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Research article

Boron reduces cell wall aluminum content in rice (*Oryza sativa*) roots by decreasing H_2O_2 accumulation

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ABSTRACT

When boron (B) deficiency and aluminum (Al) toxicity co-exist in acidic soils, crop productivity is limited. In the current study, we found that 3 μM of B pretreatment significantly enhances rice root elongation under Al toxicity conditions. Pretreatment with B significantly decreases the deposition of Al in rice apoplasts, suppresses the synthesis of cell wall pectin, inhibits cell wall pectin methylesterase (PME) activity and its gene expression, and increases the expression of *OsSTAR1* and *OsSTAR2*, which are responsible for reducing the Al content in the cell walls. In addition, B pretreatment significantly increases *OsALS1* expression, thereby facilitating the transfer of Al from the cytoplasm to the vacuoles. However, B pretreatment had no effect on Al uptake and citric acid secretion. Pretreatment with B significantly increases the activity of ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT), thus increasing the elimination rate of H_2O_2 in rice roots. Co-treatment using B and H_2O_2 does not increase root growth under Al toxicity conditions; it also improves pectin synthesis, enhances PME activity, and increases Al deposition in root cell walls. However, the co-treatment of B and H_2O_2 scavenger 4-hydroxy-TEMPO has an opposite effect. The above results indicate that applying B fertilizers in acidic soil can help decrease the side effects of Al toxicity on rice growth.

1. Introduction

The non-toxic aluminum (Al) metal in the earth in acidic soil (pH < 5), which occurs in 50% of potential arable land around the world, is easily transformed to the toxic trivalent (Al^{3+}) cationic form (Kochian, 1995). During periods of plant Al toxicity, root elongation is significantly inhibited, which in turn reduces nutrient and water uptake that ultimately limits production (Kochian et al., 2005). Al toxicity also leads to various negative effects on the normal growth of plants, including damage to the integrity of the cell membrane by increasing production of reactive oxygen species (ROS) and inhibiting or altering the functions of other elements in plants such as Mg (Deng et al., 2006), Ca (Rengel and Zhang, 2003) and Zn (Keltjens, 2005). In addition, the accumulated Al in the cytoplasm can bind to nuclei and damage DNA (Silva et al., 2002), thereby inhibiting cell division (Mohanty et al., 2004). Therefore, elucidating the underlying mechanism of Al resistance in plants may increase crop production in acidic soil.

The roots of plants are continuously exposed to Al in acidic soil, and thus over time plants develop adapted Al tolerance mechanisms, which include external and internal detoxification. The external detoxification strategy can be described as suppressing Al from entering the plant or excluding Al from the plant. These include root border cells secreting mucilage (Cai et al., 2011), modifying the lipid composition of the cell membrane (Huynh et al., 2012), changing the content of polysaccharide in the root cell wall or masking the cell wall Al-binding site using UDP-glucose (Huang et al., 2009; Yang et al., 2008), releasing organic acids from the roots, including malate (Chen et al., 2010), oxalate (Ma et al., 1997), and citrate (Liu et al., 2018), increasing the uptake of other elements such as Mg^{2+} (Chen et al., 2012), and chelating Al in the plasma membrane (Xia et al., 2013). Internal detoxification processes include removing ROS by increasing antioxidant enzymatic activity (Ma et al., 2012) or transferring the toxic Al to less toxic compartments (such as vacuoles) by increasing the activity of relevant transporters and enhancing the expression of relevant genes (Xia et al., 2010).

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The root apoplast is the main target of Al deposition, and a variety of nutrient elements or beneficial elements are involved in alleviating Al toxicity in plants by decreasing Al content in plant root cell walls (Blamey, 2001). For example, the macroelement NH_4^+ reverses the inhibition of root elongation in rice by decreasing root pectin content to reduce cell wall Al content (Wang et al., 2015). The application of the beneficial element Si to sorghum alleviates Al toxicity by forming Al-Si complexes in its roots (Kopittke et al., 2017). Therefore, increasing the exclusion of Al from root cell walls is pivotal for plants to resistant Al toxicity, and discovering plant regulatory factors is important to further understand the underlying detoxification mechanism.

Boron (B) is an essential microelement for plant growth and is involved in alleviating Al toxicity. The application of B to Al-containing hydroponic nutrient media and acidic soil significantly increases root elongation in squash (*Cucurbita pepo* L. cv. Sunbar) and alfalfa (*Medicago sativa* L. cv. Hy-Phy) (Lenoble et al., 1996a, 1996b). The application of B in nutrient solutions significantly increases root growth by decreasing Al content in the cell walls of soybean (*Glycine max*) cv. Maple Arrow and decreasing Al stress in the pea (*Pisum sativum*) (Stass et al., 2010; Yu et al., 2008). In the trifoliate orange, the addition of B significantly reduces oxidative damage, decreased cell wall Al-binding capacity by altering cell wall components and structure, thereby alleviating Al-induced inhibition of root growth (Riaz et al., 2018a; Yan et al., 2018). Recently, another study found that B promotes the alkalization of the transition zone in pea roots, thus alleviating Al toxicity (Li et al., 2018). However, the mechanisms for mitigating Al toxicity by B in different plants may vary. For example, the application of B does not alleviate Al toxicity in monocotyledonous maize (*Zea mays*) due to its low requirement for B (Wang et al., 2005). The graminaceous wheat has a lower B requirement and quickly recovers from B deprivation (Yu et al., 2010). Rice is also a graminaceous plant; however, whether B participates in alleviating Al toxicity in this species remains unclear.

The present study examined the root apices (0–1 cm) of the rice cultivar Kasalath, which is the main target of Al toxicity (Ryan et al., 1993). The aims of our study were to investigate whether B is involved in alleviating Al toxicity in rice and its underlying mechanism. This is the first study that has focused on the physiology and molecular reactions of Al in rice roots apices (0–1 cm) in the presence of B, which showed that B decreases H_2O_2 content to reduce pectin content and pectin methylation, thereby decreasing Al toxicity. This study provides a theoretical basis for the alleviation of Al toxicity in rice by applying B-containing fertilizers to acidic soils.

2. Materials and methods

2.1. Rice culture and treatments

Rice seeds of the *indica* subspecies Kasalath (Kas) cultivar were soaked in deionized water for one day and germinated on moist filter paper until germination. The seeds were transferred to a net floating in a 5-L plastic container of 0.5 mM $CaCl_2$ (pH 5.5) solution and grown until root length reached approximately 2 cm (approximately 2 d). The growth room was maintained at 30 °C with 60% relative humidity in total darkness.

The seedlings were grown in 0.5 mM $CaCl_2$ (pH 5.5) that contained 0, 1, 3, or 9 μM B. After 24 h, every set of treatments was administered to two groups; one group was grown in 0.5 mM $CaCl_2$ (pH 4.5) and the other in 0.5 mM $CaCl_2$ (pH 4.5) that contained 30 μM Al ($AlCl_3{\cdot}6H_2O$). The length of the rice roots was measured before and after 24 h of Al treatment, and the Al content in the root apices (0–1 cm) was also measured after 24 h of Al treatment. The root apices (0–1 cm) were collected with a razor after washed three times with distilled water (Fig. S1).

To explore whether H_2O_2 participates in the response of rice to Al toxicity, the following treatments were used: the seedlings were grown in 0.5 mM $CaCl_2$ (pH 5.6) with or without 3 μM B for 24 h. Every set of treatments was administered to following groups: the set without B pretreatment was treated with Al (+Al) or without Al (CK); the other set with B pretreatment was treated with Al (+B + Al), Al and H_2O_2 (+B + Al + H_2O_2), and Al and the H_2O_2 scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl {4-hydroxy-TEMPO} (+B + Al+4-TEMPO). All treatments were grown in 0.5 mM $CaCl_2$ (pH 4.5), the concentration of H_2O_2 in the solution was 50 μM, and the concentration of 4-hydroxy-TEMPO was 100 μM. The concentrations of H_2O_2 and 4-hydroxy-TEMPO (4-TEMPO) used in the present study were based on Xiong et al. (2015).

2.2. Al extraction and quantitation

Al was extracted from 10 root apices (0–1 cm) in 1 mL of 2 M HCl. The Al in the cell sap was extracted using a freeze-thaw method, whereby 10 apices (0–1 cm) were placed in 1.5-mL Ultra free-MC tubes (Millipore, Billerica, MA, USA) that contained a 0.45-μM filter. After storage at −80 °C for 24 h, the tubes were thawed at room temperature, the cell sap was collected after centrifugation, and the solution volume was recorded (Xia et al., 2010). The residues in the 1.5-mL Ultra free-MC tubes were further used to extract the Al in the apoplast using 1 mL of 2 M HCl, and the apoplast extract was collected after centrifugation. The Al in the cell wall was extracted with 1 mL 2 M HCl from approximately 2 mg of cell wall materials and collected after centrifugation. The Al in the solutions was diluted as necessary and measured by inductively coupled plasma-mass spectrometry (ICP-MS). Every set of experiments consisted of three replicates.

2.3. Root staining

Eriochrome cyanine R stain was used to visualize the Al content on the root surface. The root apices (0–1 cm) were stained in a 0.1% solution of eriochrome cyanine R (*v/w*) for 10 min at 25 °C, and then the excess dye was washed off with distilled water. A light stereomicroscope (M205 FA; Leica) was used to observe the pink color in the root apices, and images were captured (Ma et al., 2001).

Ruthenium red staining was performed to observe the pectin content in the root apices. A 0.02% (*w/v*) ruthenium red solution was used to stain root apices (0–1 cm) at 25 °C for 2 h, and any excess dye was washed off with distilled water. A light stereomicroscope (M205 FA; Leica) was used to observe the red stain in the root apices, and images were captured (Ballance et al., 2012).

CM-H_2DCFDA staining was performed to calculate the relative H_2O_2 content in the root apices based on the intensity of green fluorescence. Root apices (0–1 cm) were washed in 30 mL 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES)-KOH buffer (20 mM, pH 7.8) in darkness. Then, the apices were stained in total darkness for 24 h with 1 mL HEPES-KOH buffer (20 mM, pH 7.8) containing 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, dissolved in 0.0025% dimethyl sulfoxide (DMSO), 50 μM). Any excess dye was washed off with 30 mL of HEPES-KOH buffer (20 mM, pH 7.8) three times for 15 min each. Fluorescent images were captured, and the fluorescence intensity was analyzed according to Chen et al. (2013a).

2.4. Quantification of B in root apices

The root apices (0–1 cm) were collected and dried in the oven until constant weight, then ground into powder. Approximately 0.1 g of each powdered sample was digested in a Teflon tubes (Nalgene, Rochester) containing 1 mL of concentrated HNO_3 at 120 °C until total dryness, then dissolved with 80 mM HNO_3 and measured using ICP-MS (Noguchi et al., 1997).

2.5. Cell wall and pectin extraction

Approximately 0.01 g of fresh root apices was homogenized in 8 mL of 75% alcohol, and the homogenates were incubated for 20 min at 25 °C, then centrifuged at 16,000 *g* for 5 min. The supernatant was discarded, and the residue was washed with 8 mL of acetone, followed by a mixture of a 1:1 ratio of methanol and chloroform (*v*/*v*), and then methanol. The supernatant from each wash was discarded, and the cell walls in the final residue were dried at 60 °C.

The pectin was extracted from 2 mg of crude cell walls by incubating three times with 1 mL of hot water in a 100 °C water bath, for 1 h each time. The supernatants containing the water-soluble pectin were collected after centrifugation for 10 min at 16,000 *g* (Zhong and Lauchli, 1993).

2.6. Quantitation of pectin

The uronic acid concentration in the pectin was measured to determine pectin content. Briefly, 200 μL of pectin solution and 1 mL of 98% H_2SO_4 (containing 12.5 mM $NaB_4O_7{\cdot}10\ H_2O$) were combined in a 1.5-mL Eppendorf tube, incubated at 100 °C for 5 min, and cooled on ice. After the addition of 20 μL of 0.15% M-hydroxydiphenyl, which was dissolved in 0.5% NaOH, the reaction was allowed proceed at room temperature for 20 min, and the absorbance was recorded at a wavelength of 520 nm (Blumenkrantz and Asboe-Hansen, 1973).

2.7. Measurement of activity of pectin methylesterase (PME)

For extraction of the crude enzyme from 0.5 g fresh root apices (0–1 cm), 0.5 mL of 10 mM Tris buffer (pH 7.7) that contained 1 M NaCl was used. PME activity was measured based on the amount of methanol remaining after PME catalysis of pectin according to Anthon and Barrett (2004).

2.8. Citric acid efflux measurement

After 24 h of different treatments, the culture solution was replaced with 0.5 mM $CaCl_2$ solution, into which the seedlings soaked for 12 h. The cations in the root exudate were removed using a cation exchange column filled with 5 g Amerlite IR-120B resin (H^+ form; 16–45 mesh), and the organic acids in the root exudate were bound in an anion exchange column filled with 1.5 g AG 1-X8 resin (formate form, 100–200 mesh). To elute the organic acid from the anion column and concentrate the organic acid at 40 °C with a rotary evaporator, 15 mL of HCl were used. Then, the dried acid residue was re-dissolved in 1 mL of ultrapure water for further analysis. The concentration of citric acid was measured by HPLC according to Ma et al. (2002) and calculated based on the dry weight of the rice roots.

2.9. Quantitation of H_2O_2

The rice root apices (0–1 cm) were weighed and ground with 3 mL of 3-amino-1,2,4-triazole that was cooled to 4 °C to extract the H_2O_2. The amount of H_2O_2 was measured following its equilibration in 0.1% (*v*/*v*) $TiCl_4$ dissolved in 20% (*v*/*v*) H_2SO_4, according to Yang et al. (2007).

2.10. Assay of antioxidant enzymatic activity

The supernatant, which was regarded as the crude enzyme, was extracted with 50 mM phosphate-buffered saline (PBS) pH 7.8 containing 1 mM EDTA and 1 mM ascorbic acid from fresh rice root apices (0–1 cm) and collected after centrifugation at 4 °C. The decreasing rate of H_2O_2 in the reaction system was used to determine catalase (CAT) activity according to Dhindsa et al. (1981). The decreasing rate of ascorbate in the reaction system was used to determine the ascorbate peroxidase (APX) activity according to Nakano and Asada (1981). The production rate of tetraguaiacol in the reaction system was used to determine the peroxidase (POD) activity based on the method of Chen et al. (2013b).

Table 1
Primers used in the present study.

Gene	Sequence (5′→3′)
OsFRDL4-F	CGTCATCAGCACCATCCACAG
OsFRDL4-R	TCATTTGCGAAGAAACTTCCACG
OsSTAR1-F	TCGCATTGGCTCGCACCCT
OsSTAR1-R	TCGTCTTCTTCAGCCGCACGAT
OsSTAR2-F	ACCTCTTCATGGTCACCGTCG
OsSTAR2-R	CCTCAGCTTCTTCATCGTCACC
OsNRAT1-F	GAGGCCGTCTGCAGGAGAGG
OsNRAT1-R	GGAAGTATCTGCAAGCAGCTCTGATGC
OsALS1-F	GGTCGTCAGTCTCTGCCTTCTC
OsALS1-R	CCTCCCCATCATTTTCATTTGT
OsPME11-F	GGAGATCTTCGTGAAGCCTG
OsPME11-R	GTTGATGAACTTGTCGGCCT
OsPME12-F	GAGTCGGTGATCGGAGGG
OsPME12-R	CCACCTAGCACGCATCAAC
OsPME27-F	CTCAAGACGCTCTACTACGC
OsPME27-R	CGCAACATTTCCACTCG
OsHistone-F	GGTCAACTTGTTGATTCCCCTCT
OsHistone-R	AACCGCAAAATCCAAAGAACG

2.11. Quantitative real-time PCR (qRT-PCR)

Total RNA in the root apices (0–1 cm) was extracted using TRIzol reagent, and cDNA amplification was performed using a PrimeScript RT reagent kit (Takara Bio, Kyoto, Japan) according to the kit specifications. qRT-PCR was conducted according to Zhu et al. (2016). The primers used in the present study are shown in Table 1 (Huang et al., 2009, 2012; Kengo et al., 2011; Xia et al., 2010, 2013; Yang et al., 2013). Three replicates were used for each sample. The $2^{-\Delta\Delta CT}$ method was used to calculate relative gene expression levels (Livak and Schmittgen, 2001). The *OsHistone* gene was used as reference for data normalization.

2.12. Statistical analysis

All experiments in the present study were performed in triplicate. The data were analyzed by one-way ANOVA. Mean values were compared using the Tukey test. Different letters in the figures denote significantly different mean values at $P < 0.05$.

3. Results

3.1. B alleviates Al toxicity in rice

Pretreating the rice roots with 3 and 9 μM of B for 24 h significantly enhanced root elongation compared with the 0 and 1 μM B pretreatment under Al toxicity conditions (Fig. 1), indicating that an appropriate B concentration mitigates Al toxicity in rice. Based on the above results, the 3 μM B concentration was used in the subsequent experiments.

3.2. B alleviates Al toxicity by reducing the amount of apoplastic Al

The phenotype of the rice roots confirmed that the 3 μM B pretreatment alleviates Al-induced inhibition of root growth (Fig. 2A). The B concentration in the rice roots significantly increased after pretreatment with B, regardless of treatment with Al (Fig. 2B), indicating that pretreatment with B resulted in an increase in B concentration in rice roots and further confirmed that B is involved in alleviating Al toxicity. In addition, the amount of Al in the rice root apices (Fig. 2C) and the Al

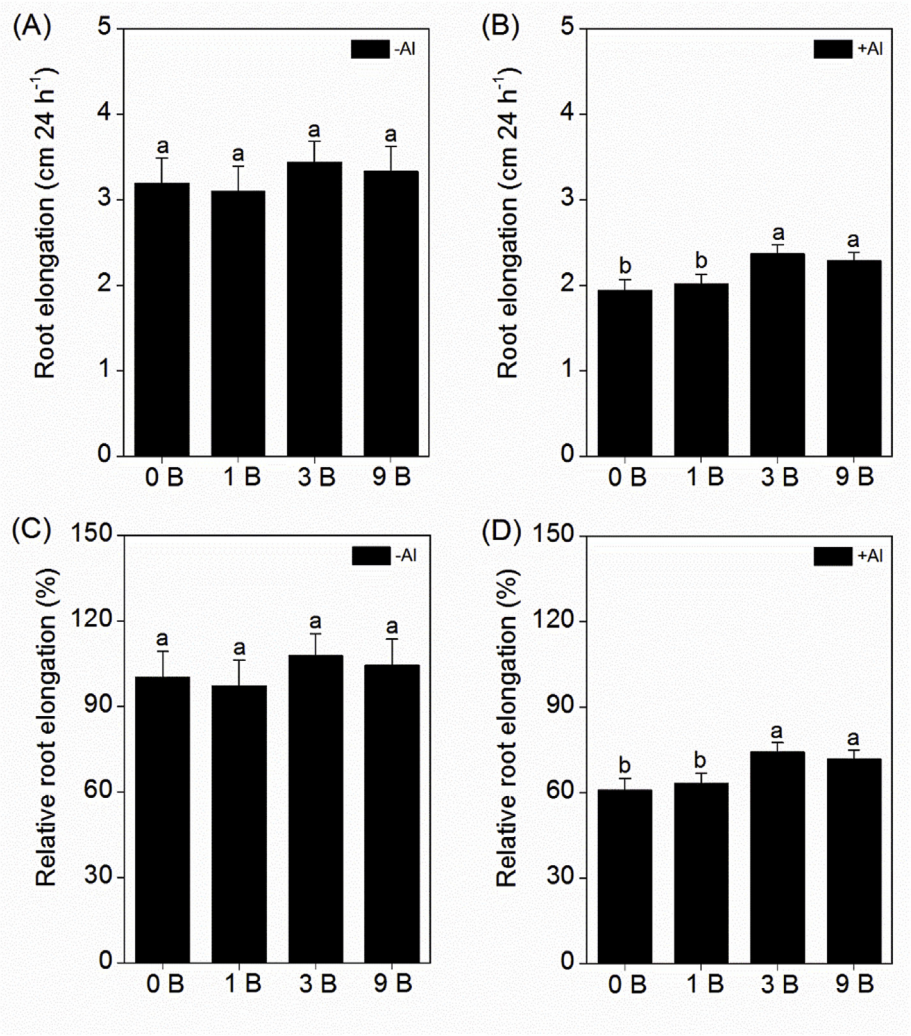

Fig. 1. The effect of B pretreatment on root elongation under (A) -Al and (B) +Al conditions and relative root elongation under (C) -Al and (D) +Al conditions in the Kas cultivar. The data were expressed as the mean ± SD ($n = 10$). Columns with different letters are significantly different at $P < 0.05$.

deposited on the surface of root apices (Fig. 2D, the pink color denotes the Al that accumulated) significantly decreased after pretreatment with 3 μM of B under Al toxicity conditions, indicating that B alleviates Al toxicity in rice by decreasing root Al content.

Most of the Al in plant roots accumulates in the cell wall, and a previous study has shown that Al removal significantly mitigates Al toxicity (Ma et al., 2004). We also extracted the cell wall from the root apices and found that the Al content in the cell wall also significantly decreased after pretreatment with B (Fig. 2E), together with a decrease in the Al concentration in the apoplast sap (Fig. 3F), indicating that B takes part in excluding Al from the root cell wall.

3.3. Pectin is involved in alleviating Al toxicity

Pectin content and its demethylation levels are the main factors that determine the number of negative charges and thus affect its capacity to bind with Al cations (Horst et al., 2010). In our study, Al significantly increased the amount of pectin and activity of PME in the root apices (Fig. 3A and B). However, these parameters in the root apices that were pretreated with B significantly decreased in the presence of Al (Fig. 3A and B). Pectin can be visualized using ruthenium red staining, and our results showed that Al treatment significantly deepens the pink color in the root apices, and pretreatment with B results in a faded, pink color (Fig. 3C), further confirming that B significantly decreases the pectin content in the rice root apices.

There are 35 members in the *OsPMEs* gene family in rice, and some of them are responsible for Al tolerance in different rice cultivars (Yang et al., 2013). In our study, *OsPME11*, *OsPME12*, and *OsPME27* were selected, and Al treatment significantly induced their expression. However, the B pretreatment significantly inhibited their expression under Al toxicity conditions (Fig. 3D–F), implying that B decreases the low methyl esterification pectin content in rice by inhibiting the relevant genes expression.

The *OsSTAR1* and *OsSTAR2* genes encode proteins that are associated with transporting UDP-glucose to the cell wall, ultimately covering up the Al-binding sites (Huang et al., 2009). In our study, the expression of both genes was stimulated by Al, and B pretreatment further enhanced their expression (Fig. 4), suggesting that B takes part in decreasing Al absorption in the cell wall to mitigate Al toxicity.

The secretion of citric acid from the roots is another important strategy in alleviating Al toxicity in rice (Ishikawa et al., 2000; Ma et al., 2002). In our study, the expression of *OsFRDL4*, which is involved in citric acid secretion, and the amount of citric acid in the root exudate both significantly increased under Al toxicity conditions (Fig. 5). However, no significant difference between Al and B + Al treatments was observed (Fig. 5), indicating that B pretreatment failed to stimulate citric acid secretion to decrease Al deposition in the rice roots.

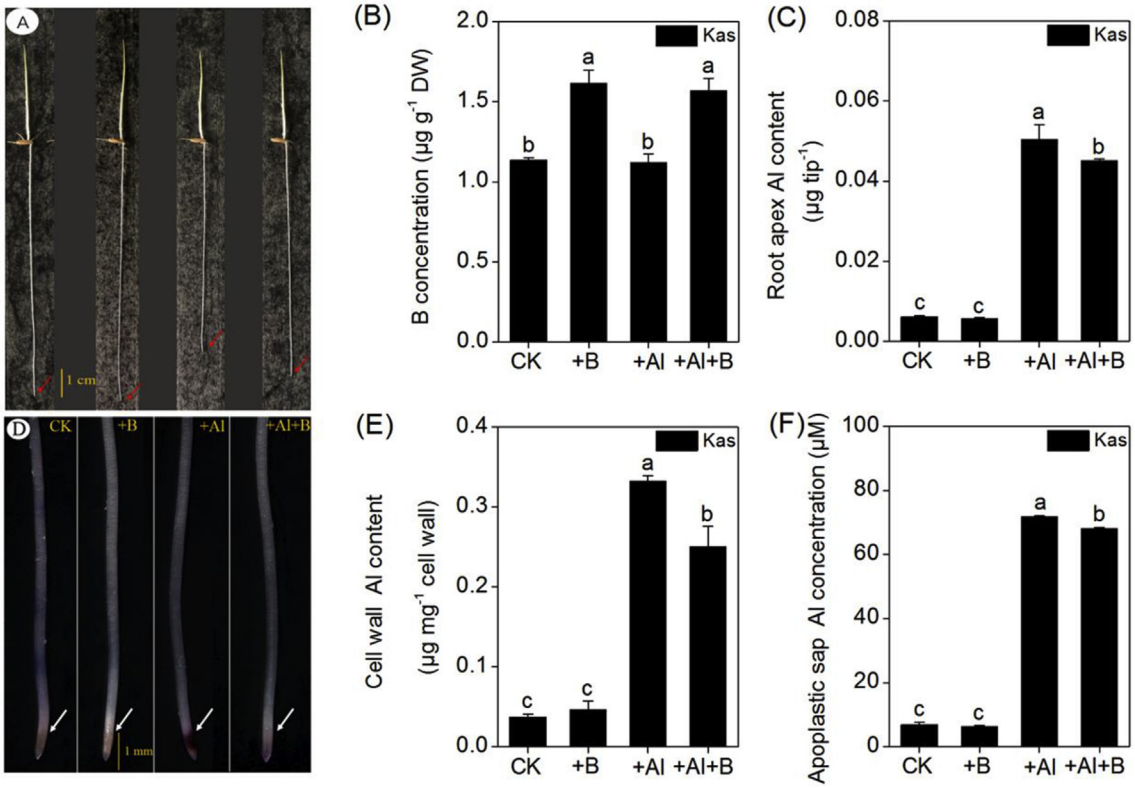

Fig. 2. The effect of B pretreatment on (A) rice phenotype, (B) B concentration in the root tips, (C) the Al content in root tips, (D) the Al deposition in the root surface in the root tips, (E) the Al content in the cell wall in root tips, and (F) the Al concentration in apoplast sap in the root tips of the Kas cultivar. For the Al deposition in the root surface experiment, the data were expressed as the mean ± SD (n = 10), and the pink color indicates the Al deposition on the root surface. For the other three experiments, the data are expressed as the mean ± SD (n = 4). Columns with different letters are significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. B increases Al transport from the cytoplasm to the vacuoles

The cell sap was collected by the freeze-thaw method, and the results showed that the Al content of the cell sap remarkably increased with Al treatment (Fig. 6A). However, B pretreatment failed to reduce cell sap Al content (Fig. 6A), suggesting that B does not regulate Al uptake. The expression of *OsNRAT1*, which encodes the Al transporter in rice (Xia et al., 2010), was also induced by Al but failed to decrease with B pretreatment (Fig. 6B), which further confirmed the results of Al in the cell sap (Fig. 6A). However, the expression of *OsALS1*, which is responsible for transporting Al in the cytoplasm into the vacuoles (Huang et al., 2012), was remarkably induced by B pretreatment under Al toxicity conditions (Fig. 6C), indicating that although B failed to reduce Al levels in the cell cytoplasm, it enhanced Al transport from the cytoplasm to the low-toxicity vacuoles.

3.5. B pretreatment reduces H_2O_2 content

H_2O_2 is a side product of oxidation reactions that lead to oxidative damage in plants, as well as acts as a signaling molecule at low concentrations to regulate plant physiological activity (Shafi et al., 2015). To measure the changes in the amounts of H_2O_2 in the root tip, two methods were used in the present study. The fluorescent probe method was used, which revealed that the relative content of H_2O_2 (as indicated by the green fluorescence) in the rice tip significantly increased after 24 h of Al treatment, and B pretreatment significantly decreased H_2O_2 content (Fig. 7A and B). The results of the colorimetric method also generated similar findings for H_2O_2 (Fig. 7C), i.e., Al increased the H_2O_2 content, and B pretreatment decreased the H_2O_2 content.

The antioxidant enzymes that are associated with H_2O_2 catalysis were also measured in the present study. The activity of APX, CAT, and POD in the rice root apices was dramatically stimulated by Al treatment, which was further enhanced by B pretreatment (Fig. 8). These results coincide with the changes in H_2O_2 content in the rice root tips (Fig. 7), implying that B reduces the amount of H_2O_2 by increasing the amount of antioxidant enzymes.

3.6. B pretreatment decreases H_2O_2 content to decrease cell wall Al content

A previous study demonstrated that H_2O_2 is also involved in stimulating the synthesis of pectin and increasing PME activity in rice roots (Xiong et al., 2015), and thus, we hypothesized that B decreases pectin content in cell walls and inhibits PME activity, which are related to changes in H_2O_2 content in rice roots. The results indicated that the treatment of the H_2O_2 scavenger 4-TEMPO together with B pretreatment significantly increased root growth and decreased Al content in the root apices (Fig. 9). However, H_2O_2 treatment, together with B pretreatment, generated the opposite effect under Al toxicity conditions (Fig. 9), indicating that there might be crosstalk between B and H_2O_2 in mitigating Al toxicity.

Treatment with the H_2O_2 scavenger 4-TEMPO together with B pretreatment significantly decreased the amounts of pectin, cell wall Al, and apoplastic Al concentration, as well as PME activity, under Al toxicity conditions (Fig. 10). However, H_2O_2 treatment, together with B pretreatment, generated the opposite results (Fig. 10), indicating that B decreases H_2O_2 content in rice, which in turn reduced the amount of Al in the cell wall, ultimately alleviating Al toxicity.

4. Discussion

B is an important micronutrient for plant growth and is involved in the plant responses to Al toxicity. In sunflower, the application of B

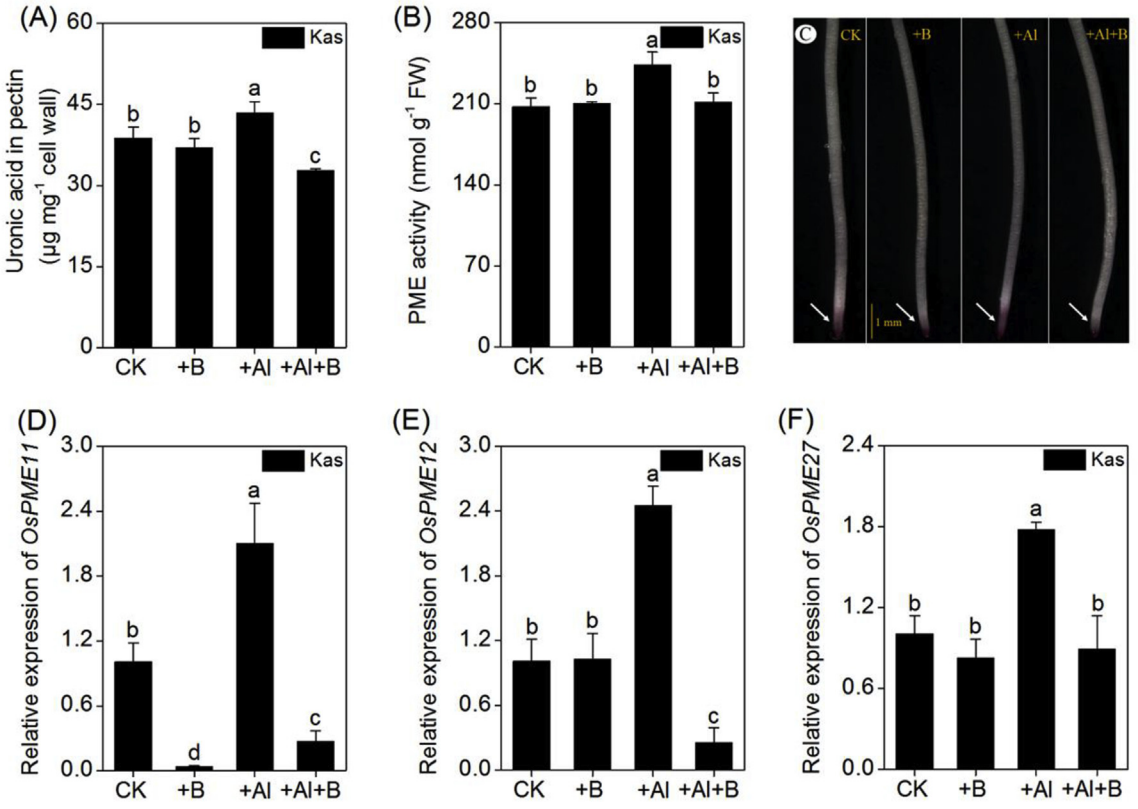

Fig. 3. The effect of B pretreatment on the (A) pectin content, (B) pectin methylesterase (PME) activity, (C) pectin deposition on the root surface, and the expression of (D) *OsPME11*, (E) *OsPME12*, and (F) *OsPME27* in the root tips of the Kas cultivar. The data were expressed as the mean ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

significantly enhances the synthesis of glutathione (GSH) to combat damage caused by oxidative stress and in turn ameliorates Al toxicity (Ruiz et al., 2006). In addition, $O_2^{\bullet -}$ and MDA levels in the trifoliate orange roots significantly decrease after B application under Al toxicity conditions, alleviating Al-induced oxidative damage (Riaz et al., 2018a). A sufficient supply of B significantly alleviates root inhibition in cucumber (*Cucumis sativus*) by decreasing oxidative stress instead of excluding Al from the root apices. B application also induces the synthesis of glutathione to decrease cell death in maize (*Zea mays*) (Corrales et al., 2008), and the application of 200 μM B slightly mitigates root growth in the wheat cultivar "Kalyansona" under Al toxicity conditions (Zakirhossain et al., 2004), indicating that B can also alleviate Al toxicity in monocotyledonous plants. In the present study, pretreatment with B before Al treatment significantly enhanced rice root growth (Figs. 1 and 2A), increased B concentration in rice root apices (Fig. 2B), and decreased Al content in the root apices (Fig. 2C and D), indicating that B prevents the deposition of Al in the roots, thereby alleviating Al toxicity. Therefore, although the B requirements

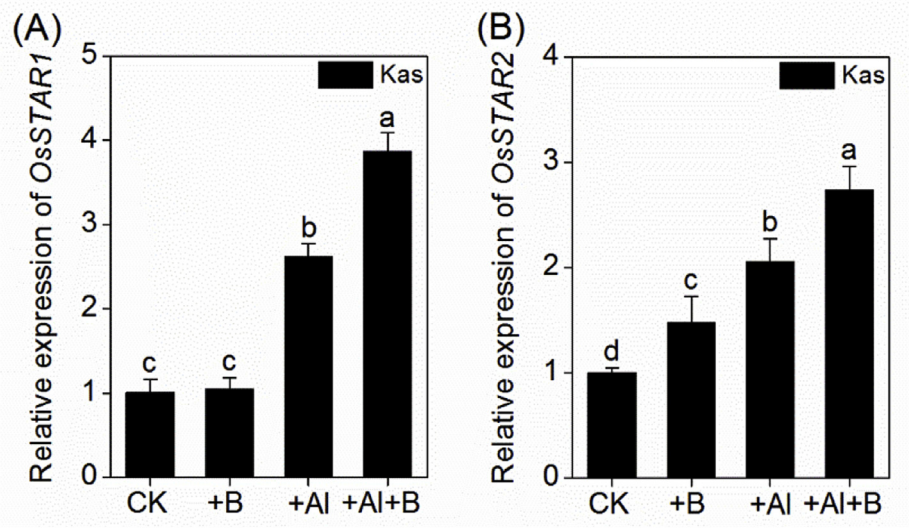

Fig. 4. The effect of B pretreatment on the expression of (A) *OsSTAR1* and (B) *OsSTAR2* in the root tips of the Kas cultivar. The data were expressed as the mean ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

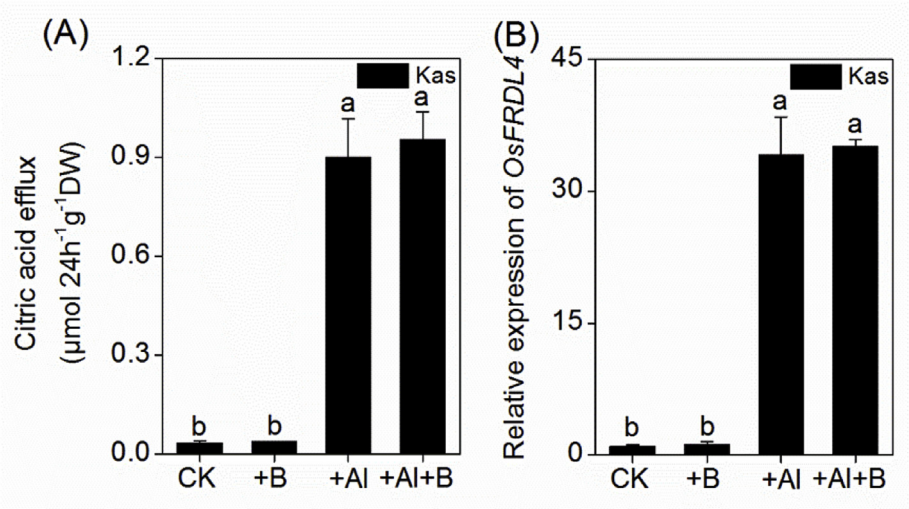

Fig. 5. The effect of B pretreatment on (A) the secretion of citric acid from Kas roots and (B) the expression of *OsFRDL4* in the root tips of the Kas cultivar. The data were expressed as the mean ± SD (n = 4). Columns with different letters are significantly different at $P < 0.05$.

for rice are low, the appropriate supplementation with B can also help in resisting Al toxicity.

The cell wall is the main deposition site for Al in plants, and it has previously been demonstrated that excluding Al from it dramatically mitigates Al toxicity (Li et al., 2016; Yang et al., 2011). Pectin possesses negative charges in its carboxylic groups that bind with Al cations and modulate Al sensitivity in plants (Horst et al., 2010; Schmohl and Horst, 2010). Pectin content can be spatially and temporally regulated (Bush et al., 2001; Peaucelle et al., 2008). In the dicots, B deficiency increases the amount of the low methyl ester of pectin and apoplast Al, which then increase Al toxicity (Stass et al., 2005). Although the pectin content in graminaceous plants is 3–7 times lower than dicotyledonous plants (Smith and Harris, 1999), it also plays a vital role in determining graminaceous plant Al resistance based on genotypic differences such as in rice and maize (Eticha et al., 2005; Yang et al., 2008). Treatment with NH_4^+ without controlling the pH significantly decreases pectin content in the rice cultivar WYJ7, thus decreasing Al content in the root cell wall and alleviating Al toxicity compared to nitrogen treatment using NO_3^- (Wang et al., 2015). The signaling molecule H_2S dramatically inhibits the synthesis of pectin and excludes Al from the root cell wall, thus ameliorating Al toxicity in Nipponbare and Kasalath rice cultivars (Zhu et al., 2018). In our study, the amounts of pectin (Fig. 3A and C), the Al in the cell wall (Fig. 2E), and the Al in the apoplast (Fig. 2F) all significantly decreased after pretreatment with B under Al toxicity conditions, implying that B alleviates Al toxicity by decreasing pectin content in rice.

Pectin contributes to Al deposition in plants, but the degree of pectin methyl-esterification also determines the quantities of carboxylic groups that bind Al (Rangel et al., 2010). The main component that carries carboxyl groups in the pectin is homogalacturonan, which is synthesized in the Golgi bodies in higher methylated forms. Once these are secreted and adhere to the cell wall, the methyl groups in homogalacturonan are removed by PME catalyzation, exposing the free carboxyl groups (Micheli, 2001; Willats et al., 2006). Previous studies have demonstrated that Al treatment markedly enhances the activity of PME and improves the binding capacity between the cell wall and Al, thus increasing root growth inhibition. For example, Eticha et al. (2005), Yang et al. (2008), and Li et al. (2016) found that Al-sensitive maize, rice, and pea all have higher low-methylated pectin content than Al-tolerant varieties, as well as a higher PME activity. A recent study demonstrated that the application of B significantly decreases pectin content and the degree of pectin methyl-esterification (DM%), reduces the relative content of carboxyl C associated with carboxylic acid, decreases the Al content in the cell wall, ultimately alleviating Al toxicity in trifoliate orange (Yan et al., 2018), indicating that B is involved in regulating the activity of PME to modify pectin methylation levels.

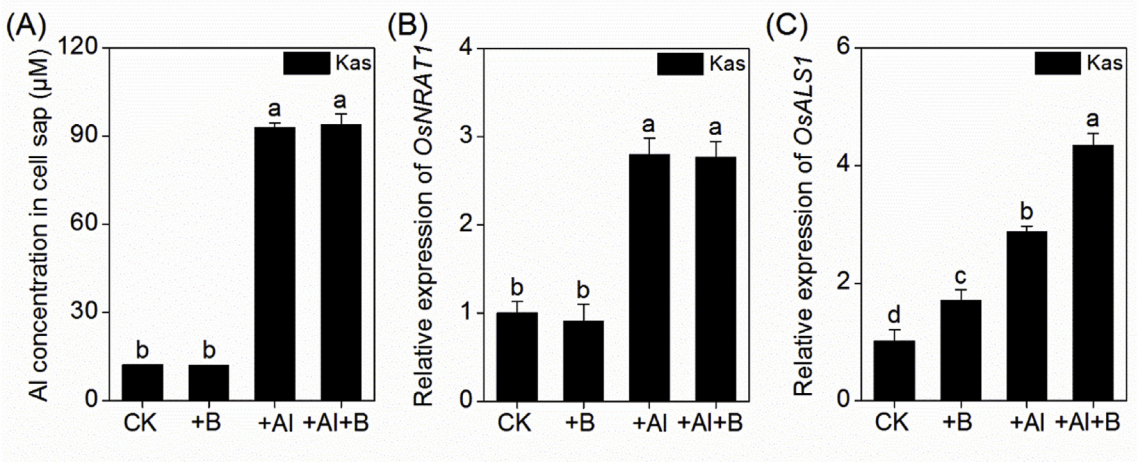

Fig. 6. The effect of B pretreatment on (A) the cell sap Al content, and the expression of (B) *OsNRAT1* and (C) *OsALS1* in the root tips of the Kas cultivar. The data were expressed as the mean ± SD (n = 4). Columns with different letters are significantly different at $P < 0.05$.

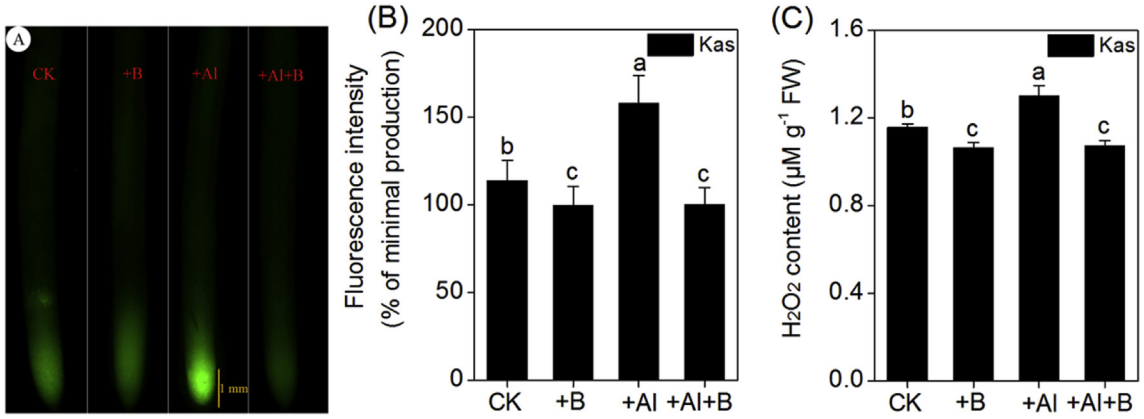

Fig. 7. The effect of B pretreatment on (A) the H_2O_2 content in rice root apices (0–1 cm; indicated by green fluorescence), (B) relative fluorescence intensity (% of minimal production), and (C) the H_2O_2 content in root tips. For the H_2O_2 content experiment, the data are expressed as the mean ± SD (n = 4); for the fluorescence intensity experiment, the data were expressed as the mean ± SD (n = 10), scale bar = 1 mm. Columns with different letters are significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

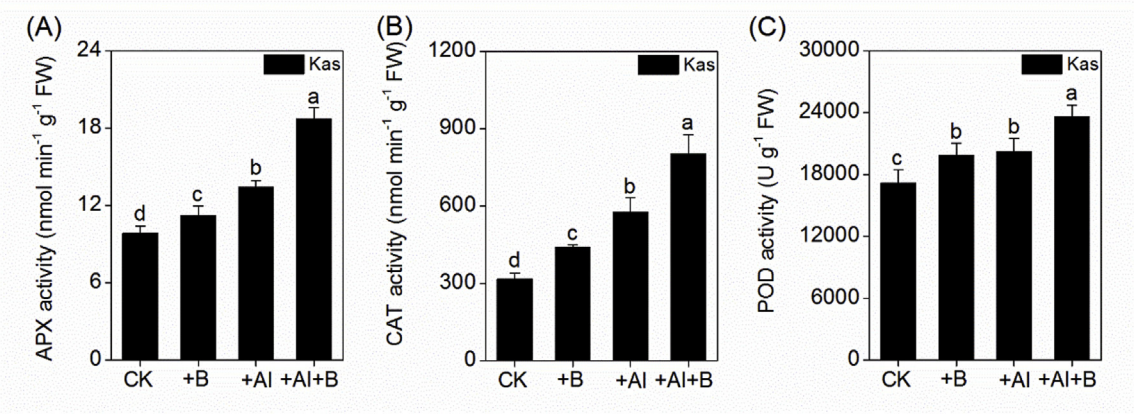

Fig. 8. The effect of B pretreatment on the activity of (A) APX, (B) CAT, and (C) POD in the root tips of the Kas cultivar. The data were expressed as the mean ± SD (n = 4). Columns with different letters are significantly different at $P < 0.05$.

The expression of the *OsPME* family member genes also exhibited different behavior among Al-tolerant rice cultivars. The overexpression of *OsPME14* in the Al-tolerant rice cultivar Nipponbare resulted in an increase in sensitivity to Al stress due to the significant increase in apoplastic Al content (Yang et al., 2013). Short-term treatment with PME and Al in maize roots prominently increases the content of Al in roots that restrains root growth (Horst et al., 2007). The addition of NO scavenger c-PTIO significantly inhibits the activity of PME and increases pectin methylation levels in wheat roots, which in turn decreases root Al content and increases root growth under Al toxicity conditions (Sun et al., 2016). The above studies all demonstrate that the activity of PME determines Al tolerance of plants and can be regulated by other factors. In the present study, the activity of PME and the expression of selected *OsPMEs* that associate with Al toxicity were all

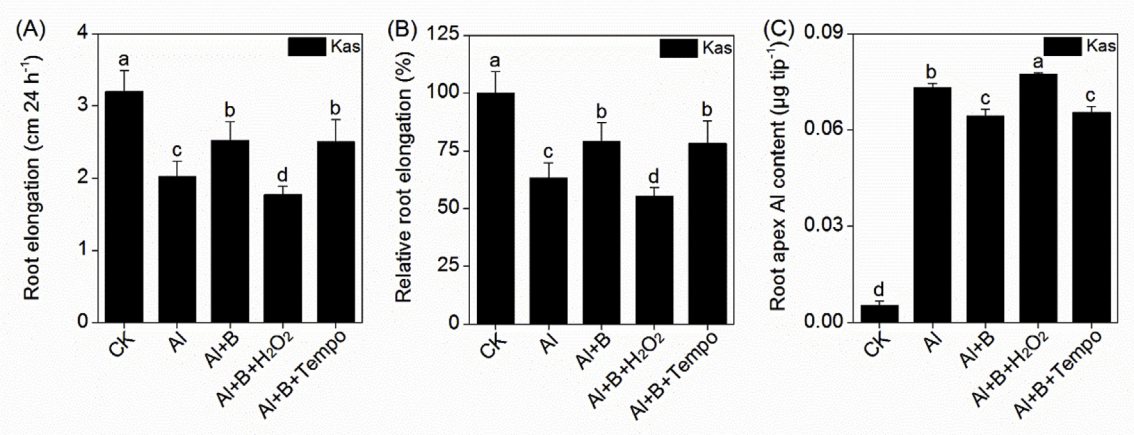

Fig. 9. The effect of different treatments on the (A) root elongation, (B) relative root elongation, and (C) Al content in the root tips of the Kas cultivar. The data were expressed as the mean ± SD (n = 10). Columns with different letters are significantly different at $P < 0.05$. Tempo: 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl.

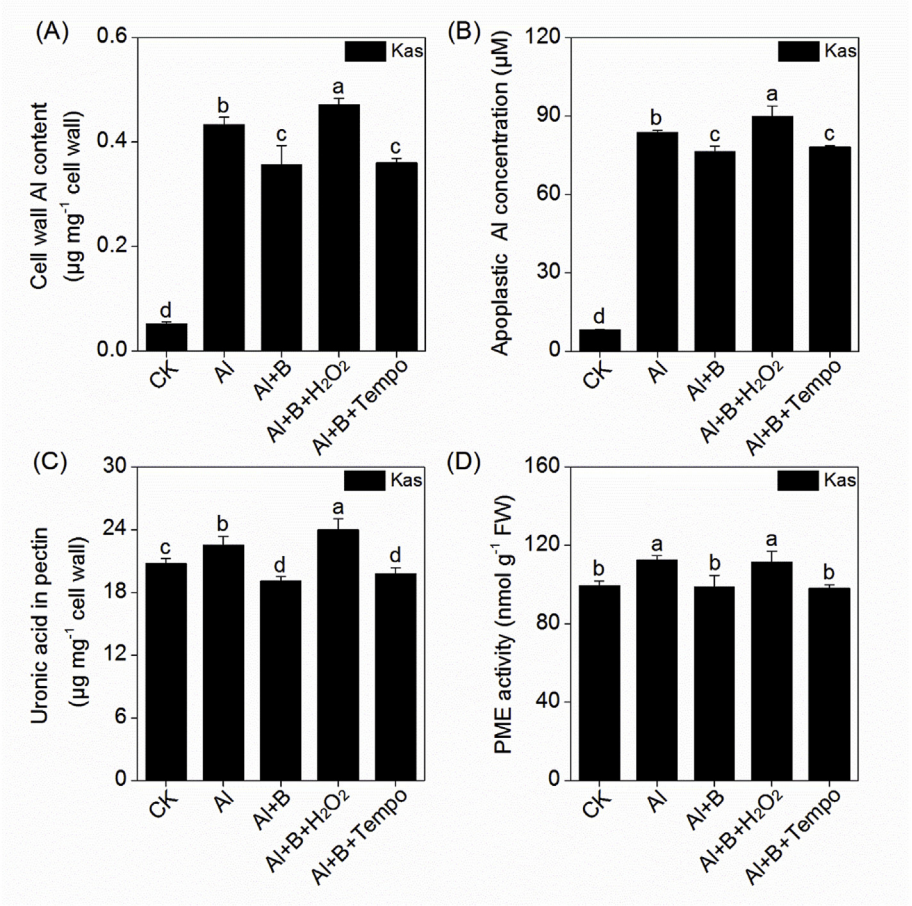

Fig. 10. The effect of different treatments on the (A) cell wall Al content, (B) apoplastic Al concentration, (C) pectin content, and (D) PME activity in the root tips of the Kas cultivar under 30 μM Al^{3+} conditions. The data were expressed as the mean ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

inhibited by B pretreatment under Al toxicity conditions (Fig. 3B and D-F), which consequently reduced the negative charges in pectin and excluded Al from the cell walls.

Masking the Al-binding sites to mitigate Al stress has also been found to be effective in rice. *OsSTAR1* and *OsSTAR2* are two genes associated with detoxifying Al that is regulated by the C_2H_2-type zinc finger transcription factor *ALUMINUM RESISTANCE TRANSCRIPTION FACTOR 1* (*ART1*) in rice (Yamaji et al., 2009). *OsSTAR1* and *OsSTAR2* are both expressed in the roots and encode a nucleotide-binding and transmembrane domain, respectively. The STAR1/STAR2 complex is localized in vesicle membranes in rice root cells and is responsible for transferring UDP-glucose to the cell wall. It is also associated with masking the Al binding sites. Disrupting either gene leads to weakening the ability to tolerate Al in rice, which can be rescued by UDP-glucose application (Huang et al., 2009). In our study, *OsSTAR1* and *OsSTAR2* expression increased after treatment with Al, and pretreatment with 3 μM B under Al toxicity conditions further increased their expression (Fig. 4), implying that B also masks the Al binding sites in rice roots, thereby reducing Al toxicity.

Increasing the secretion of citric acid from the roots and complexation with Al in the rhizosphere to reduce Al uptake is important for rice to survive under Al stress conditions (Kochian et al., 2015). The *OsFRDL4* gene encodes a plasma membrane-localized citrate transporter that is responsible for transferring the citric acid out of root cells and reducing Al toxicity by depolymerizing and excluding Al deposition in the cell wall or complexation with Al in the growth medium to reduce Al uptake (Yokosho et al., 2011). The positive correlation between *OsFRDL4* expression and Al tolerance capacity among different rice cultivars further confirmed the importance of citric acid in resisting Al toxicity in rice (Yokosho et al., 2016). However, although Al increased the expression of *OsFRDL4* and enhanced citric acid secretion, pretreatment with B failed to further induce *OsFRDL4* expression and citric acid secretion (Fig. 5), indicating that increasing citric acid secretion is not effective in alleviating Al toxicity.

The *OsNRAT1* gene encodes an Al transporter in rice roots and is responsible for Al uptake (Xia et al., 2010). In our study, no dramatic difference in *OsNRAT1* expression and cell sap Al content was observed with or without B pretreatment under Al toxicity conditions (Fig. 6A and B), indicating that B is not helpful in alleviating Al toxicity by decreasing Al uptake. However, the expression of *OsALS1*, which encodes the transporter responsible for the transfer of Al in the cytoplasm to the vacuole (Huang et al., 2012), exhibited a significant increase under Al toxicity conditions and was further induced by B pretreatment (Fig. 6C), suggesting that although B does not decrease the cell sap Al content, it stimulates the transfer of Al from the cytoplasm to the vacuoles, which are areas of low Al toxicity, thus decreasing Al toxicity in rice.

H_2O_2 is a byproduct of oxidation reactions in plants and often acts as a signaling molecule that participates in regulating plant development at low concentrations such as regulating the synthesis of cell walls, stimulating the production of stress-induced proteins, and accumulating the deposition of compatible solutes (Miller et al., 2010; Shafi et al., 2015; Vierling and Kimpel, 1992). H_2O_2 is eliminated from cells by a series of enzymatic and non-enzymatic processes, and APX, CAT, and POD are the main antioxidant enzymes involved in catalyzing H_2O_2 to remove it from cells and alleviate peroxidation damage (Gill and Tuteja, 2010; Inostroza-Blancheteau et al., 2011; Tanveer and Shabala, 2018). The toxicity of Al in plants is associated with increasing levels of H_2O_2 and leads to peroxidation injury in plants (Tamás et al., 2004). The appropriate addition of B to the growth media could assist plants in mitigating peroxidation damage by regulating the activity of antioxidant enzymes under Al toxicity conditions. For example, the

application of B significantly enhances CAT and APX activity, decreases H_2O_2 content in trifoliate orange under Al toxicity conditions, which in turn reduces root cell injury (Riaz et al., 2018b). Similar to previous studies, the accumulation of H_2O_2 in the rice root apices decreased with B pretreatment under Al conditions, compared to a single Al treatment (Fig. 7) and was accompanied by a significant increase in APX, CAT, and POD activity (Fig. 8), indicating that B enhanced the activity of antioxidant enzymes to remove H_2O_2 in rice, and then decreased the peroxidation damage induced by excess H_2O_2.

It has also been demonstrated that H_2O_2 is involved in regulating the components and physiological properties of plant cell walls such as the stiffening of the coleoptile cell wall in maize (Schopfer, 1996), the form of lignin in cultured spruce cells (Kärkönen et al., 2009), and the synthesis and modification of pectin in rice roots (Xiong et al., 2015). The pectin content, especially the low methylation pectin content in rice roots, significantly increased after treatment with H_2O_2, accompanied by an increase in PME activity, although treatment with the H_2O_2 scavenger 4-TEMPO resulted in the opposite effect (Xiong et al., 2015). Considering how pretreatment with B in our study regulated the pectin content, PME activity, and H_2O_2 accumulation in rice roots, we hypothesized that in addition to alleviating peroxidation damage induced by Al toxicity after pretreatment with B, the decrease in the amount of the signaling molecule H_2O_2 in rice roots might also be involved in regulating Al deposition in the cell walls. Further studies also showed that B + Al and B + 4-TEMPO + Al treatments increased root elongation compared to Al only treatment (Fig. 9) and was accompanied by a decrease in the amount of Al in the lower cell wall, the apoplastic Al concentration, the pectin content, and PME activity (Fig. 10). However, B + H_2O_2 + Al treatment resulted in the opposite effect (Figs. 9 and 10). Therefore, the existing of H_2O_2 significantly increased pectin content and PME activity, then increased the capacity of pectin binding with Al, and finally increased Al content in rice. However, the addition of B significantly decreased the accumulation of H_2O_2 in rice, which then reduced cell wall Al content and alleviated Al toxicity.

In conclusion, this study is the first to demonstrate that B alleviates Al toxicity in rice. B not only decreases the deposition of Al in the cell wall by inhibiting pectin synthesis and suppressing PME activity, but also increases the compartmentalization of Al from the cytoplasm to the vacuoles. In addition, B decreases H_2O_2 content in rice roots, which in turn decreases cell wall Al content that ultimately alleviates Al toxicity.

Contribution

C.Q.Z., J.H.Z., L.F.Z., B.A., Y.F.L. and L.P.W. performed research; C.Q.Z. analyzed data and wrote the draft; W.J.H., Z.G.B., A.Y.H., Q.D.L., J.H., H.S. and Q.Y.J. revised the article; C.Q.Z., and J.H.Z. designed the research; and J.H.Z. wrote the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.02.022.

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