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A mutation in *Arabidopsis BSK5* encoding a brassinosteroid-signaling kinase protein affects responses to salinity and abscisic acid

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ABSTRACT

As the most recently characterized group of plant hormones, brassinosteroids (BR) are involved in a number of physiological responses. Although many key components of the BR signaling pathway have been isolated and characterized, there is little information on detailed characterization of brassinosteroid-signaling kinase (BSK) proteins. In this study, *Arabidopsis* BSK5 was isolated and functionally analyzed. BSK5 transcripts were detected in various tissues, and were induced by abiotic stresses including salt and drought, as well as phytohormones of BR and abscisic acid (ABA). *Arabidopsis* loss-of-function mutant *bsk5* exhibited sensitivity to salinity and ABA. Mutations of the BSK5 gene also altered the expression of several stress-regulated genes. We suggest that BSK5 responds to other signals as well as BR.

1. Introduction

Plants have evolved sophisticated signal perception and transduction networks that enable them to cope with adverse environmental stresses [1]. Phytohormones play essential roles in mediating cellular events associated with plant responses to various stresses during all developmental stages [2,3].

Many physiological studies, as well as characterization of brassinosteroid (BR) biosynthetic and signaling mutants, have demonstrated that BRs play important roles in modulating plant development and response to environmental stresses [4–6]. In *Arabidopsis*, *brassinosteroid insensitive 1* (*BRI1*) mutant *bri1* showed multiple developmental deficiencies, including a severely dwarfed stature, male sterility and non-etiolation of dark-grown seedlings [4]. Suppression of the BR biosynthetic gene *DET2* produced sensitivity to salt stress, which was observed in *BRI1 kinase inhibitor 1* (*BKI1*)-overexpressing plants [7,8]. With identification of increasing numbers of mutants, the diverse roles of BR will be more clearly established.

BR is associated with other phytohormones such as abscisic acid (ABA) in regulating plant growth and development [9]. Microarray data analyses show that numerous genes are co-regulated by BR and ABA [10]. It is generally accepted that ABA plays critical roles in seed maturation, dormancy and germination, whereas BRs act antagonistically with ABA in control of germination [7,11]. In *Arabidopsis*, germination of BR receptor kinase mutant *bri1* is more sensitive than wild type plants to ABA, whereas *BRI1*-overexpression enhances the retardation effects of ABA on seed germination [7]. Other studies revealed that mutations in possible components of the ABA signaling pathway altered sensitivity to BR [12,13].

The nature of BR signal transduction has been gradually revealed by molecular genetic approaches [14]. With more components of the signaling pathways of BR being found and characterized, the mechanisms of BR signaling in plant responses are being better understood. In the present work, we established that *brassinosteroid-signaling kinase 5* (*BSK5*) was activated by BR and ABA as well as salt stress. Repression of *BSK5* alters response to abiotic stresses in *Arabidopsis*. The possible role of *BSK5* in response to salt stress and ABA was further examined. We show that BSK5 is required for salt stress and ABA-mediated drought stress tolerance.

2. Materials and methods

2.1. Plant materials and multiple stress treatments

Seeds of *Arabidopsis* (Columbia, Col-0) and mutant line *bsk5* (SALK_051739C) were surface sterilized with bleach and thoroughly washed three times with sterile water before incubation

Abbreviations: BR, brassinosteroid; ABA, abscisic acid; qRT-PCR, quantitative reverse transcription polymerase chain reaction; MS medium, Murashige and Skoog medium; GFP, green fluorescent protein.

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in a growth chamber following 3 days of cold treatment. For stress treatments, 4-week-old plants were exposed to various solutions containing 200 mM NaCl, 6% PEG, 1 μM BR, 10 μM IAA and 50 μM ABA, respectively. For cold treatment, 7-day-old plants were placed in a 4 °C incubator with normal illumination. All plant materials were harvested and stored at −80 °C.

2.2. RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Isolation of total RNA from plant materials was performed using an RNA extraction kit (Takara) according to the manufacture's recommendations. The cDNA synthesis and RT-PCR were conducted as previously described [15]. The real-time qRT-PCR analyses were performed on an ABI7300 system with SYBR Premix Ex Taq II (Takara) by previously described methods [16]. All primers used in the study are listed in Supplementary Table 1.

2.3. Subcellular localization

Expression vectors with green fluorescent protein (GFP) tags were constructed for subcellular localization analysis. The coding region of *BSK5* was amplified by RT-PCR with specific primers (Supplementary Table 1) and fused to the N-terminal end of GFP under control of CaMV 35S promoter. Subcellular localization of GFP expression in onion epidermal cells was monitored by confocal microscopy 16 h after particle bombardment transformation as previously described [15].

2.4. Genotyping of T-DNA insertion mutants

The T-DNA insertion point of the *bsk5* homozygous mutant was identified by PCR and homozygous mutant plants were confirmed by RT-PCR with the gene-specific primers in Supplementary Table 1.

2.5. Seed germination and root growth assays

For germination assays, seeds of Col-0 and *bsk5* were placed on ½ MS medium containing various concentrations of ABA (0–1 μM) or NaCl (0–200 mM). Experiments with the ABA biosynthetic inhibitor norflurazon (NF) (Sigma) were performed under the same conditions, whereby NF was added at a final concentration of 100 μM to the solutions with or without NaCl. For root growth assays, 7-day-old seedlings were grown on vertical agar plates in the presence or absence of ABA (5 μM or 10 μM), or NaCl (75 mM or 150 mM). Root lengths were measured after 14 days.

2.6. Measurement of stomatal apertures, transpiration rates and survival rates

To measure stomatal apertures in Col-0 and *bsk5* plants, leaves from 4-week-old plants were detached from plants sprayed with zero or 10 μM ABA for 2 h. Stomata were photographed with a microscope coupled to a CCD camera and 30 stomatal apertures were examined in each treatment. To detect the rate of water loss under dehydration conditions, detached leaves from 4-week-old plants were exposed to air at room temperature and weighed at the designated times. Plants survival abilities were determined as previously described [17].

3. Results

3.1. Identification of BSK5 in Arabidopsis

Semi-quantitative RT-PCR was performed to investigate tissue specific expression pattern of *BSK5*. *BSK5* was predominantly detected in leaves and roots (Fig. 1A). As a key component in BR signaling pathway, it was necessary to study whether *BSK5* responds to BR. The result showed that *BSK5* was clearly induced by BR and ABA (Fig. 1B). Abiotic stresses including salinity and drought also enhanced the transcriptional level of *BSK5* to different degrees. Subcellular localization analysis suggested that BSK5 was mainly

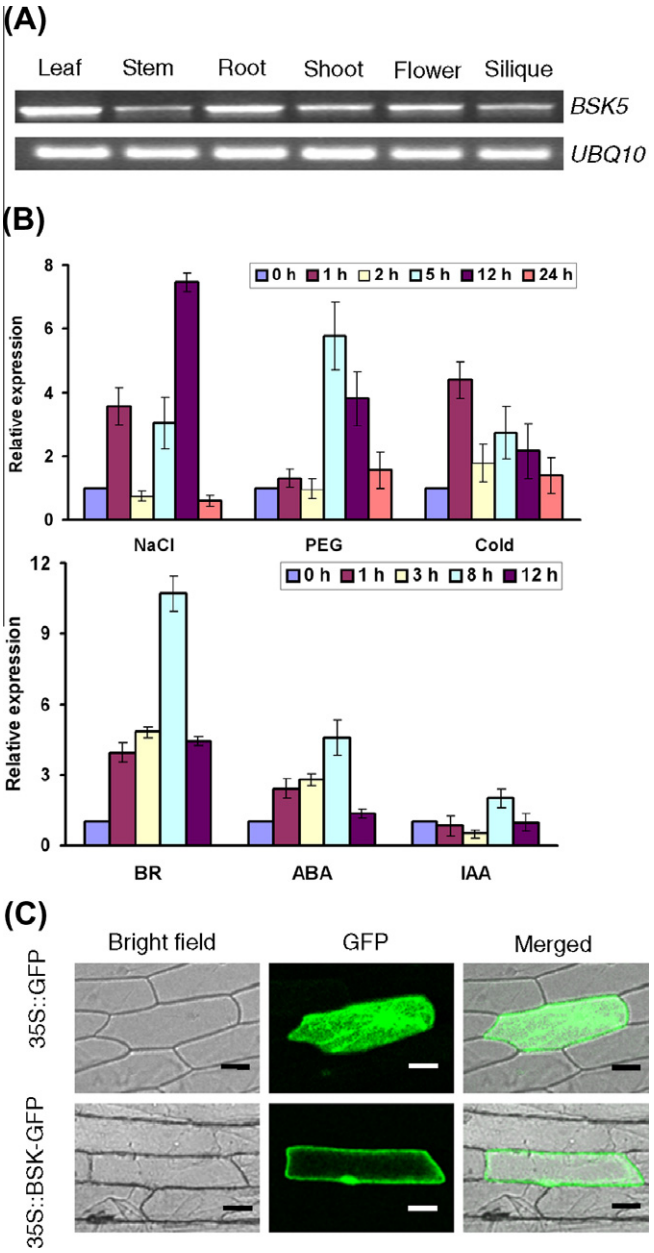

Fig. 1. Expression patterns and subcellular localization of BSK5. (A) Semi-quantitative RT-PCR analysis of the expression pattern of *BSK5*. *UBQ10* was used as control. (B) Effect of various abiotic stresses and phytohormones on transcript level of *BSK5*. The expression of BSK5 under different stimuli was normalized to the expression of *UBQ10*. Means and SD were calculated from three independent experiments. (C) Subcellular localization of BSK5 protein. The 35S::BSK5-GFP and 35S::GFP control vectors were transiently expressed in onion epidermal cells by particle bombardment. Results were visualized by confocal microscopy. Bars = 10 μm.

distributed at the plasma membrane and nucleus (Fig. 1C). These results suggest that BSK5 may play multiple roles in response to diverse environmental signals.

3.2. The bsk5 mutant is more sensitive to salt stress

A homozygous mutant line was established in order to evaluate the function of BSK5 in abiotic stress response. Sequence analysis of the mutant confirmed the insertion site in the 2nd exon of *BSK5* and the insertion resulted in a lack of full-length *BSK5* transcripts, as indicated by RT-PCR analyses using BSK5-specific primers listed in Supplementary Table 1 (Fig. 2A). Under standard culture conditions we observed no significant difference in the growth or morphology between mutant and wild-type Col-0 plants. However, mutant seedlings displayed hypersensitivity to salt compared to Col-0 (Fig. 2B). In the presence of 150 mM NaCl, the germination percentage of mutant plants was reduced to nearly 20% compared to 58% for Col-0. High salt also significantly retarded root growth of *bsk5* compared to Col-0 (Fig. 2C and D), thus suggesting that BSK5 may function as a positive regulator in response to salt stress.

3.3. Mutant bsk5 plants are hypersensitive to ABA in growth and stomatal closure of guard cells

Parallel assays were performed to examine ABA sensitivity of *bsk5*. Germination of *bsk5* mutant seed was severely affected by exogenous ABA (Fig. 3A and B), and root growth (Fig. 3C), and cotyledon greening (Fig. 3D) were more inhibited by ABA than in Col-0. ABA modulates stomatal closure by guard cells in order to avoid water loss during drought stress [18]. As shown in Fig. 3E and F, treatment of *bsk5* leaves with ABA caused more pronounced stomatal closure than in Col-0. These results indicated that the loss of function in *bsk5* increased ABA sensitivity in stomatal closure, suggesting that BSK5 is a negative regulator of ABA response and regulates diverse ABA responses in development and stress responses. Further study revealed that *bsk5* plants lost water more slowly than Col-0 (Fig. 3G). According to the drought tolerance assay, only 16% of Col-0 survived without irrigation after 4 weeks, whereas about 55% of the *bsk5* mutant plants recovered after rewatering (Fig. 3H). These results further support BSK5 as a negative regulator of drought tolerance via regulation of ABA-mediated stomatal closure.

3.4. bsk5 mutants have altered ABA- and stress-induced gene expression

To investigate the role of ABA sensitivity in *bsk5*, real-time qRT-PCR was performed to analyze the expression of stress-response genes. As shown in Fig. 4A, several stress marker genes, including *RD29A*, *DREB1A*, *KIN1* and *ABI5*, were highly up-regulated in *bsk5* under ABA treatment. In addition, two ABA biosynthetic key enzymes, *ABA3* and *NCED3*, were significantly up-regulated in *bsk5* in the presence of NaCl (Fig. 4B). We hypothesized that the salt-stress sensitivity of the mutant resulted from an ABA-dependent process. To consider this point, NF was used for germination assays on media containing high levels of NaCl. As shown in Fig. 4C, a supplement of 100 μM NF almost fully restored the germination percentage of

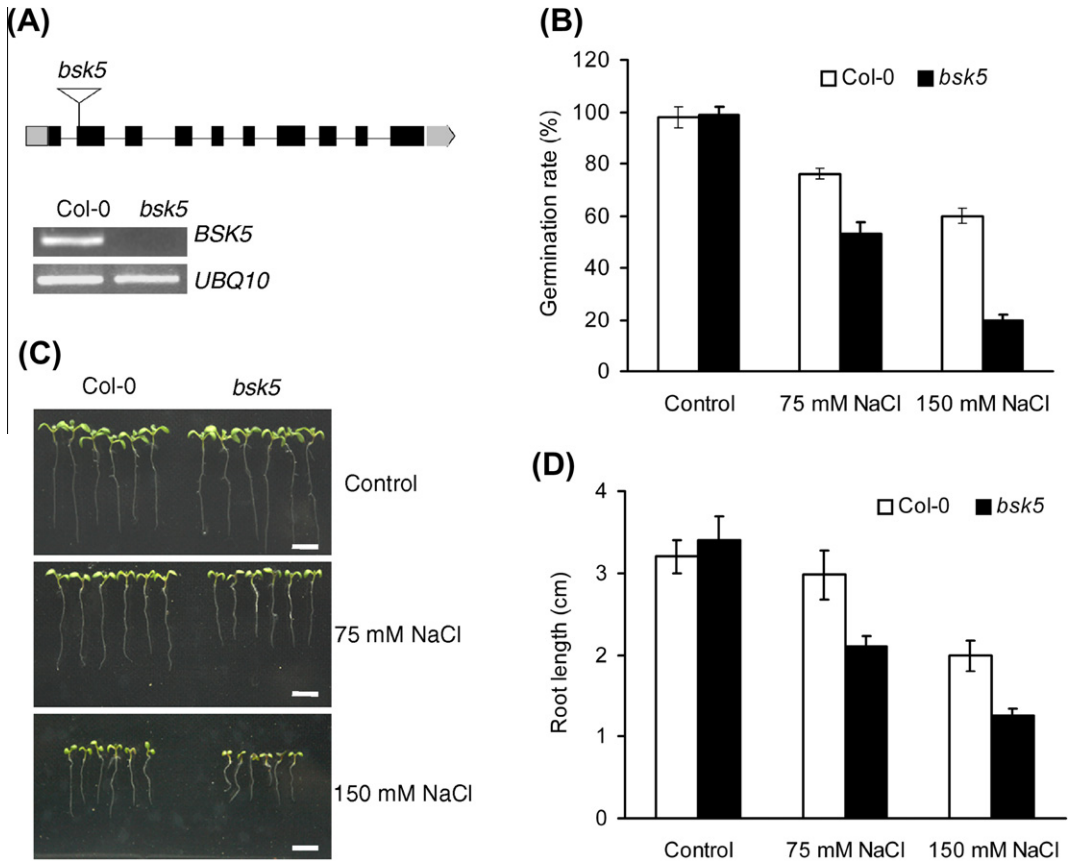

Fig. 2. Response of mutant *bsk5* to salt stress. (A) Verification of *bsk5* mutant. T-DNA was inserted in the 2nd exon downstream of the ATG start codon. *BSK5* was not detected in mutant plants. Expression levels of *UBQ10* served as a control. (B) Germination rates of seeds after 7 day of growth in the presence or absence of NaCl. (C) Phenotypic comparison of root lengths in ½ MS medium with or without NaCl. Images were recorded on day 7 after transfer of 7-day-old seedlings from ½ MS medium to plates containing NaCl. Bars = 1 cm. (D) Effect of different NaCl concentrations of on root growth in Col-0 and mutant plants. Data are means ± SD (n = 30).

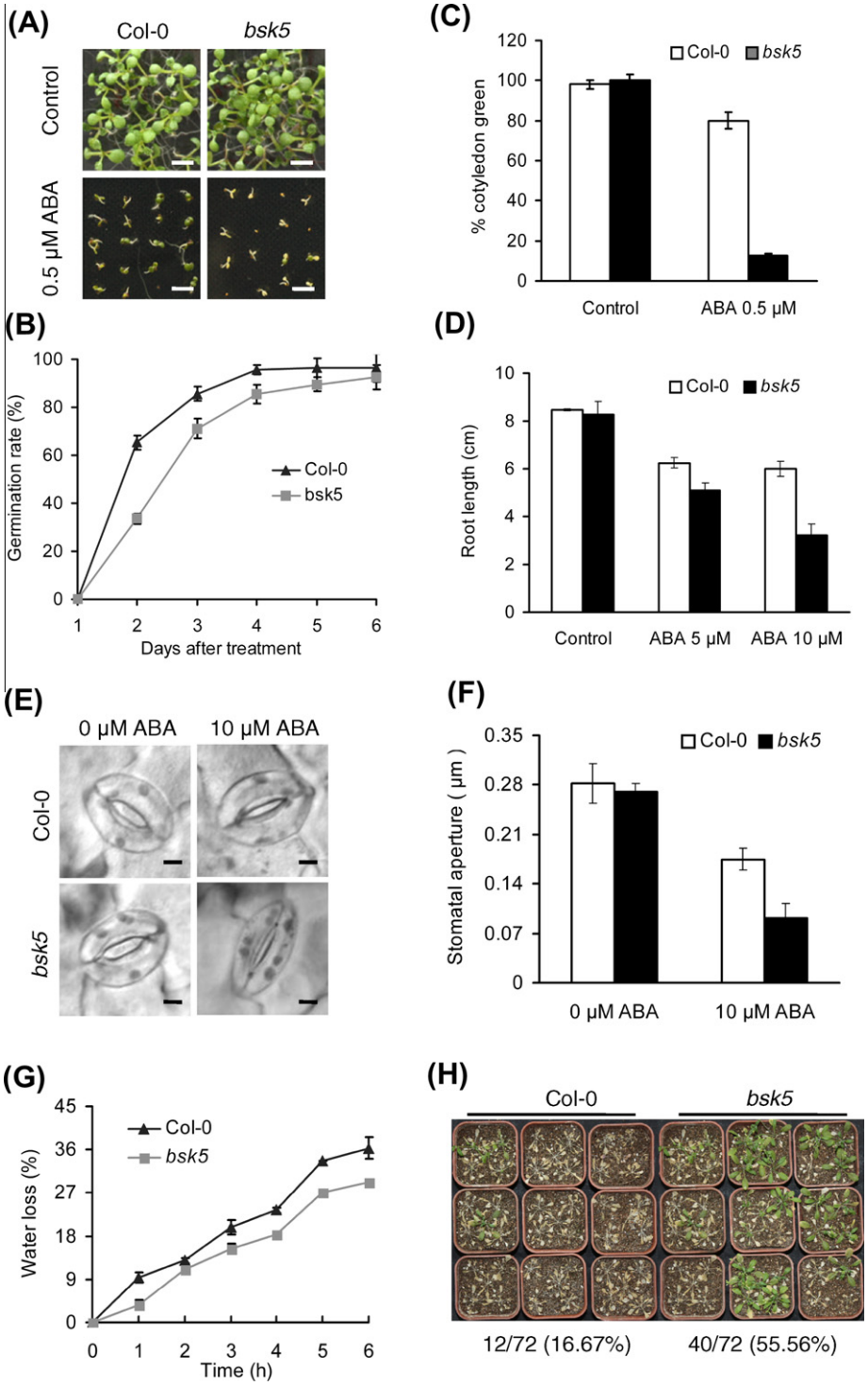

Fig. 3. Response of mutant *bsk5* to ABA and drought stresses. (A) Growth of Col-0 and mutant plants on ½ MS medium containing zero or 0.5 μM ABA. Bars = 0.5 cm. (B) Germination rates of Col-0 and mutant. Seedlings were grown with or without 0.5 μM ABA. Germination rates were determined daily after stratification. Data represent means ± SD (n = 90). (C) Comparison of green cotyledon percentages of Col-0 and mutant plants in conditions used in A. (D) Effects of ABA on root growth of Col-0 and mutant plants. Data represent means ± SD (n = 50) from three independent experiments. (E) Stomatal movement profiles of Col-0 and mutant plants. Stomatal guard cells were observed in the epidermal sheets treated with 0 μM or 10 μM ABA. Bars = 10 μm. (F) Stomatal closure of guard cells as a result of ABA treatment. Data are mean ratios of width to length ± SD of three independent experiments (n = 30). (G) Determination of detached leaf water loss rates. Water loss rates are indicated as percentages of the initial fresh weight (%). Results are shown as the means ± SD for three independent experiments. (H) Determination of survival rates after drought stress. Healthy 14-day-old Col-0 and mutant plants were withheld from water for 15 days and then re-watered. The photographs were taken 36 h after re-watering. Three independent experiments were conducted.

Col-0 under salt-stress conditions. However, addition of NF did not enhance the germination percentage of *bsk5* as much as Col-0 under same conditions (Fig. 4C). These results suggest that the inhibition of seed germination by salt in *bsk5* mutant is partially mediated by ABA.

4. Discussion

BRs control a broad range of responses in plants in addition to its important roles in plant development. However, studies reveal-

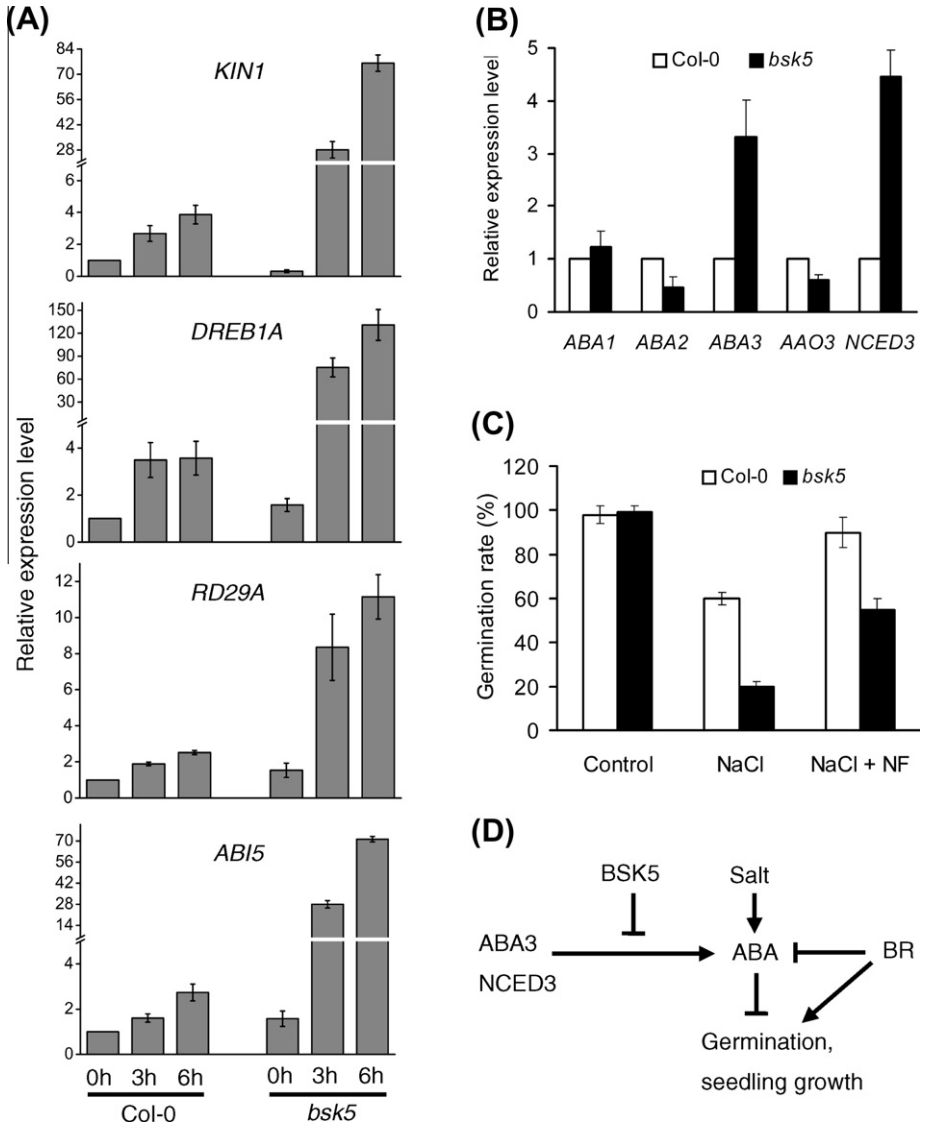

Fig. 4. (A) Expression levels of *DREB1A*, *RD29A*, *ABI5* and *KIN1* in Col-0 and mutant plants under normal conditions and 1 μM ABA treatment. Measurement was performed by real-time qRT-PCR. Values represent means ± SD with three biological replicates. (B) Relative quantitative analysis of ABA biosynthetic gene expression in Col-0 and mutant plants in the presence of NaCl. Total RNA was isolated from 7-day-old seedlings treated with 200 mM NaCl for 6 h. Data represent means ± SD from three independent experiments. (C) Germination rates of Col-0 and mutant in the presence or absence of NaCl and with or without NF scored 7 days after sowing. Data represent means ± SD ($n = 90$). (D) A model for BSK5 functions in salt stress response. BSK5 positively modulates salt stress response and suppresses salt induced ABA biosynthesis through regulation of *ABA3* and *NCED3* expression. Positive effects are indicated by arrows and bars indicate repression.

ing the role of BR signaling in modulating plant responses to different environmental stresses are still limited. There are reportedly 12 BSK proteins in *Arabidopsis* and they mainly exert a role in signal transduction via BR signaling [14]. Here we present new evidence that BSK5 is responsive to diverse stimuli. The real-time qRT-PCR results revealed that *BSK5* was induced by various abiotic stresses and phytohormones (Fig. 1B). The expression patterns of BSK5 suggest specific and differential roles in plant development and stress signaling. Moreover, loss-of-function mutant *bsk5* showed salt stress and ABA sensitivities, which were similar to those observed in *bri1* mutant plants [7]. This further supports involvement of BR signaling in salt stress and ABA response.

Salt stress causes hyperosmolarity, which in turn induces ABA biosynthesis to coordinate the adaptive response [3,19]. The level of ABA is tightly controlled by biosynthesis and catabolism. Here, we show that ABA biosynthetic genes *ABA3* and *NCED3* were clearly induced in *bsk5* plants (Fig. 4B). Thus, higher levels of ABA may be accumulated in *bsk5* plants under salt stress compared with Col-0. In view of ABA sensitivity in *bsk5*, this suggests that *BSK5* mutation not only alters ABA sensitivity, but also modifies the ABA biosynthetic pathway. Further examination showed that NF partially counteracts salt-induced inhibition in *bsk5* mutant (Fig. 4C). Hence, germination deficiency in *bsk5* under saline conditions may partially be due to ABA accumulation (Fig. 4D). A previous report showed that BSK5 overexpression suppressed developmental deficiencies in *bril* mutant, suggesting a positive role of BSK5 in BR signaling [14]. Thus, repression of BSK5 may lead to BR-deficiency that affects salt and ABA stress responses as observed in the *bri1* mutant [7]. Several studies have shown that BR has the opposite effect of ABA in seed germination [20–22]. As expected, we also found that exogenous BR largely restored the ability of *bsk5* mutant to germinate in the presence of ABA (data not shown). Our results suggest that BSK5 modulates salt stress response through antagonistic actions of BR and ABA in seed germination (Fig. 4D).

Very recent studies showed that stomatal development can be regulated by BR. Overexpression of BR biosynthetic or pathway

genes, such as *BRI1*, *BIN2* and *DET2*, significantly increased the number of stomata in hypocotyls, whereas the number was significantly reduced in these mutants plants [23,24]. However, stomatal development was not affected by suppression of *BSK5*. The number and size of stomata in *bsk5* mutant plant hypocotyls and leaves were almost identical to those in Col-0 (data not shown). BSK5, therefore, is possibly not responsible for stomatal development in BR signaling. Alternatively, it may be due to a redundant function in the BSK protein family. Previous reports show that ABA-mediated stomatal closure is important for drought tolerance in plants [25,26]. Here, stomatal closure was more sensitive to ABA, and lower water loss was detected in *bsk5* mutant plants compared with Col-0 (Fig. 3F and G). Furthermore, the drought tolerance assay showed that *bsk5* mutant plants were more tolerant to water shortage (Fig. 3H), hence suggesting that BSK5 may play a key role in ABA-related stomatal closure under drought stress. Induction of stress-responsive genes is one of the most important mechanisms for adaptation in plants. As shown in Fig. 4A, expression of several stress- and ABA-response genes was obviously up-regulated in *bsk5* relative to Col-0. Among them, the *ABI5* transcription factor is crucial for ABA inhibition of germination, concurring with germination deficiency in *bsk5* mutant [27]. In addition, it has been reported that overexpression of stress-response genes, such as *DREB1A* and *RD29A*, enhance drought tolerance in transgenic plants [28,29]. Thus, the dramatic induction of stress-response genes, such as *RD29A*, *DREB1A* and *KIN1* in *bsk5* corresponded to a drought tolerance phenotype. Thus BSK5 may negatively modulate drought stress response through control of stress response gene expression and stomatal closure in an ABA-dependent manner.

The function of BSK5 in response to salt and ABA stresses provides new evidence for understanding the diverse roles of BR signaling components in *Arabidopsis*.

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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.2012.08.118.

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