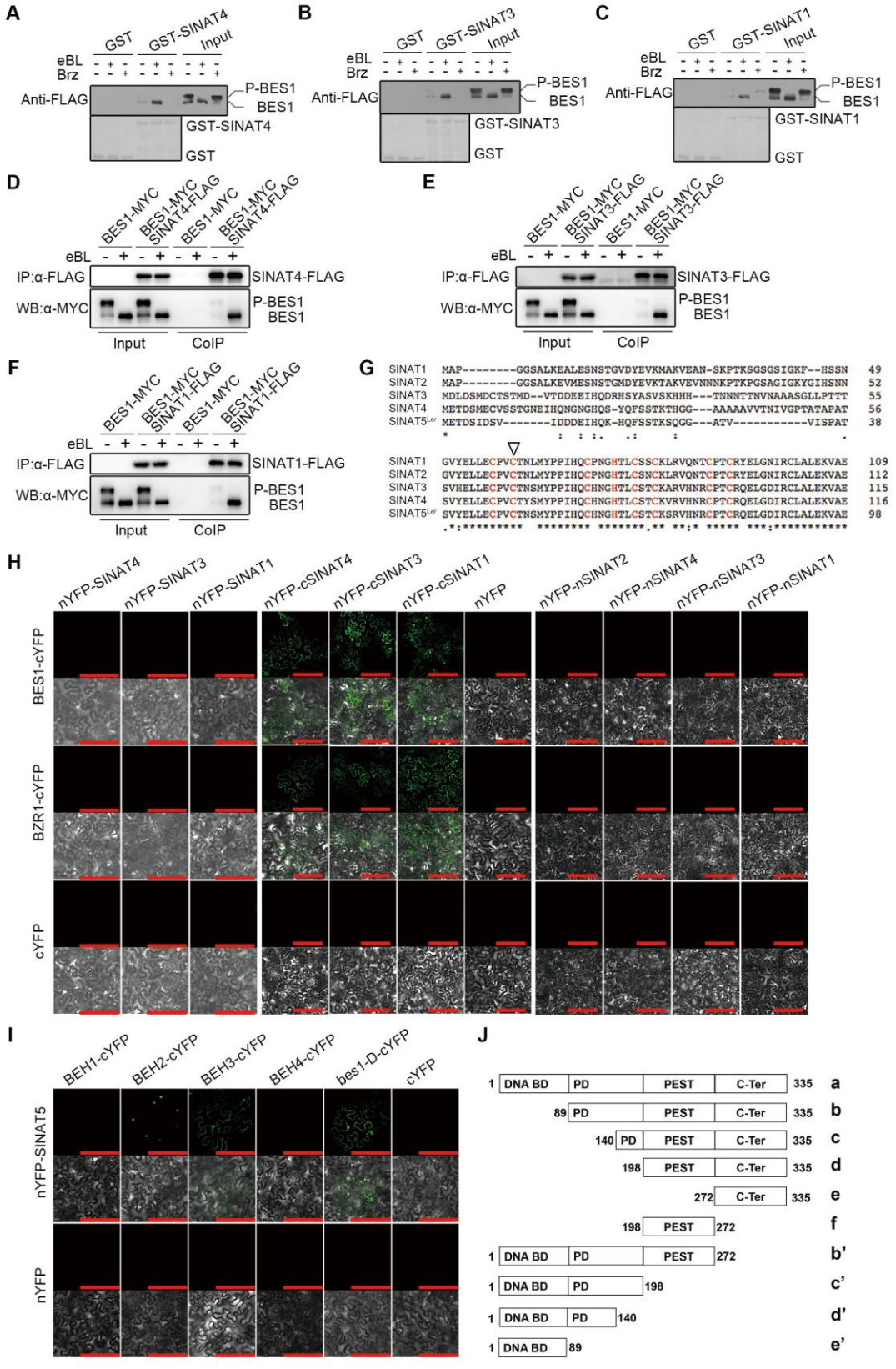


Figure S2, related to Figure 2. SINAT4, SINAT3, and SINAT1 function as E3 Ub-ligases to mediate BES1 ubiquitination and degradation.

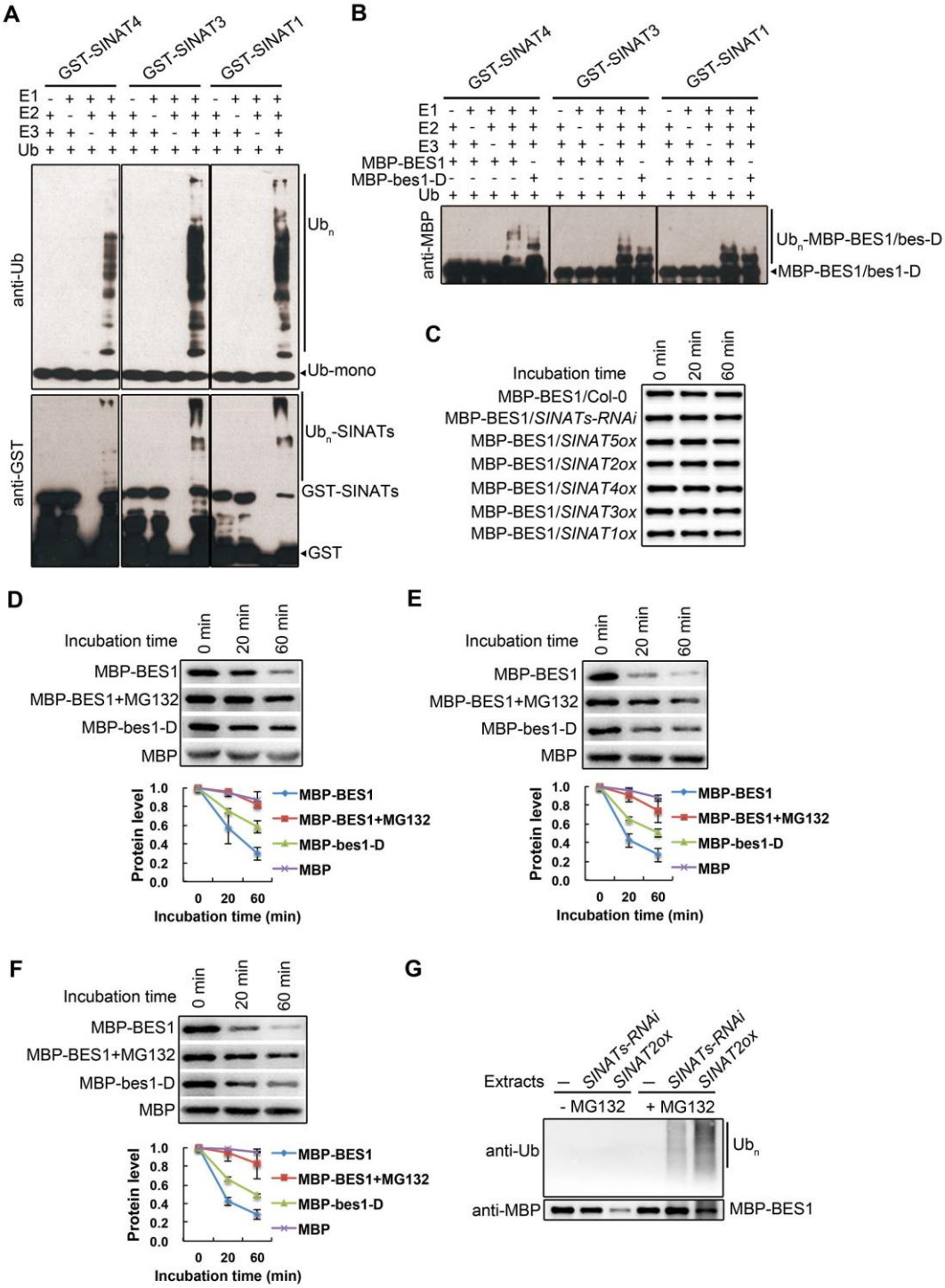


Figure S3, related to Figure 3. *SINATs* are ubiquitously expressed.

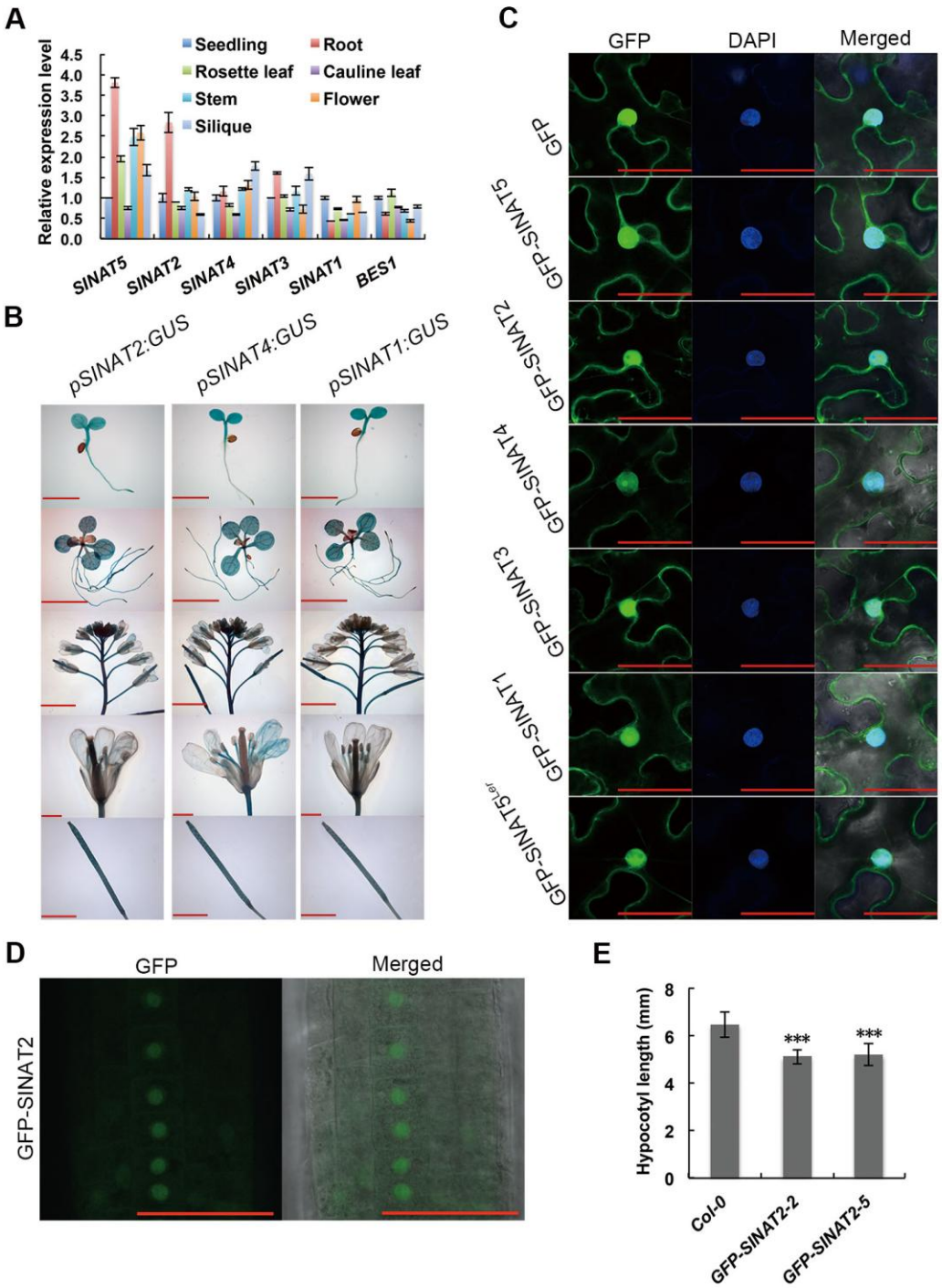


Figure S4, related to Figure 3. Over-expression of *SINAT2, 4, 3, or 1,* but not *SINAT5,* inhibits BR signaling.

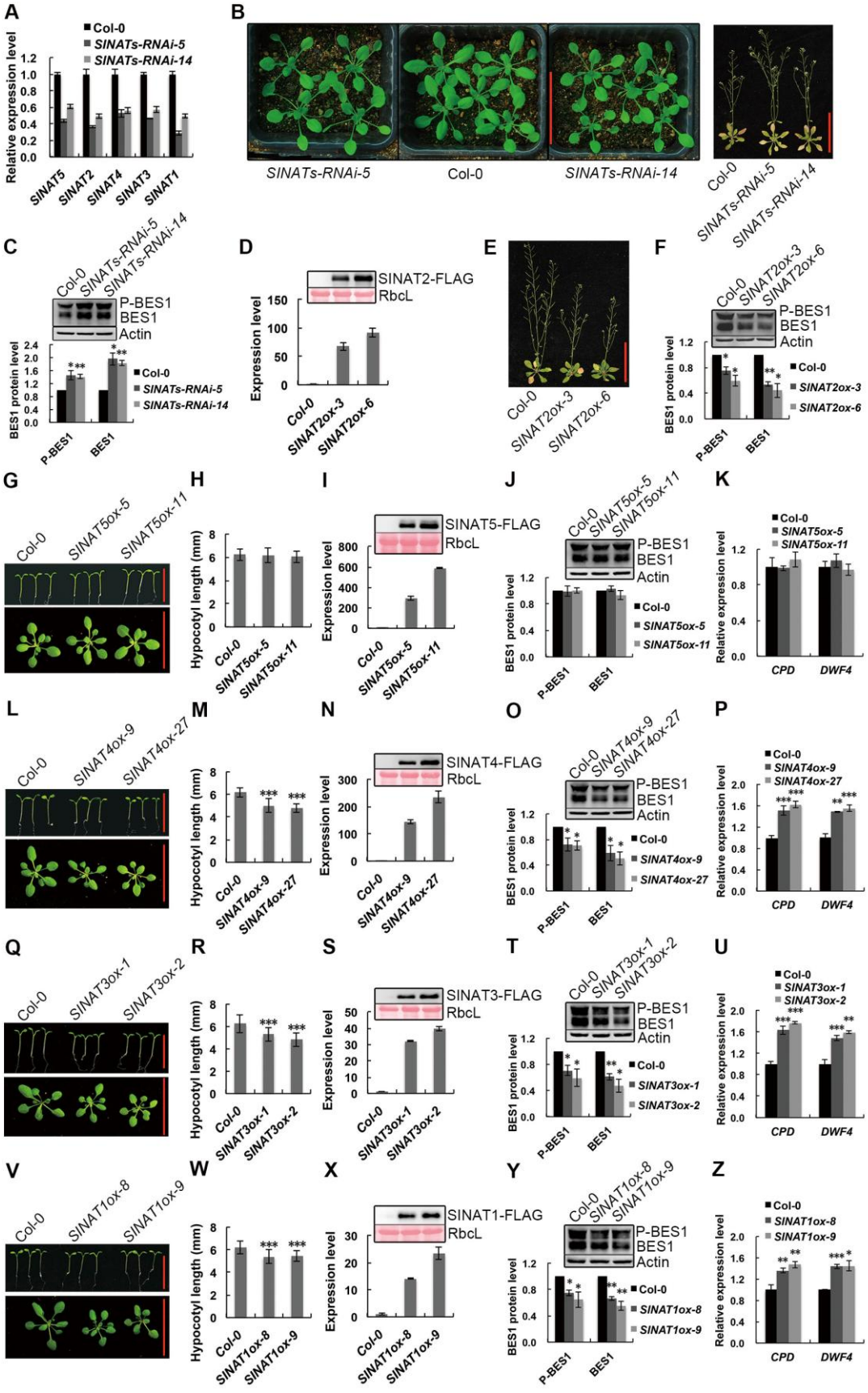


Figure S5, related to Figure 5. Light, not BRs, controls the protein levels of SINAT4, 3, and 1.

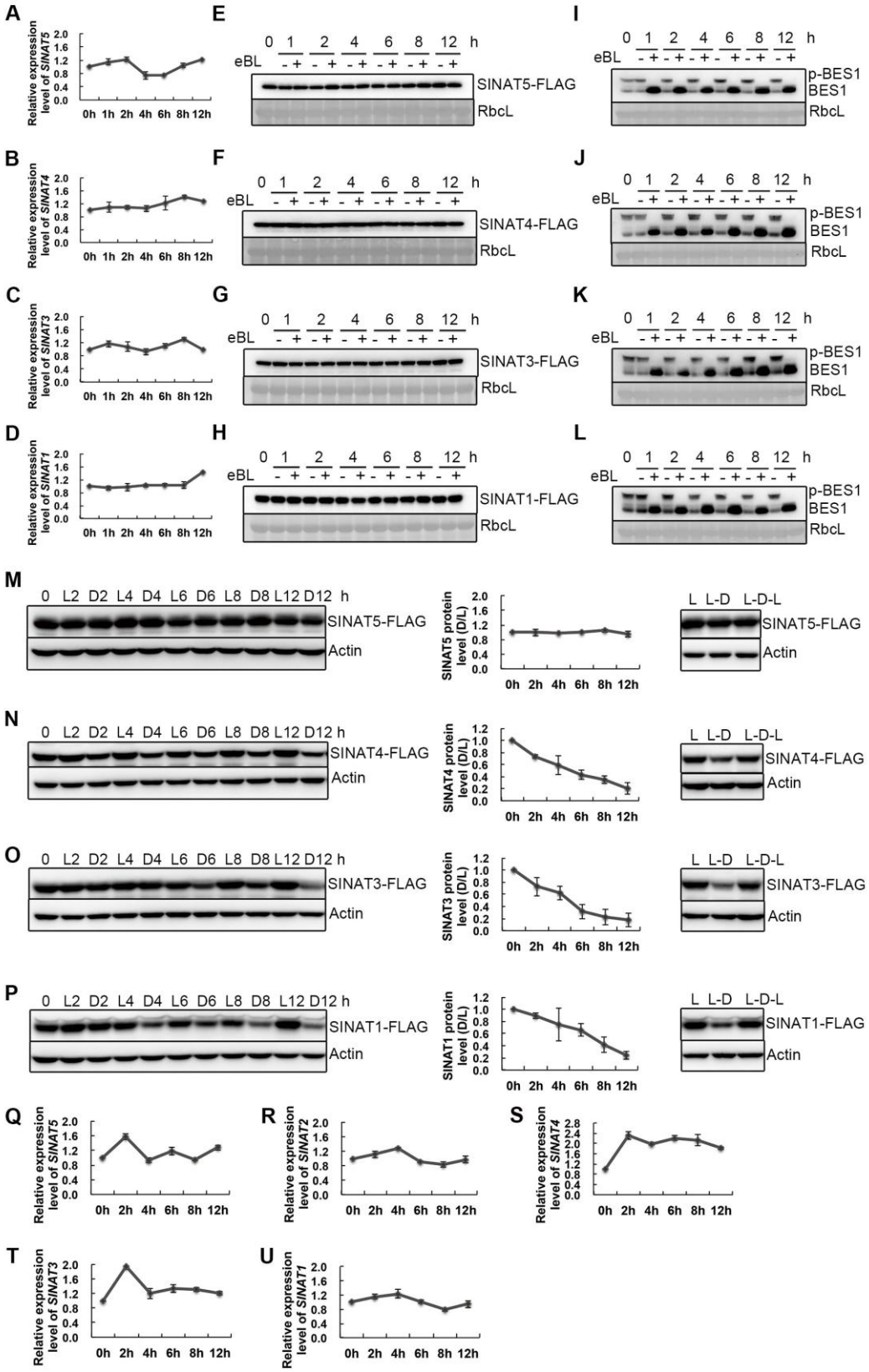
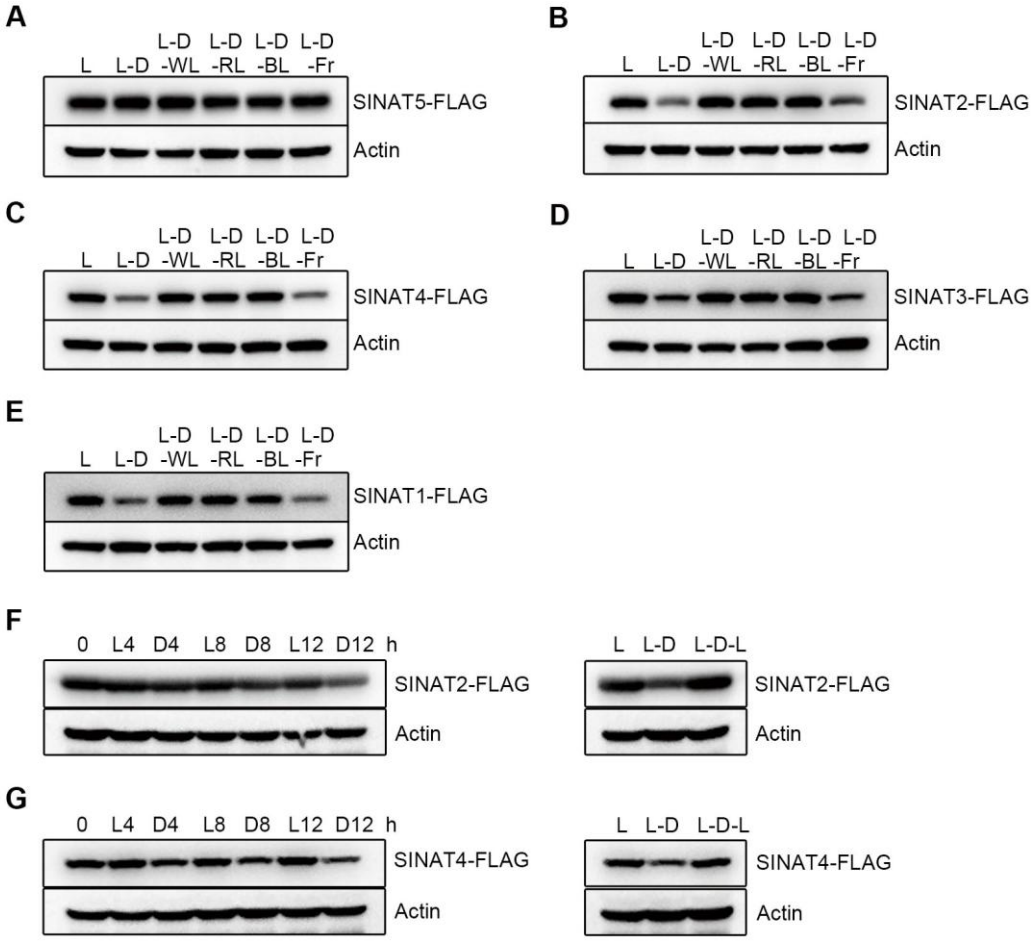


Figure S6, related to Figure 5. Both red light and blue light but not far-red light enhance the levels of SINATs and the response of SINATs to light was independent of COP1.



Supplemental Figure Legends

Figure S1, related to Figure 1. SINATs interact with BES1 and its homologs.
(A-C) SINAT4, SINAT3, and SINAT1 mainly interacted with dephosphorylated BES1 in the semi-*in vivo* pull-down assays. GST and GST-SINAT4, 3, and 1 fusion proteins purified from *E. coli* were used to pull down interacting proteins. The pulled-down BES1-FLAG from the total protein extract of the *BES1-FLAG* over-expression line was detected by western blotting with anti-FLAG antibodies. GST and GST-SINAT4, 3, and 1 stained with Ponceau S were used for equal loading.
(D-F) SINAT4, SINAT3, and SINAT1 mainly interacted with the dephosphorylated BES1 in the Co-IP assays. Plants expressing both the *35S:SINAT-FLAG* and the *35S:BES1-MYC* were used. Plants solely expressing the *35S:BES1-MYC* were used as a negative control. The SINAT-FLAG was IPed with anti-FLAG agarose, and the Co-IPed BES1-MYC was detected by western blotting with anti-MYC antibodies.
(G) Amino acid alignment of partial sequences of SINAT1, 2, 3, 4, and SINAT5^{Ler}. Red letters indicate conserved cysteine and histidine residues in the RING domains. Triangle indicates the mutant cysteine of mSINAT2.
(H) The C-terminal regions (SINA domains), but not the RING domains, of SINAT4, 3, and 1 interacted with BES1 and BZR1 in the BiFC assays. Scale bars: 200 μM.
(I) SINAT5 interacted with BES1 homologs BEH2 and BEH3 and bes1-D in the BiFC assays. Scale bars: 200 μM.
(J) Schematic diagram of various truncated BES1s. Numbers indicate the amino acid positions of these BES1 variants.

Figure S2, related to Figure 2. SINAT4, SINAT3, and SINAT1 function as E3 Ub-ligases to mediate BES1 ubiquitination and degradation.
(A) Detection of self-ubiquitination of SINAT4, SINAT3, and SINAT1 *in vitro*. Ubiquitin attached protein bands were detected by western blotting with anti-Ub antibodies (upper gels) and anti-GST antibodies (lower gels).
(B) Detection of the ubiquitination of BES1 and bes1-D by SINAT4, SINAT3, and SINAT1 *in vitro*. The full-length MBP-BES1 and MBP-bes1-D fusion proteins were used as substrates for the *in vitro* ubiquitination assays. Anti-MBP antibodies were used in western blotting for detecting substrates.
(C) Cell-free degradation assays using MBP as a substrate for the negative control of Figure 2D. The protein level of MBP was determined by western blotting with anti-MBP antibodies.
(D-F) Cell-free degradation assays showed the proteasome-dependent degradation of MBP-BES1 and delayed degradation of MBP-bes1-D in the over-expression lines of *SINAT4* (D), *SINAT3* (E), and *SINAT1* (F). MBP was used as a substrate for the negative control. Upper panels: The protein levels were detected by western blotting with anti-MBP antibodies. Lower panels: Quantitative results for western blotting in upper panels. The initial protein levels were defined as "1". The data are means ± SD (n = 3).
(G) Detection of BES1 ubiquitination in the semi-*in vivo* ubiquitination assays. Recombinant MBP-BES1 protein was incubated with protein extracts from the *SINATs-RNAi* line or the *SINAT2*-over-expression line in degradation buffer with or without MG132 for 2 hrs. MBP-BES1 and MBP-BES1-Ub proteins were detected by western blotting with anti-Ub antibodies (upper gels) and anti-MBP antibodies (lower gels).

Figure S3, related to Figure 3. *SINATs* are ubiquitously expressed.

(A) qRT-PCR analysis of *SINATs* and *BES1* transcripts in the wild-type Columbia (Col-0). The expression level in seedlings was defined as "1". The data are means ± SD (n = 3).

(B) GUS staining of the *pSINAT2, 4, or 1:GUS* reporter lines. GUS activity was observed in the four-day-old seedlings (scale bars: 2 mm), fifteen-day-old seedlings (scale bars: 5 mm), and in the inflorescences (scale bars: 5 mm), flowers (scale bars: 1 mm), and siliques (scale bars: 5 mm) of adult plants.

(C) Subcellular localization of GFP, GFP-SINAT5, 2, 4, 3 and 1, and GFP-SINAT5^{Ler} in tobacco pavement cells. Scale bars: 50 μM.

(D) Subcellular localization of GFP-SINAT2 in the *Arabidopsis* root tip cells. Scale bars: 50 μM.

(E) Hypocotyl length of the *35S:GFP-SINAT2* transgenic lines grown on 1⁄2 MS medium under dim light (25 μmol m^{-2} s^{-1}) for nine days. The data are means ± SD (n > 20). ***p < 0.001.

Figure S4, related to Figure 3. Over-expression of *SINAT2, 4, 3, or 1,* but not *SINAT5,* inhibits BR signaling.

(A) Expression levels of *SINATs* in the *SINATs-RNAi* lines.

(B) Phenotypes of the *SINATs-RNAi* lines grown on soil for three weeks (left panel) or six weeks (right panel). Scales bars: 5 cm (left) and 10 cm (right).

(C) Endogenous BES1 detected with anti-BES1 antibodies in the wild type and the *SINATs-RNAi* lines. ACTIN was used for equal loading. The protein levels of phosphorylated and dephosphorylated BES1 in the wild type were defined as "1". The data are means ± SD (n = 3). *p < 0.05, **p < 0.01.

(D) Relative expression levels of *SINAT2*. The expression level in Col-0 was defined as "1". The data are means ± SD (n = 3). SINAT2-FLAG was detected by western blotting with anti-FLAG antibodies. The Rubisco large subunit (RbcL) stained with Ponceau S was used for equal loading.

(E) Phenotypes of the *SINAT2* over-expression lines grown on soil for six weeks. Scales bar: 10 cm.

(F) Endogenous BES1 detected with anti-BES1 antibodies in wild type and the *SINAT2* over-expression lines. ACTIN was used for equal loading. The protein levels of phosphorylated and dephosphorylated BES1 in the wild type were defined as "1". The data are means ± SD (n = 3). *p < 0.05, **p < 0.01.

(G), (L), (Q), and (V) Seedling phenotypes of the *SINAT5* over-expression (G), *SINAT4* over-expression (L), *SINAT3* over-expression (Q), and *SINAT1* over-expression (V) lines grown on 1⁄2 MS medium under dim light (25 μmol m^{-2} s^{-1}) for nine days (upper) and in soil for three weeks (lower). Scales bars: 1 cm (upper) and 5 cm (lower).

(H), (M), (R), and (W) Hypocotyl lengths of nine-day-old seedlings in (G), (L), (Q), and (V), respectively. The data are means ± SD (n > 20). ***p < 0.001.

(I), (N), (S), and (X) Relative expression levels of *SINAT5* (I), *SINAT4* (N), *SINAT3* (S), and *SINAT1* (X) in their corresponding over-expression lines and wild type. The expression level of each *SINAT* in Col-0 was defined as "1". The data are means ± SD (n = 3). SINAT5-, 4-, 3-, and 1-FLAG in their over-expression lines were detected by western blotting with anti-FLAG antibodies. The Rubisco large subunit (RbcL) stained with Ponceau S was used for equal loading.

(J), (O), (T), and (Y) The protein level of endogenous BES1 in seedlings of the wild-type and the *SINAT5* (J), *SINAT4* (O), *SINAT3* (T), and *SINAT1* (Y) over-expression lines. BES1 was detected by western blotting with anti-BES1 antibodies. Endogenous ACTIN detected with anti-ACTIN antibodies was used for equal loading. The protein levels of phosphorylated and dephosphorylated BES1 in the

wild type were defined as "1". The data are means ± SD (n = 3). *p < 0.05, **p < 0.01.
(K), (P), (U), and (Z) Relative expression levels of *CPD* and *DWF4* in seedlings of the wild-type and the *SINAT5* (K), *SINAT4* (P), *SINAT3* (U), and *SINAT1* (Z) over-expression lines. The expression levels of *CPD* and *DWF4* in Col-0 were defined as "1". The data are means ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

Figure S5, related to Figure 5. Light, not BRs, controls the protein levels of SINAT4, 3, and 1.
(A-D) Relative transcript levels of *SINAT5* (A), *SINAT4* (B), *SINAT3* (C), and *SINAT1* (D) in Col-0 treated with 5 μM eBL. The data are normalized to mock treatment at each time point. The data are means ± SD (n = 3).
(E-H) Protein levels of SINAT5 (E), SINAT4 (F), SINAT3 (G), and SINAT1 (H) with or without eBL treatment. The experimental conditions were the same as in Figure 5, (B).
(I-L) The phosphorylation status of BES1 detected with anti-BES1 antibodies in samples used in (E-H). The Rubisco large subunit (RbcL) stained with Ponceau S was used for equal loading.
(M-P) Protein levels of SINAT5 (M), SINAT4 (N), SINAT3 (O), and SINAT1 (P) under different light conditions. The experimental conditions were the same as in Figure 5, (H).
(Q-U) Transcript levels of *SINAT5* (Q), *SINAT2* (R), *SINAT4* (S), *SINAT3* (T) and *SINAT1* (U) in Col-0 with dark treatment. The experimental conditions were the same as in Figure 5 (H, left panel). The data are normalized to that in light-grown seedlings at each time point. The data are means ± SD (n = 3).

Figure S6, related to Figure 5. Both red light and blue light but not far-red light enhance the levels of SINATs and the response of SINATs to light was independent of COP1.
(A-E) SINAT5 (A), SINAT2 (B), SINAT4 (C), SINAT3 (D), and SINAT1 (E) protein levels under different light conditions. The *35S:SINAT5*, *2*, *4*, *3* and *1-FLAG* seedlings were grown under continuous light for five days and either remained in the light (L) or were transferred to the dark (L-D) for six hours. Then the dark-treated seedlings were transferred to the white light (L-D-WL), red light (L-D-RL), blue light (L-D-BL), or far-red light (L-D-Fr) for another six hours. Endogenous ACTIN was used for equal loading.
(F-G) SINAT2 (F) and SINAT4 (G) protein levels in *cop1-4* mutant under different light conditions. The *35S:SINAT2-FLAG/cop1-4* and *35S:SINAT4-FLAG/cop1-4* seedlings were used. Left panels: seedlings grown under continuous light for five days and either remained in the light (L) or were transferred to the dark (D) for the indicated time period. Right panels: L, continuous light for five days; L-D, the continuous light-grown seedlings were transferred to the dark for six hours; L-D-L, the seedlings transferred to the dark for six hours were then transferred back to the light for another six hours. Endogenous ACTIN was used for equal loading.

Supplemental Tables

Table S1, related to STAR methods. Primers for quantitative RT-PCR.

Genes	**Oligonucleotide primers (from 5' to 3')**
SINAT5 (AT5g53360)	Forward: GCATAATCGCTCTTGAGAA Reverse: TCACCTACAGCAGCACACTC
SINAT2 (AT3g58040)	Forward: CCTACAAAGCCAGGGAGTG Reverse: CCCAAATTTTGGTACCGG
SINAT4 (AT4g27880)	Forward: GGTAATGGAGACAGATAGTATGG Reverse: CGAAGAGAATTGGTAAGATTGG
SINAT3 (AT3g61790)	Forward: CTGCTTCTGGGCTTCTCC Reverse: CGGTTGTGAACCCTGGC
SINAT1 (AT2g41980)	Forward: CAACAAAATCAGGTAGTGGCT Reverse: CAATGTGTGGCCGTTCG
BES1 (AT1g19350)	Forward: AACCACACTGTAAGTTAC Reverse: ACCGTTGATTTGAGTTAA
CPD (AT5g05690)	Forward: TTACCGCAAAGCCATCCAAG Reverse: TCCATCATCCGCCGCAAG
DWF4 (AT3g50660)	Forward: CATTGCTCTCGCTATCTTCTTC Reverse: GACTCTCCTAGTTCCTTCTTGG
PRE1 (AT5g39860)	Forward: GGCAATCTTCAAGTGCTCCAAGG Reverse: ACTTTCGAGGCTGATGCCTTATC
PRE5 (AT3g28857)	Forward: ACGGCGTCGTTCTGATAAGGTG Reverse: AGTCAAGAAGCTGCGACAAACG
IAA19 (AT3g15540)	Forward: TGGTTCGAGCCAAGGCTATGATG Reverse: CATCTTTCAAGGCCACACCGATGC
ACS5 (AT5g65800)	Forward: ACGAGTCCACGTCGTTTATAGCC Reverse: TAGATCGCTCCCACACGGAAAC
Saur_AC1 (AT4g38850)	Forward: CAGAAGAAGAAGAGATATGTGGTG Reverse: GTATTGTTAAGCCGCCCATTG
ATEXP8 (AT2g40610)	Forward: TGGTGCAATCCTCCTCTTCAGC Reverse: TGGTACTCTTCGGAAAGAGACAGG
LHY1 (AT1g01060)	Forward: GAGCTTGGCAACGAATTGAAGAAC Reverse: AAAGCTTGGCAAACAGGGATGC
IBH1 (AT2g43060)	Forward: AGAGGCTGAGGAATCTTGTTCCG Reverse: CCGTCTCTTCCATCAGCTTTGACG
U-box (At5g15400)	Forward: TCTTCTTCTGCTACATCTACTCTC Reverse: AGTGTGTGAACCCGTGAAC