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Overexpression of miR172 suppresses the brassinosteroid signaling defects of *bak1* in Arabidopsis

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ABSTRACT

BRI1-Associated Receptor Kinase 1 (BAK1) is a leucine-rich repeat serine/threonine receptor-like kinase (LRR-RLK) that is involved in multiple developmental pathways, such as brassinosteroid (BR) signaling, plant immunity and cell death control in plants. Because the roundish and compact rosette leaves of *bak1* mutant plants are characteristic phenotypes for deficient BR signaling, we screened genetic suppressors of *bak1* according to changes in leaf shape to identify new components that may be involved in BAK1-mediated BR signaling using the activation-tagging method. Here, we report *bak1-SUP1*, which exhibited longer and narrower rosette leaves and an increased BR sensitivity compared with those of *bak1*. Analyses of the T-DNA insertional site and the gene expression that was affected by the T-DNA insertion revealed that a microRNA, namely, miR172, over-accumulates in *bak1-SUP1*. Detailed phenotypic analyses of *bak1-SUP1* and a single mutant in which the *bak1* mutation was segregated out (*miR172-D*) revealed that the overexpression of miR172 promotes leaf length elongation in adult plants and increases the root and hypocotyl growth during the seedling stage compared with that of wild type plants. Taken together with its increased BR sensitivity, these results suggest that miR172 regulates vegetative growth patterns by modulating BR sensitivity as well as by the previously identified developmental phase transition.

1. Introduction

Throughout plant life, plant development is affected by complex temporal and spatial networks of regulatory gene expression patterns. Phytohormones are central coordinators that regulate these aspects in various ways [1]. The plant-specific steroidal hormones, brassinosteroids (BR) are involved in diverse physiological processes, such as cell elongation, photomorphogenesis, xylem differentiation, and pollen tube development through BR-regulated gene expressions [2]. BRs are perceived by the twenty-five leucine-rich repeat serine/threonine receptor-like kinase, Brassinosteroid-Insensitive 1 (BRI1), in the plasma membrane [3]. BRI1-Associated Receptor Kinase 1 (BAK1) was first identified as an interacting protein with BRI1 [4,5]. As *bak1* mutants are semi-dwarfed with the round and compact rosette architecture that is characteristic of many BR-biosynthetic or BR-signaling mutants and show reduced BR sensitivity, BAK1 is thought to be a co-receptor of BRI1 for mediating BR signaling. BAK1 was also known to form complexes with another ligand-binding receptors, FLAGELLIN SENSITIVE 2 (FLS2) and Elongation Factor-Tu Receptor 1 (EFR1), which bind to bacterial flagellin and elongation factor Tu, respectively [6] and are involved in plant innate immunity [7,8]. Therefore, *bak1* mutants also have impaired responses upon infection by several types of pathogenic organisms [9]. In addition, based on the enhanced cell death phenotype of the double mutants of *BAK1* and its closest homolog *BKK1*, BAK1 is thought to be involved in the negative regulation of cell death [10]. These results suggest that BAK1 functions in multiple pathways and has a great potential to elucidate the regulatory mechanisms of plant development. Therefore, the mechanism of how certain specific complexes containing BAK1 are temporarily or spatially partitioned in plants is being pursued in many research fields.

MicroRNAs (miRNAs) are 20- to 24-nucleotide small non-coding RNAs (sRNAs) that affect almost all aspects of plant development [11]. Mature miRNAs are generated through several steps: *MIRNA* genes are first transcribed into the primary miRNA by RNA polymerase II, forming secondary stem and loop hairpin structures

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[12]. DCL1 cleaves the hairpin structure, generating short functional miRNAs [13]. The regulatory functions of miRNAs are operated through a post-transcriptional cleavage or a translational inhibition of the target mRNAs [14,15]. The antagonistic action between miR156 and miR172 and their corresponding target genes encoding Squamosa Promoter Binding Protein-like (SPL) and Apetala 2 (AP2)-like transcriptional factors, respectively, is one of best known examples of a transit developmental phase [16,17]. The formation of leaves from the boundary regions of the meristem is also regulated by *CUC1* and *CUC2*, targets of miR164 [18]. TCPs modulate the expression of miR164. MiR165/miR166 is involved in the control of HD-ZIP III proteins, such as PHAVOLUTA, PHABULOSA and REVOLUTA [19].

The expressions of miRNAs are up- and down-regulated by phytohormones. In ABA-treated germinating Arabidopsis seeds, the expression of miR159 was increased. The overexpression of miR159 led to the cleavage of MYB33 and MYB101, causing hyposensitivity to ABA. These results suggest that miR159 is involved in resetting the ABA responses [20]. In Arabidopsis, miR167 decreases the expression of *ARF6* and *ARF8*, regulating the reproductive maturation of the plant [16]. In addition, miR167 induced by auxin decreases the expression of both *ARF8* and *OsGH3-2* in rice culture cells. This effect of auxin mediated by miR167 and resulting down-regulation of *ARF8* and *OsGH3-2* can regulate the cellular free auxin level [21]. More than one class of plant hormones often simultaneously regulates the same miRNAs. The fact that the expression of miR167 is inversely regulated by auxin and ABA implies that cross-talk between auxin and ABA may occur by altering the expression level of miR167. The mRNAs of the other ARFs *ARF10*, *ARF16*, and *ARF17* are cleaved by miR160 [21]. MiR393, through the regulation of F-box proteins, including TIR1, and miR164, through the cleavage of *NAC1* mRNA, also provide an additional layer of auxin signal regulation throughout plant development [22,23]. The expression of miR159 is induced by gibberellin (GA), leading to the repression of *GAMYB*, resulting in the reduction of the expression of *LEAFY*, which promotes flowering [24]. In comparison, miR319 and miR166 are repressed by GA, leading to the additional accumulation of class III homeodomain-leucine-zipper (HD-ZIP) transcriptions factors [25]. However, few reports have been focused on how brassinosteroids (BR) affect the expression of miRNA or how certain miRNA are related to BR responsiveness. In this study, we found that the overexpression of miR172 increases the sensitivity to BR, leading to the partial suppression of the leaf phenotypes of *bak1*.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana Wassilewskija (WS-2) and *bak1-2* [4] were used as the wild type and background for suppressor screening, respectively. For the gross observation of the phenotypic changes, the seeds were sown directly onto Sunshine #5 soil. Otherwise, the seeds were plated onto a 1/2 MS (Duchefa) plate containing 0.8% phytoagar after sterilization with 75% ethanol containing 0.05% Tween-20 for 15 min. All plants were grown at 22 °C under long-day condition (16 h L/8 h D).

2.2. Suppressor screening of bak1-2

Bak1-2 plants were transformed with *Agrobacterium tumefaciens* (GV3101) containing the *pSKI015* plasmid by the floral dipping method. The selection of transgenic plants using commercially available Finale (AgrEvo, Montvale, NJ) was performed as described in detail in Weigel et al. [26].

2.3. Analyses of BR sensitivity

The sterilized seeds of each line were plated onto 1/2 MS (Duchefa) 0.8% phytoagar media supplemented with the brassinolide (BL) in indicated concentrations as described in Yun et al. [27]. Three sets of plates were positioned vertically at 22 °C under long-day conditions (16 h L/8 h D). The root and hypocotyl lengths were measured from 20 to 30 seedlings grown for 7 days. All of the experiments were repeated three times. For the examination of the phosphorylation status of BES1, the total proteins were extracted from 10-day-old seedlings that were grown with or without 1 μM brassinolide (BL) using 2× SDS sample buffer. The protein were separated by 10% SDS–PAGE. A Western blot analysis was performed with the anti-BES1 primary antibodies (customized by the Young In Frontier Co., Seoul, Korea) and peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, Pierce). The phosphorylated and de-phosphorylated BES1 bands were visualized using the ECL system (GE Healthcare).

2.4. RT-PCR analysis

The RNA samples were purified using the RNAiso Plus (Takara) reagent following the manufacturer's protocol and were treated with RNase-free RQ1 DNase (Promega). The first-strand cDNA was synthesized with the Superscript^{III}-MMLV reverse transcriptase (Invitrogen) using oligo-d(T_{15}) as the primer. The second-strand synthesis was performed using the same aliquot of first-strand cDNA as the template. The expression of each gene was normalized to that of β-*Tubulin*. All of the primer sequences that were used in this study are provided in Supplementary Table 1.

2.5. Analysis of miR172 expression

For the small RNA extractions, each tissue sample was ground and incubated with TRI reagent solution following the manufacturer's protocol (Ambion). The small RNA samples (15 μg each) were resolved on denatured 15% polyacrylamide gel containing 7.5 M urea and then blotted onto a Hybond-N+ nylon membrane (GE Healthcare). The nylon membrane was soaked in ULTRAhyb-Oligo Hybridization Buffer (Ambion) and incubated at 42 °C. The radiolabeled single stranded DNA oligonucleotide probes were generated using γ-[^{32}P] ATP (3000 Ci/mmol, PerkinElmer) and T4 polynucleotide kinase (New England Biolabs) and added to the hybridization solution. The sequences of the oligonucleotides were 5′-ATGCAGCATCATCAAGATTCT-3′ for miR172 and 5′-TCATCCTTGCGCAGGGGCCA-3′ for U6 snRNA. After overnight hybridization, the blots were washed, dried, and then analyzed using the Storm 860 phosphoimager (Molecular Dynamics) and ImageQuant TL software (GE Healthcare).

3. Results and discussions

3.1. Genetic screening of the putative bak1 suppressor, bak1-SUP1, which is involved in BR signaling

Since the identification of BAK1 as a versatile co-receptor various receptor-complexes, much efforts has been conducted to find downstream components that may specify the different developmental pathways. Botrytis-Induced Kinase 1 (BIK1), a receptor-like cytoplasmic kinase that is required for resistance to *Botrytis cinerea* [28], interacts with BAK1 as well as FLS2 [29]. The BAK1-interacting receptor-like kinase 1 (BIR1) is another example of a BAK1-interacting protein that belongs to the RLKX group with five LRRs [30]. The calcium-dependent phospholipid-binding protein BON1, which was first identified as a BIR1-interacting protein, also

interacts with BAK1 [31]. In these resulting complexes, BAK1 could phosphorylate both BON1 and BIR1 in vivo [31]. Recently, BIR2 and BIR3 were reported to be co-immunoprecipitated with BAK1, acting negatively to regulate BAK1-mediated plant immunity [32]. Compared with the fact that few proteins have been reported to interact with BAK1 in the cytoplasm after the activation of the receptor complexes containing BAK1/FLS2, there have been no reports concerning BAK1 interacting proteins in the BRI1/BAK1 complex for BR signaling.

At the beginning of this research, we wanted to screening genetic suppressors of *bak1* to identity new components that may be involved in BAK1-mediated BR signaling. Therefore, we used the activation-tagging method and subsequently generated screening pools by transforming the *bak1-2* mutant with the *pSKI015* binary vector construct containing 4 copies of the *35S CaMV* enhancer element, which has been used to identify gain-of-function mutations in many genes [26]. The gross morphology of *bak1-2* included shorter leaf and petiole lengths compared with those of the corresponding wild type, Ws-2 [4]. These phenotypes are typical for BR-deficient and BR-insensitive mutant alleles. Although the phenotypic strength of *bak1-2* is even weaker than that of *bri1-301*, the weakest allele of *BRI1* [33], it was not difficult to identify suppressed *bak1* phenotypes from the screening pool. We found several putative suppressors of *bak1-2* that displayed longer and narrow leaves than those of *bak1-2* in the T1 and T2 generations. One of these suppressors, named *bak1-SUP1*, was further analyzed in this study.

The original *bak1-SUP1* plant had longer leaves than those of *bak1-2* in the T1 generation. We obtained additional seeds from a single *bak1-SUP1* plant in the T1 generation and further analyzed the phenotypic differences. Compared with *bak1-2* and its corresponding wild type WS, *bak1-SUP1* had longer leaves and less compactness in the rosette area (Fig. 1A). We also noticed that flowering time of the *bak1-SUP1* plant was earlier than that of *bak1-2* and the WS wild type (Fig. 1B).

To further determine whether this putative *bak1-2* suppressor plant was involved in BR-related signaling processes, we performed a root growth inhibition assay to monitor its BR sensitivity in response to BL, the most bioactive BR. While the wild type showed a sharp decrease in root elongation in response to the increasing concentrations of BL, *bak1-2* displayed a much less sensitive phenotype to the inhibition of root growth by BL. The root growth inhibition pattern of *bak1-SUP1* by BL was similar to that of wild type (Fig. 1C). To confirm the BR sensitivity of *bak1-SUP1*, we examined the BES1 phosphorylation status in response to BL [34]. The majority of the BES1 in wild type were dephosphorylated by the BL treatment, which activates BES1 transcriptional activity. However, an even larger proportion of phosphorylated BES1 remained in *bak1-2*. Compared with these patterns, *bak1-SUP1* showed an intermediate BES1 phosphorylation status; the amount of active dephosphorylated form of BES1 in *bak1-SUP1* was increased compared with that of *bak1-2*, but was still less than that of the wild type (Fig. 1D). These results suggest that the reduced BR sensitivity of *bak1-2* was rescued in *bak1-SUP1*. As previously known, genetic suppressors of *bri1* that are essential BR signaling components also showed increased BR sensitivity [33–35]. We therefore, decided to further pursue the function of the gene that was affected in *bak1-SUP1*.

3.2. Expression of miR172a-2 was up-regulated in bak1-SUP1

To identify the T-DNA insertional site in *bak1-SUP1*, TAIL-PCR was performed, and the resulting PCR product, which was amplified through the successive annealing with nesting primer sets, was sequenced [36]. The T-DNA was inserted in the non-coding region that was flanked with *At5g04270* and *At5g04280*, 143 bp upstream of the first exon of *At5g04280* encoding a putative RNA binding protein (Fig. 2A). Because the original *bak1-SUP1* was identified in the T1 generation, it was expected to be a gain-of-function mutant. Therefore, we examined which neighboring gene would be overexpressed by the 4 copies of *35S CaMV* enhancer elements in the activation-tagging vector. Despite several approaches using different primer pairs in the RT-PCR analyses, we did not detect any specific gene that was up-regulated in *bak1-SUP1* among the four genes nearby the T-DNA insertion site (Fig. 2B).

Concomitantly with the identification of the T-DNA insertional site, we performed fine quantitative analyses of the *bak1-SUP1* phenotype in various aspects. We found that *bak1-SUP1* showed

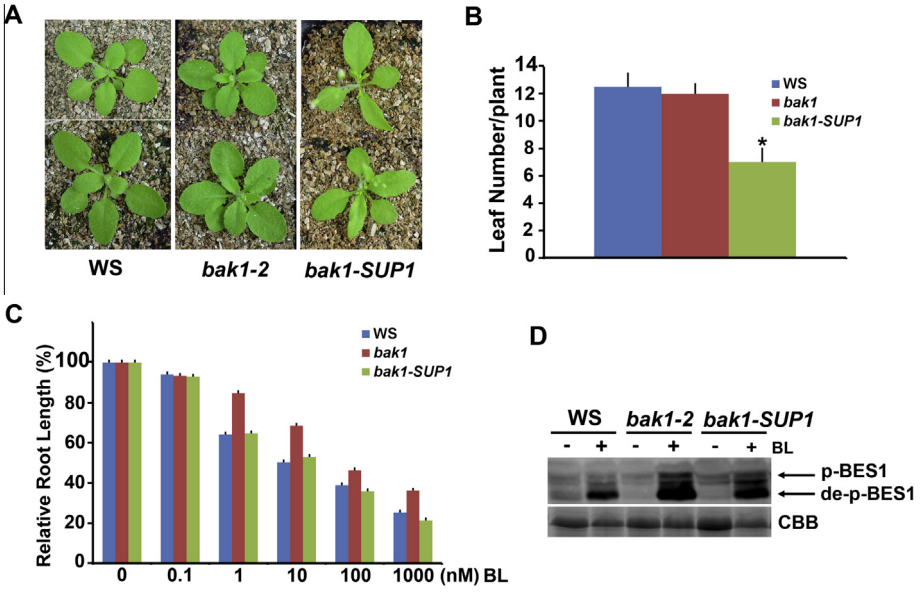

Fig. 1. Genetic suppressor of *bak1-2*, *bak1-SUP1*, was identified from the activation-tagged *bak1-2* pools. (A) Gross morphology of *bak1-SUP1* compared with that of the wild type WS and *bak1-2*. The pictures were taken of 4-week-old plants. (B) *Bak1-SUP1* showed an early flowering phenotype as determined by the number of leaves before bolting. The error bars indicate the standard deviation ($^{*}p \leqslant 0.0001$ by paired *t*-test compared with *bak1-2*). (C) and (D) The BR sensitivity of *bak1-SUP1* was determined via the inhibition of root elongation (C) or BES1 dephosphorylation (D) in response to BL compared with those of the wild type and *bak1-2*.

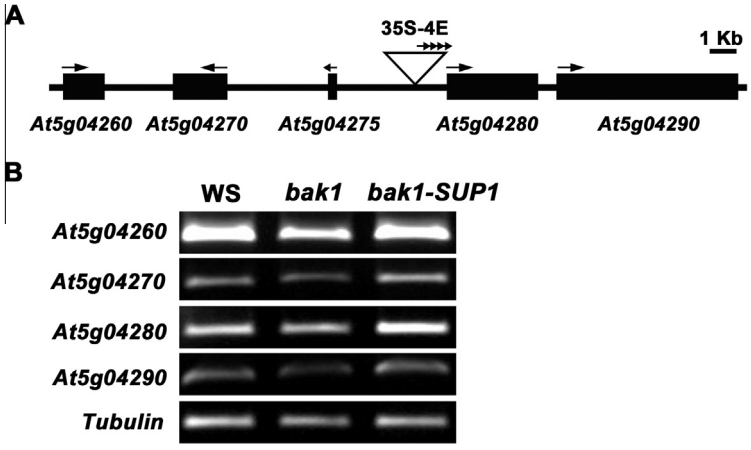

Fig. 2. Identification of the *35S CaMV* enhancer elements-containing T-DNA insertion sites in *bak1-SUP1*. (A) Schematic diagram of the activation-tagged locus of *bak1-SUP1* is shown in the region flanked with *At5g04280* and *At5g04270*. The arrows mark the translational direction of each gene. (B) The expression of the four neighboring genes encoding functional proteins near the activation-tagged locus were analyzed in *bak1-SUP1* and compared with those of the wild type and *bak1-2* by semi-quantitative RT-PCR.

a longer leaf length and shorter leaf width than those of *bak1-2*, resulting in a similar leaf shape as that of the wild type. However, the petiole length of *bak1-SUP1* was shorter than that even of *bak1-2* (Fig. 3A). The height of *bak1-SUP1* decreased during later developmental stages than did that of *bak1-2*, indicating that the overall vegetative growth might be halted earlier in *bak1-SUP1* (Fig. 3B). These features of *bak1-SUP1*, except for leaf shape, are unique compared with those of the *bri1* suppressors, because most of the *bri1* suppressors have a promoted elongation in both the leaf and petiole and a higher status [33–35]. In addition, we interestingly found that flowers of *bak1-SUP1* were abnormal: the sepals and petals were shorter and narrower compared with those of the wild type flowers (Fig. 3C). The siliques that were produced from the *bak1-SUP1* flower were shorter and bumpy without the abscission of floral organs (Fig. 3A and D). We noticed that these phenotypes were previously reported in the mutants of class A floral organ identifying genes [37]. All of the genes encoding class A proteins are in loci that are different from that of the T-DNA insertional site in *bak1-SUP1*. In addition, we did not detect any affected gene encoding functional proteins that was flanked by T-DNA in *bak1-SUP1*. Therefore, these observations led us to re-analyze the sequences of the non-coding region in which T-DNA was placed in

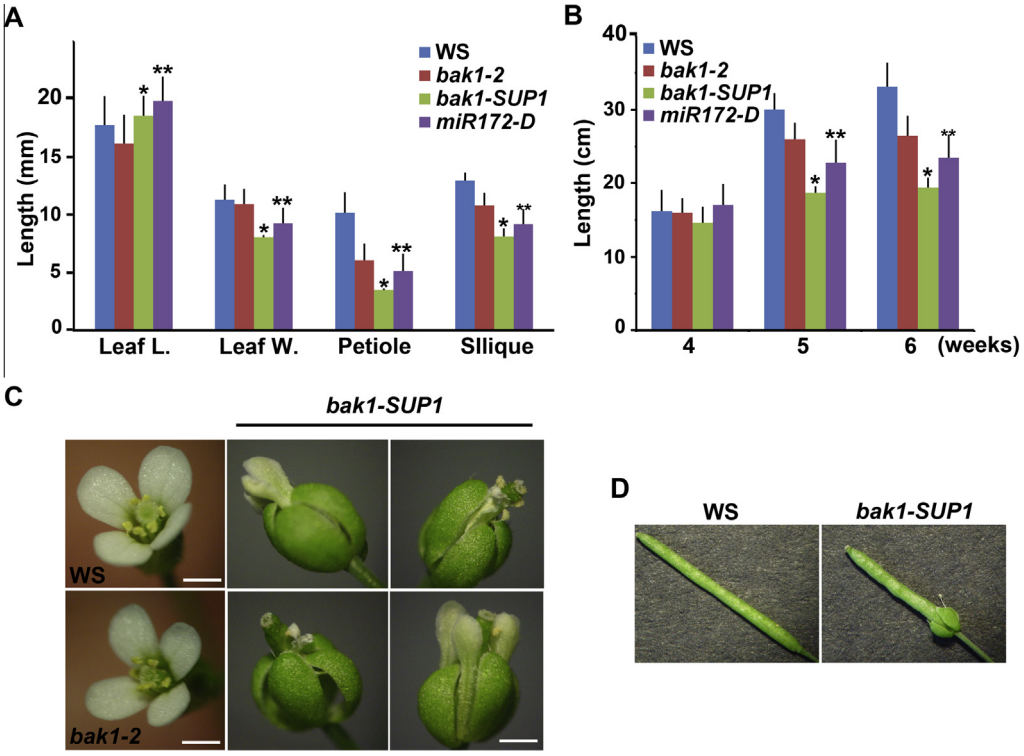

Fig. 3. Detailed phenotypic analyses of *bak1-SUP1*. (A) and (B) The growth criteria were measured from the indicated plants. (A) The leaf length, leaf width, and petiole length were measured from the 4-week-old plants ($N = 69$ from 23 plants in each line), and the silique length was measured from the 6-week-old plants ($N = 20$ from 10 plants in each line). (B) The plant height was measured from 4-, 5-, 6-week-old plants ($N = 12$ at each week). The experiments were repeated three times. The error bars denote the standard errors. ($^{*}p = 0.0001$ compared with bak1-2, $^{**}p = 0.0001$ compared with *bak1-SUP1*). (C) The flower phenotypes of *bak1-SUP1* compared with those of WS and *bak1-2*. The scale bar denotes 1 mm. (D) A comparison of the silique phenotypes between WS and *bak1-SUP1*.

bak1-SUP1 to identify possible regulatory regions that affect the expression of class A genes in flowering. We found that *At5g04275* was designated as the *EAT* (early activation tagged), encoding a precursor transcript of miR172a-2 [37]. This finding prompted us to extract small RNA and to examine the expression level of miR172 in *bak1-SUP1* compared to that of *bak1-2* and the WS wild type. We finally observed that the expression of miR172 dramatically increased in *bak1-SUP1* compared with that of *bak1-2* and WS (Fig. 4A). These results suggest that the 4 copies of *35S CaMV* enhancer-containing T-DNA that were inserted near the *At5g04280* led to the overexpression of *At5g04275*, resulting in the additional accumulation of miR172. AP2 and AP2-like proteins as targets of miR172 [37,38]. Therefore, we examined the expression of *AP2* and its homolog *TOE1* by RT-PCR analyses. We observed that these two genes did not change much in *bak1-SUP1* compared with wild type and *bak1-2* (Fig. 4B). This result was consistent with the previous report that miR172 regulates AP2 through translational repression [38].

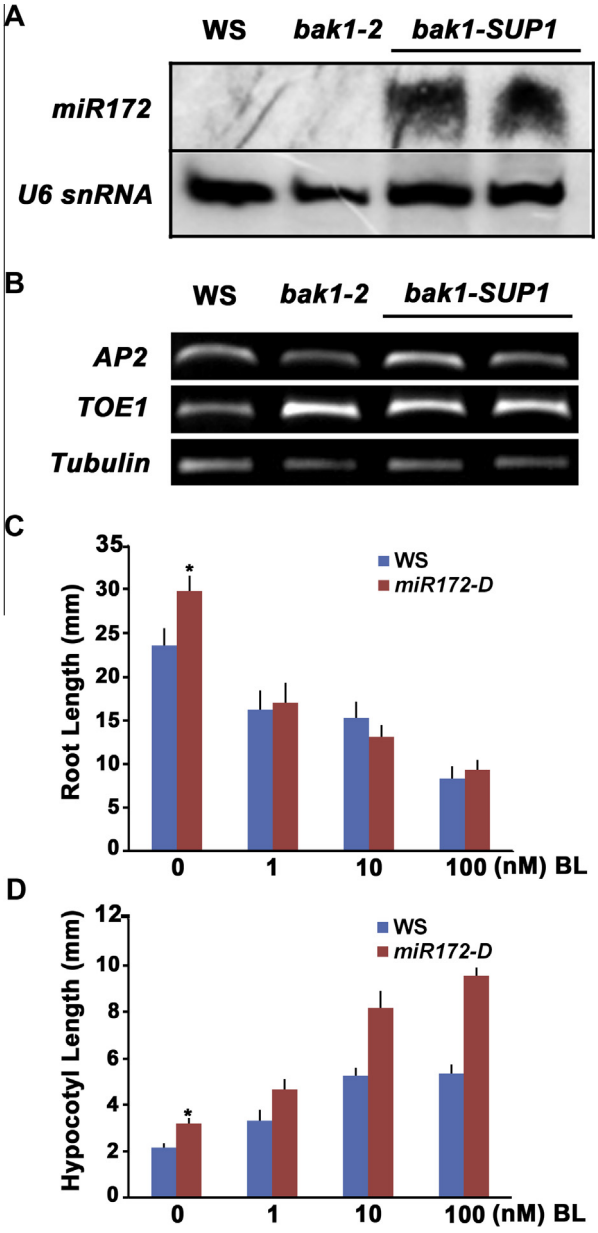

Fig. 4. Growth promotion and increased BR sensitivity were shown by the overexpression of miR172 in *bak1-SUP1*. (A) A small RNA blot analysis that was probed with miR172 showed that miR172 was overexpressed in *bak1-SUP1* compared with that of the wild type and *bak1-2*. (B) The expression levels of *AP2* and *TOE1* were analyzed in *bak1-SUP1* and compared to those of the wild type and *bak1-2*. (C) and (D), The root and hypocotyl lengths of the *miR172-D* plants in response to BL were measured and compared with those of the wild type plants.

3.3. Single gain-of-function mutant miR172-D promotes seedling growth by the activation of BR signaling

So far, miR172 has been known to be involved in developmental phase transition [11]. The repression of flowering by AP2 and AP2-like proteins, such as SCHLAFMUTZE (SMZ), SCHNARCHZAPFFEN (SNZ), TARGET OF EAT 1 and TARGET OF EAT 2 (TOE1 and TOE2, respectively) has been reported [37,38]. These genes are all targets of miR172. In comparison, reduced miR172 activity prolongs juvenile features and delay flowering. Based on these previous results, the early flowering phenotype of *bak1-SUP1* (Fig. 1B) is likely due to the overexpression of miR172.

In addition to early flowering, as *bak1-SUP1* showed suppressed *bak1* phenotypes in terms of leaf architecture and BR sensitivity, we wanted to further validate the effects of the overexpression of miR172 on plant development. Therefore, we deleted the *bak1-2* mutation by crossing the original *bak1-SUP1* with the WS wild type. From the genotyping of individual plants in the F2 generation, we identified a single dominant mutant from which *bak1-2* mutation segregated out. We named this mutant *miR172-D*. Compared with *bak1-SUP1*, *miR172-D* showed better growth in all of the criteria, such as leaf length and width, petiole length, total height, silique and pedicel lengths (Fig. 3A and B). However, except for the leaf length, the other growth criteria of *miR172-D* did not exceed those of the wild type. Therefore, it is possible that the phenotypic changes in *bak1-SUP1* might be simple additive effects due to a lack of BAK1 and an overexpression of miR172. These results suggest that the growth-promoting activity of miR172 is exposed more clearly in growth-inhibited genotypes, such as *bak1*. In the wild type, miR172 is likely to exert its function during phase transition.

We further analyzed the BR sensitivity of the root and hypocotyl of *miR172-D* compared with the corresponding wild type plant. *Mir172-D* showed promoted root and hypocotyl elongation under normal conditions compared with those of the wild type, indicating that the overexpression of miR172 also promotes seedling growth. When treated with BL, the root elongation of the *miR172-D* was more rapidly inhibited, and the hypocotyl elongation of the *miR172-D* was more increased in response to the increasing concentration of BL (Fig. 4C and D). Taken together with the normal growth pattern of the adult *miR172-D* shown in Fig. 3A and B, these results suggest that the overexpression of miR172 regulates not only the phase transition and flower organ development but also the vegetative growth pattern, which may be due to increases BR sensitivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.011.

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