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Negative regulation of abscisic acid signaling by the *Brassica oleracea* ABI1 ortholog

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ABSTRACT

ABI1 (ABA Insensitive 1) is an important component of the core regulatory network in early ABA (Abscisic acid) signaling. Here, we investigated the functions of an ABI1 ortholog in *Brassica oleracea* (BolABI1). The expression of *BolABI1* was dramatically induced by drought, and constitutive expression of *BolABI1* confers ABA insensitivity upon the wild-type. Subcellular localization and phosphatase assays reveal that BolABI1 is predominantly localized in the nucleus and harbors phosphatase activity. Furthermore, BolABI1 interacts with a homolog of OST1 (OPEN STOMATA 1) in *B. oleracea* (BolOST1) and can dephosphorylate ABI5 (ABA Insensitive 5) *in vitro*. Overall, these results suggest that *BolABI1* is a functional PP2C-type protein phosphatase that is involved in the negative modulation of the ABA signaling pathway.

1. Introduction

Abscisic acid (ABA) plays a major role in the cellular response to environmental stresses [1]. A phosphorylation cascade fundamentally affects early ABA signaling, as shown by genetic and biochemical studies [2]. Two type-2C protein phosphatases (PP2C), ABI1 and ABI2 (ABA Insensitive 1 and 2), have been extensively studied in Arabidopsis and play roles in the regulation of seed germination, stomatal closure, ion channel activity, and ABA-responsive gene expression [3–6]. The release of regulators (such as sucrose non-fermenting-related kinase (SnRK)-2 subfamily members) from the PP2C-SnRK2 complex is central to the core regulation of the phosphorylation cascade in early ABA signaling [2].

PP2C is one of four protein serine/threonine phosphatase (PP1, PP2A, PP2B, and PP2C) families in plants [5]. In Arabidopsis, 76 genes comprise the PP2C family [6]. ABI1 and ABI2, the pioneering members of plant group A PP2C proteins in Arabidopsis, were isolated using a genetic screen of mutagenized seeds that were resistant to the ABA inhibition of seed germination [3]. The subsequent identification of loss-of-function alleles of ABI1 and ABI2 and the generation of double mutants confirmed that ABI1 and ABI2 function partially redundantly as inhibitors of ABA signaling [7,8]. It is reported that ABI1 and ABI2 contribute nearly 50% of ABA-induced PP2C activity in Arabidopsis [5,9].

In addition to Arabidopsis, many homologous genes belonging to PP2C gene families have been studied in various plant species [6,10–14]. Many PP2C genes are encoded by the rice genome, but only 10 have been categorized into the group A subfamily [6]. Only two genes encoding group A PP2C proteins have been identified in moss (*Physcomitrella patens*), both of which take part in the repression of desiccation tolerance [12]. FsPP2C1, the first PP2C isolated from beech (*Fagus sylvatica*) seeds, functions as a negative regulator of ABA signaling [10]. Conversely, FsPP2C2, another PP2C in beech, functions as a positive regulator of ABA [14]. When overexpressed in Arabidopsis, the maize PP2C (ZmPP2C) decreases plant tolerance to salt and drought [13]. In addition to functioning as an important hub in ABA responses, recently isolated fruit PP2C proteins provide evidence of a pivotal role for group A PP2C in the modulation of fruit-ripening processes [11].

Cabbage (*Brassica oleracea* var. capitata L.) is one of the most common vegetable species in open field cultivation and is very popular with consumers in northern China. However, environmental stresses (especially drought stress and abnormal decreases in temperature) seriously affect cabbage yield and quality, with consequent effects on farmer income. Recent studies have shown that the above mentioned problems are mainly related to ABA signaling [15,16]. Although very little is known about ABA signaling, the ABA signaling mechanisms in *B. oleracea* are being elucidated [16]. Our recent studies have revealed that ABA Insensitive 5 and OPEN STOMATA 1 orthologs in *B. oleracea* (BolABI5 and BolOST1, respectively) are involved in the modulation of plant ABA and drought stress responses [16,17]. In this study, Arabidopsis overexpressing

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BolABI1 were used to investigate the effects of this protein on plant responses to ABA. Taken together, our data reveal that BolABI1 is a functional PP2C-type protein phosphatase and delineate a novel ABA signaling pathway in *B. oleracea*.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatments

Plants were grown in a controlled environment as previously described [16]. For protoplast isolation, plants were grown in a growth chamber under 45% humidity and a 12-h daylight cycle at 20 °C (night) and 23 °C (day). For abiotic stresses and exogenous hormone treatments, eleven-day-old cabbage (*B. oleracea* var. capitata L.) seedlings were treated as previously described [16]. Briefly, the seedlings were treated with 300 mM NaCl, −1.7 MPa PEG 8000, drought and 0.1 mM ABA followed by sampling at 0, 4, 8, 12, 16, 20 and 24 h.

2.2. Plasmid construction

To produce Flag-tagged BolABI1, the full-length coding sequence (CDS) of BolABI1 was amplified using following primers: BolABI1-BamF (5′-CGCGGATCCATGGAGGAAGTATCACCGGCGGTTTC-3′) and BolABI1-woSalR, (5′-ACGCGTCGACAGGCTTTTTGATCTTGAGTTTC-3′). After sequence confirmation, the BolABI1 fragment was then cloned into the *Bam*HI and *Sal*I sites of the binary expression vector p1307-Flag.

To generate GFP-tagged BolABI1, the full-length CDS of BolABI1 was excised from p1307-Flag-BolABI1 and inserted into the Cam-35S-GFP vector between the *Bam*HI and *Sal*I sites.

To construct GST-tagged BolABI1, the full-length CDS of BolABI1 was removed from p1307-Flag-BolABI1 and cloned into the pGEX-6p-1 vector (GE Healthcare) between the *Bam*HI and *Sal*I sites. To obtain GST-tagged BolABI5-N, the CDS of the N-terminal 248 aa of ABI5 was inserted into the pGEX-6p-1 vector (GE Healthcare) between the *Bam*HI and *Sal*I sites using following primers: ABI5-10BamF (5′-CGGGATCCTCAGAGCGAGAAGTAGAGTCGTC-3′) and ABI5-N-SalR (5′-ACGCGTCGACATCACCGGTTCCTTGAAACAC-3′).

To detect the interactions among BolABI1, BolOST1, ABI5 and BolABI5 using the firefly luciferase complementation technique, the CDS of BolABI1was cloned into the p2305-cLUC vector between the *Kpn*I and *Sal*I sites using the following primers: BolABI1-KpnF (5′-GGGGTACCATGGAGGAAGTATCACCGGCGGTTTC-3′) and BolABI1-woSalR. The CDSs of BolOST1 (GenBank Accession No. KF577724), ABI5 and BolABI5 were cloned into the p2305-nLUC vector between the *Bam*HI and *Sal*I sites using the primers: for BolOST1, BolOST1-BamF (5′-CGCGGATCCATGGACCGACCAGCAGTGAGTG-3′) and BolOST1-woSalR (5′-ACGCGTCGACCATTGCGTACACAATCTCTC-3′); for ABI5, ABI5-BamF (5′-CGCGGATCCATGGTAACTAGAGAAACGAAGTTG-3′) and ABI5-SalR (5′-ACGCGTCGACTTAGAGTGGACAACTCGGGTTC-3′); and for BolABI5, BolABI5-BamF (5′-CGCGGATCCATGATGTCTGGACGAGAAGTAG-3′) and BolABI5-woSalR (5′-ACGCGTCGACGAGAGGGCAACTAGGGTTCCTC-3′).

2.3. Real-time quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNAs from samples were extracted using RNAiso Plus reagent (TaKaRa). Five micrograms of RNA were reverse transcribed using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's protocol. Real-time quantitative RT-PCR was performed using a CFX 96 real-time PCR machine (Bio-Rad, Hercules, CA, USA) and the SYBR Premix Ex Taq kit (TaKaRa) to monitor the double-stranded amplified products as previously described [16]. The thermal cycling used consisted of a hold at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 10 s and 72 °C for 30 s. The samples were maintained at 95 °C for 10 s, 85 °C for 5 s and 95 °C for 10 s to perform a melt-curve analysis. The relative level of *BolABI1* was normalized to the expression level of cabbage *ACTIN2* and expressed relative to the level in the mock-treated seedlings or young leaves. The following primer pairs were used for the amplification: for *BolABI1*, 5′-AGCGAGTGACGGTGTTTG-3′ and 5′-CATCTCCAGCCACCATGTTC-3′, and for cabbage *ACTIN2*, 5′-GCAGACCGTATGAGCAAAG-3′ and 5′-GCTGAGGGAAGCAAGAATG-3′.

2.4. Subcellular localization and firefly luciferase complementation assay

Plasmids encoding Cam-BolABI1-GFP and the firefly luciferase complementation assay-related cLUC/nLUC vectors were isolated and purified using a Plasmid Maxiprep Kit (Vigorous Biotechnology) and introduced into Arabidopsis leaf mesophyll protoplasts according to the PEG-Ca^{2+} protocol developed by the Sheen lab [18]. Briefly, leaf mesophyll protoplasts were isolated via the digestion of from 0.5 to 1 mm leaf strips in a cellulase/macerozyme solution (1.5% cellulase R10 (Yakult Honsha, Tokyo, Japan), 0.4% macerozyme R10 (Yakult Honsha, Tokyo, Japan), 0.4 M mannitol, 20 mM KCl, 20 mM MES (pH 5.7), 10 mM $CaCl_2$, 5 mM mercaptoethanol and 0.1% BSA). The protoplasts was washed twice with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES, pH 5.7), and resuspended in MMg solution (0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES (pH 5.7)) to a density of from 1 to 2×10^5/ml immediately before transfection. For transfection, 20 μg plasmid was added to 200 μl protoplasts in a microfuge tube and mixed well with 220 μl PEG-Ca^{2+} solution (4 g PEG 4000, 3 ml H_2O, 2.5 ml 0.8 M mannitol and 1 ml 1 M $CaCl_2$). After 15 min of transfection, the protoplasts were washed twice with W5 solution, resuspended at a density of approximately 2–4×10^4/ml in W5 solution and incubated in a growth chamber for from 12 to 18 h.

For the subcellular localization analysis, BolABI1-GFP fluorescence was detected, and images were collected under an inverted fluorescence Zeiss LSM 510 META confocal microscope after incubation for 12–18 h as previously described [19].

The firefly luciferase complementation assay was performed as previously described [20]. Briefly, 10 μl 3.3 μM luciferin (Sigma–Aldrich) solution was added to transfected protoplasts, and luciferase (LUC) activity was measured. A Tecan Infinite M200 Microplate Reader (Tecan Austria GmbH) was used to measure the relative LUC activity according to the Magellan standard 7.1 protocol provided by the manufacturer.

2.5. Recombinant protein purification and phosphatase assay

The recombinant protein purification and phosphatase assay were performed as previously described [19]. Briefly, GST fusion constructs of BolABI1 and ABI5-N were transformed into *Escherichia coli* strain BL21(DE3), induced with 0.3 mM isopropyl-thio-β-galactoside (IPTG) and purified with Glutathione Sepharose™ 4B (GE Healthcare) according to manufacturer's instructions. The purified proteins were used for the subsequent phosphatase assay. ^{32}P-labeled ABI5-N was obtained via an *in vitro* kinase assay. Twenty micrograms of Ni-NTA agarose (Qiagen) conjugated with PKS5 and 50 μg ABI5-N were mixed in a 50-μl system with kinase assay reaction buffer (20 mM Tris–HCl pH 7.2, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 μM ATP and 2 mM DTT) supplemented with 5 μCi [γ-^{32}P]-labeled ATP. The phosphatase assay was initiated by mixing 10 μg GST-BolABI1 and 15 μg ^{32}P-labeled ABI5-N in a 25-μl system containing phosphatase assay reaction buffer

(20 mM Tris–HCl (pH 7.2), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 2 mM DTT). The reaction was terminated by adding SDS loading buffer and heating at 95 °C for 5 min after incubation at 30 °C for 1 h. The reaction products were resolved using 12% SDS–PAGE, stained with CBB (Coomassie Brilliant Blue) R250, and exposed on a storage phosphor screen. The phosphatase activity was visualized using a Typhoon 9410 Phosphorimager (Amersham Biosciences, GE Healthcare).

3. Results and discussion

3.1. Isolation and sequence analysis of BolABI1 in B. oleracea

A cDNA fragment (1281 bp) of *BolABI1* was isolated from cabbage (*B. oleracea* var. capitata L.) via RT-PCR; this fragment encodes a protein with 426 amino acids (GenBank Accession No. KF577723) and a calculated molecular mass and predicted pI of 46.8 kDa and 7.01, respectively. Sequence alignment revealed that BolABI1 has the same motif arrangement as ABI1, including a PA (phosphatidic acid)-binding site at the N-terminus (R-65), a SnRK3 interaction domain, a PP2C domain, a conserved Trp residue (W-290) that contacts ABA in the PYL (Pyrabactin Resistance 1-LIKE)-binding pocket, a conserved Phe residue (F-296) that mediates the PYL-PP2C contact, and an ABA box and an NLS-like (monopartite nuclear localization signal) motif at the C-terminus [21–23] (Fig. 1A). Phylogenetic analysis using the neighbor-joining method showed that BolABI1 shares an even deeper evolutionary homology with Arabidopsis ABI1 (Fig. 1C). Arabidopsis ABI1 has been shown to function as a negative regulator of ABA-mediated processes [9]. BolABI1 and Arabidopsis ABI1 exhibited a high degree of sequence identity at the amino acid level (Fig. 1A and C), which prompted us to further elucidate the role of BolABI1 in plants.

3.2. Expression of BolABI1 occurs mainly in young leaves and is induced by drought stress

Gene expression patterns can provide important clues for biological significance [19]. The inducible expression of *ABI1* by exogenous ABA and stress treatments, including low temperature,

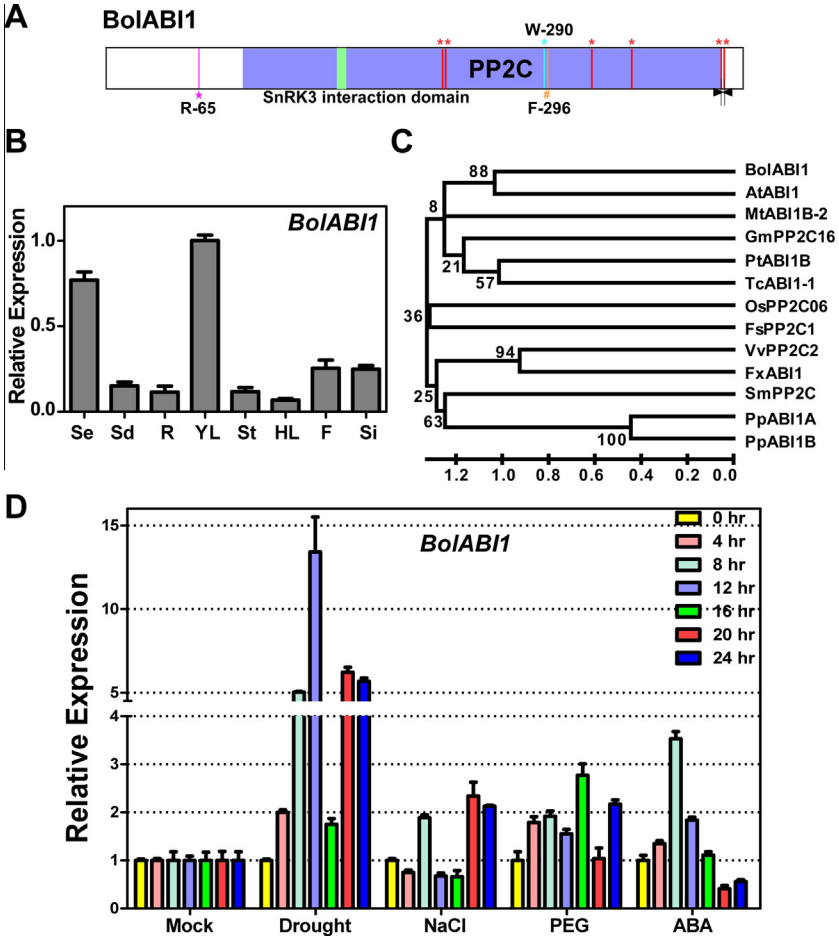

Fig. 1. Domain organization, sequence analysis and expression patterns of *BolABI1*. (A) Schematic diagram of BolABI1 protein domains. The PA binding site (R-65) is shown by the pink line, the PP2C domain is shown by the blue box, the SnRK3 interaction domain is shown by a the green box, the conserved Trp (W-290) and Phe (F-296) residues are shown by the light blue and light red lines, respectively, the key amino acids constituting the ABA box are shown by the starred red line and the NLS-like motif is shown by the black arrowhead. (B) Sequence relationships between BolABI1 and its orthologs. The dendrogram shown was generated using the neighbor-joining method and MEGA 5.1 (http://www.megasoftware.net/). BolABI1, *Brassica oleracea* ABI1-like protein; AtABI1, *Arabidopsis thaliana* ABA Insensitive 1 protein; MtABI1B-2 (XP_003603175.1); GmPPC16 (XP_003544590.1); PtABI1B (CAM84257.1); TcABI-1 (EOY29968.1); OsPP2C06 (Q0JLP9.1); FsPP2C1 (CAB90633.1); VvPP2C2 (CBI35919.3); FxABI1 (AFZ94860.1); SmPP2C (XP_002982468.1); PpABI1A (BAG12299.1); PpABI1B (BAG12298.1). (C) *BolABI1* expression in various tissues. Se, seeds; Sd, seedling; R, root; YL, young leaves; St, stem; HL, heading leaves; F, flower; Si, silique. The relative expression of *BolABI1* was normalized to the expression of cabbage *ACTIN2* and expressed relative to the level in leaves. (D) qRT-PCR analysis of the expression pattern of *BolABI1* under various environmental stress conditions. The relative expression of *BolABI1* was normalized to the expression of cabbage *ACTIN2* and expressed relative to the level in mock-treated seedlings. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dehydration and high salt, has been observed in wild-type and many ABA signaling mutants [24]. To elucidate the role of *BolABI1* in plants, we examined its expression pattern throughout the plant life cycle and in response to various environmental stimuli. As shown in Fig. 1B, the transcript of *BolABI1* was highly accumulated in seeds and leaves. When exposed to drought stress and ABA treatment, the expression of *BolABI1* was significantly induced within 8 h (Fig. 1D). The inducible expression of *BolABI1* by drought increases the concentration of this transcript to a steady state at 12 h, followed by a rapid decrease at 16 h, and a further increase after 20 h (Fig. 1D). Treatment with exogenous ABA led to an increase in *BolABI1* transcript levels until a steady state was reached after 8 h (Fig. 1D). The expression of *BolABI1* was not significantly stimulated by high salt stress or PEG treatment (Fig. 1D). Gene expression patterns often provide insight into gene function; the inducible expression pattern of *BolABI1* suggests that this gene participates in plant ABA or drought responses.

3.3. BolABI1 confers insensitivity to ABA upon the wild-type plants when overexpressed

In Arabidopsis, ABI1 is a pivotal negative regulator of ABA signaling [9]. When constitutively expressed, transgenic plants harboring *ABI1* exhibited ABA-insensitive phenotypes in seed germination, root growth and stomatal closure [25]. To determine the significance of *BolABI1* to the ABA response, we constructed transgenic lines overexpressing *BolABI1* (Col-0::*Flag-BolABI1*) and examined their responses to exogenous ABA. Three independent transgenic lines of the T3 generation were chosen to test the sensitivity of seed germination to ABA. As expected, *BolABI1* overexpression lines exhibited a high frequency of seedlings with green cotyledons under 0.6 μM ABA treatment (Fig. 2A–C). The percentage of germination under various ABA concentrations was also determined. As shown in Fig. 2E, all of the tested Col-0::*Flag-BolABI1* plants exhibited insensitivity to the indicated ABA concentrations. The protein level of Flag-BolABI1 was also measured

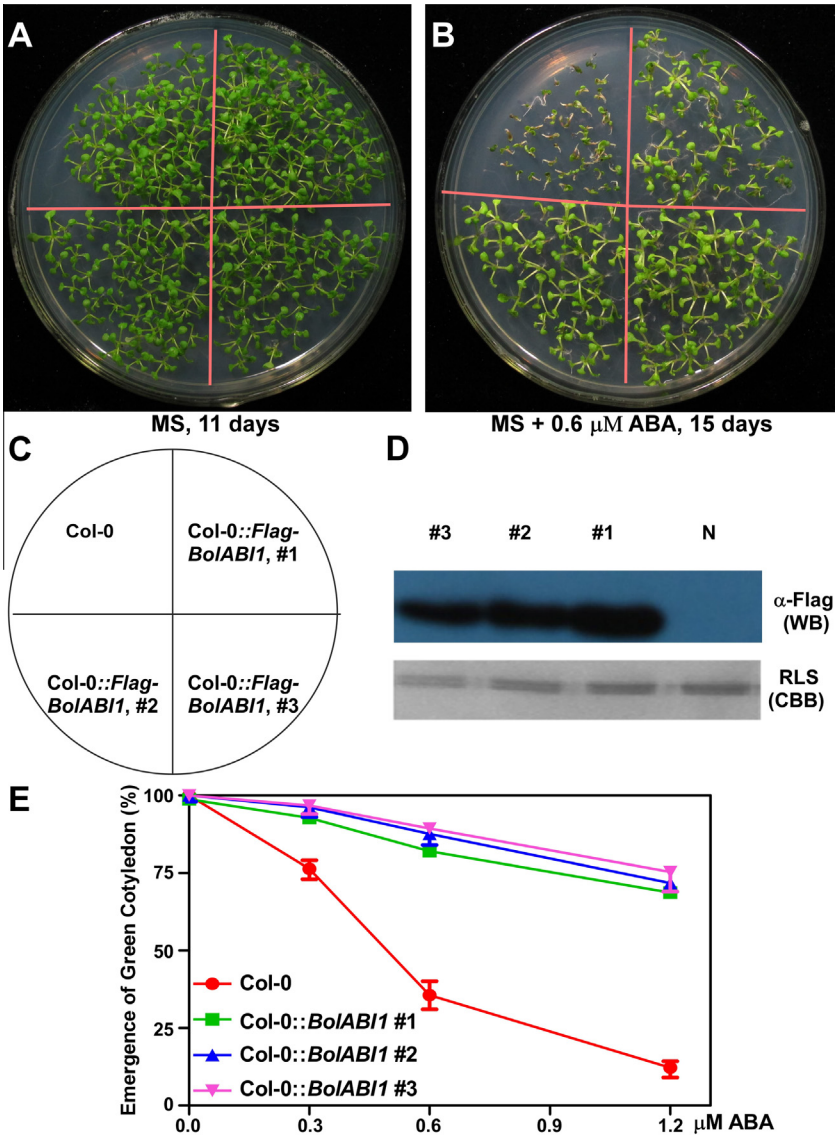

Fig. 2. Constitutive expression of *BolABI1* confers insensitivity to ABA upon the wild-type plants during germination. (A) and (B) Sensitivity of seeds to ABA. The seeds of Col-0 and transgenic Col-0 lines carrying *Flag*-tagged *BolABI1* (Col-0::*Flag-BolABI5*) were germinated on MS medium (A) and MS medium supplemented with 0.6 μM ABA (B) for the indicated number of days. A schematic diagram of the plants that were sown is shown in (C). (D) Western blots of Flag-BolABI1 protein levels in the transgenic lines. CBB R250-stained Rubisco large subunit (RLS) served as a loading control. (E) The emergence rate of green cotyledons from Col-0 and Col-0::*Flag-BolABI1*-#1, #2, and #3 transgenic seeds plated on MS medium supplemented with ABA. Approximately 150 seeds were used in each experiment. The error bars represent the standard deviation (seed number > 100).

(Fig. 2D). The results obtained indicate that BolABI1 functions as ABI1 in the negative regulation of ABA signaling during seed germination.

3.4. Subcellular localization of the BolABI1-GFP fusion protein

Nuclear localization of ABI1 is an essential requirement for the negative modulation of ABA signaling [22]. As mentioned above, sequence analysis of BolABI1 revealed a putative NLS-like motif at the C-terminus (Figs. 1A and 3D). Consequently, we aimed to determine the subcellular localization of BolABI1. We observed BolABI1-GFP fluorescent signals in transfected protoplasts under a fluorescence microscope. As previously reported [22], the BolABI1-GFP fusion protein was localized predominantly in the nucleus (Fig. 3A–C). The level of expressed GFP-fused BolABI1 was also determined (Fig. 3E). The obtained data suggest that BolABI1 is mainly localized to the nucleus.

3.5. Phosphatase activity of BolABI1

Members of the group A PP2C family are able to promote the release of a phosphate group from many phosphorylated substrates, including casein, SLAC1-NT and ABF1N [26,27]. Because the phosphatase activity of ABI1-like proteins is essential for their biological roles, we determined the phosphatase activity of BolABI1. Recombinant proteins comprising GST fused to BolABI1 and ABI5-N were produced in *E. coli*, and a phosphatase assay was performed *in vitro*. Consistent with previous studies [27], BolABI1 removed the phosphate group from the ^{32}P-labeled ABI5-N approximately 50-fold less than the control (Fig. 4). However, almost no phosphatase activity was detected when ^{32}P-labeled MBP was used as a substrate (data not shown). These assays indicate that the recombinant BolABI1 is an active phosphatase.

3.6. BolABI1 interacts with a homolog of the cabbage OST1-type SnRK2 protein kinase

According to the core regulatory network of the reversible phosphorylation cascade in the early ABA signaling pathway, SnRK2 release from the PP2C-SnRK2 complex is required to establish the ABA response in plants [2]. Direct protein-protein interactions are present among clade-A PP2C, OST1-type SnRK2 protein kinases and ABI5-like bZIP transcription factors [27]. Because we observed dephosphorylation of ABI5-N by BolABI1 and a high degree of sequence identity between ABI5 and BolABI5 [16] (Fig. 4A), we attempted to determine whether BolABI1 binds BolABI5 or ABI5 in plants. We assayed the interactions between BolABI1 and BolABI5 and between BolABI1 and ABI5 using the firefly luciferase complementation technique [20]. To our surprise, almost no LUC activity was detected when cLUC-BolABI1—BolABI5-nLUC or cLUC-BolABI1—ABI5-nLUC were co-expressed in protoplasts (Fig. 4C).

We recently identified a *B. oleracea* OST1 ortholog (BolOST1) involved in the plant drought response [17]. Therefore, we also attempted to detect an interaction between BolABI1 and BolOST1. Interestingly, a high level of luciferase activity was detected when BolOST1 was co-expressed with BolABI1 (Fig. 4C), but almost no activity was detected when BolABI1 was co-expressed with the nLUC empty vector or with the cLUC empty vector (Fig. 4C).

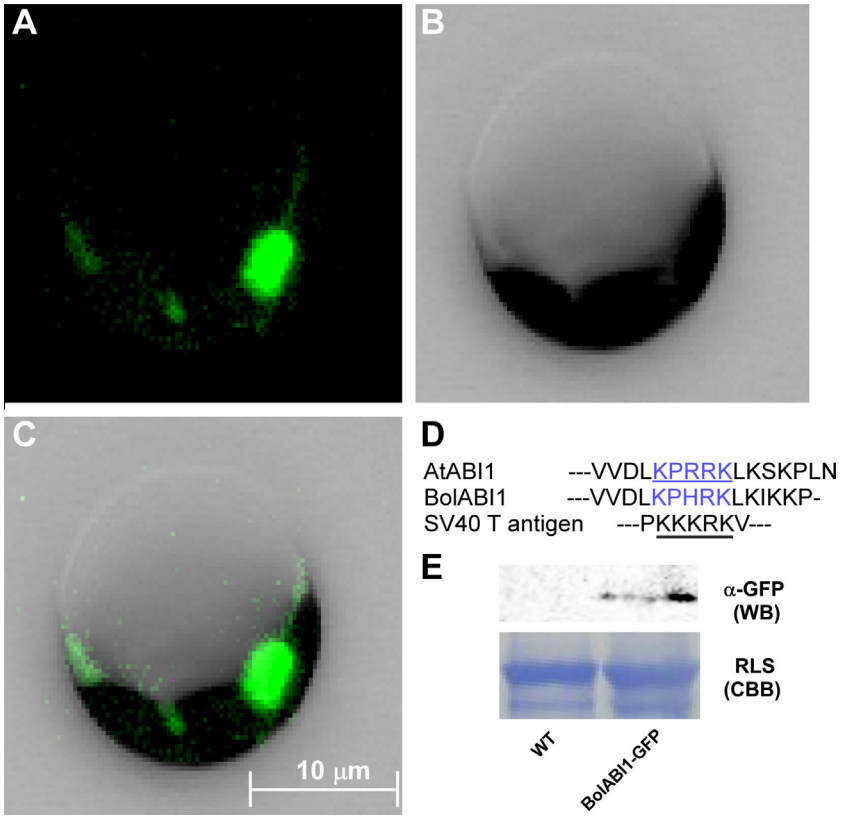

Fig. 3. Subcellular localization of the BolABI1 protein (A–C) The GFP fluorescence signal of BolABI1-GFP. (A) Green fluorescence under a dark field. (B) Cell morphology of protoplasts under a bright field. (C) Overlay of bright-field and green-fluorescence signals. Bars = 10 μm. (D) The carboxyl-terminal sequence of BolABI1 is similar to the NLS of the SV40 large T antigen. The NLS-like motif is underlined and shown in light blue. (E) Western blots of BolABI1-GFP protein levels in the transfected protoplasts. CBB R250-stained RLS in the blot served as a loading control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

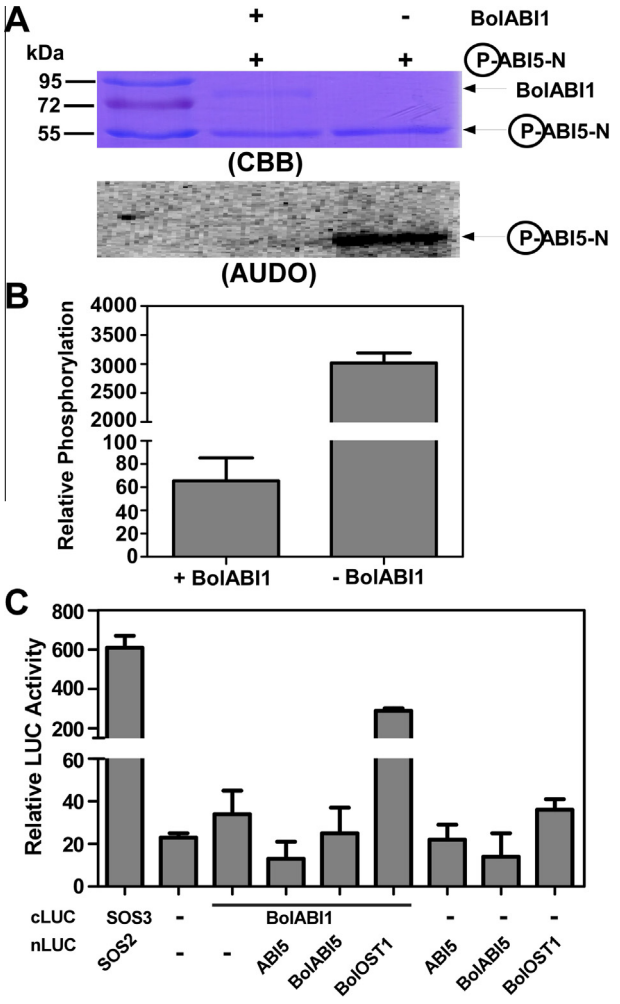

Fig. 4. Phosphatase assay of BolABI1 and the protein–protein interaction between BolABI1 and BolOST1. (A) Dephosphorylation of ABI5-N by BolABI1. The proteins are notably arrow-shaped strikingly arrowed. Upper panel, CBB R250-stained 12% SDS–PAGE gel; lower panel, phosphorylation activities of proteins (AUDO). (B) Quantification of the data shown in (A). (C) Protein–protein interaction between BolABI1 and BolOST1. The indicated combinations of the nLUC and cLUC vectors were co-expressed in protoplasts, and the relative LUC activity was measured.

In conclusion, we have functionally characterized *BolABI1*, an *ABI1* ortholog, in *B. oleracea* var. capitata L. Taken together, our data suggest that BolABI1 is a functional PP2C protein that might be equally significant in plant ABA responses as ABI1. BolABI1 dephosphorylates ABI5-N and interacts with BolOST1 (Fig. 4). However, no direct interaction between BolABI1 and BolABI5 or ABI5 was detected using the firefly luciferase complementation assay; thus, these proteins may be quite different from their homologs in Arabidopsis [27]. A comprehensive investigation of BolABI1 or a full characterization of all ABI1-like genes in *B. oleracea* is thus required to fully understand this functional relationship and to determine whether the core regulatory mechanism of early ABA signaling in cabbage is conserved.

Acknowledgments

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