

Article

Exogenous 24-Epibrassinolide Enhances Aluminum Tolerance in Oriental Melon (*Cucumis melo* var. *makuwa*), Involving a CmBES1-6–CmSTOP3/5 Regulatory Module

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Abstract

Aluminum (Al) toxicity severely limits crop growth in acidic soils. Although 24-epibrassinolide (EBR), a bioactive brassinosteroid analog, is known to regulate plant stress responses, its role in Al tolerance in oriental melon remains unclear. Here, we investigated the effects of exogenous EBR application on Al stress tolerance in oriental melon seedlings, and the molecular regulatory mechanisms underlying the EBR-induced alleviation of aluminum stress were explored. Seedlings were pretreated with 0, 0.01, 0.1, and 1 μM EBR for 24 h prior to exposure to 100 μM AlCl_3 . Our results showed that 0.01 μM EBR significantly alleviated Al toxicity by improving root vitality, promoting primary root elongation, reducing hematoxylin staining intensity in root tips, and notably increasing malate exudation. The transcriptional analysis revealed that EBR upregulated *CmBES1-6*, a core transcription factor in BR signaling, along with Al-responsive genes including *CmALMT7*, *CmALMT8*, *CmALMT12*, *CmSTOP3*, and *CmSTOP5*. Silencing of *CmBES1-6* through virus-induced gene silencing (VIGS) compromised Al tolerance, as reflected by the reduced expression of these Al-responsive genes. Yeast two-hybrid (Y2H) assays confirmed that *CmBES1-6* physically interacts with both *CmSTOP3* and *CmSTOP5*. Furthermore, yeast one-hybrid (Y1H) assays demonstrated that *CmBES1-6* directly binds to E-box cis-elements (CANNTG) in the promoters of *CmSTOP3* and *CmSTOP5*. Together, our findings suggest that exogenous EBR promotes BR signaling through *CmBES1-6*, which is associated with the upregulation of *CmSTOP3* and *CmSTOP5* and enhanced malate exudation from roots, thereby potentially contributing to improved Al tolerance in oriental melon seedlings. This work implicates a *CmBES1-6*–*CmSTOP3/5* transcriptional module in Al tolerance and provides candidate genes for the genetic improvement of Al resistance in melons cultivated in acidic soils.



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This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.**Keywords:** oriental melon; aluminum stress; 24-epibrassinolide; *CmBES1-6*; *CmSTOP3/5*; organic acid exudation

1. Introduction

Aluminum (Al) toxicity is currently considered a major abiotic constraint in acidic soils that limits the yield and quality of crops grown in acidic soils worldwide [1]. When the pH of soil is lowered below 5.5, Al that exists in the earth's crust as nontoxic silicates or oxides is solubilized into phytotoxic Al^{3+} ions and causes significant interference with root

growth and development [2]. About half of the world's arable land is at risk of acidification, and this situation is exacerbated by industrial emissions and other forms of intensive agricultural practices that induce further soil acidification [3,4]. The growth of roots is most sensitive to Al toxicity, which affects root tips, especially the transition and elongation zones [5]. Al^{3+} interferes with root elongation and lateral root initiation by binding to cell wall pectin, disrupting the cytoskeleton, and inhibiting cell division. Consequently, Al toxicity causes serious distortion in root morphology that hinders water and mineral nutrient acquisition and reduces crop yield [6,7]. In addition, Al stress increases the over-accumulation of reactive oxygen species (ROS) and enhances lipid peroxidation, thereby damaging the structure and selective permeability of cellular membranes [8].

Plants have developed two strategies to combat Al toxicity, namely external exclusion and internal tolerance [9,10]. The external exclusion strategy represents the first line of defense against Al toxicity. When roots perceive Al^{3+} as a signal molecule, root tip anion channels are rapidly activated to mediate the efflux of organic acid anions, which chelate Al^{3+} and reduce its interaction with sensitive root sites. The carboxyl groups ($-\text{COO}-$) of organic acid anions chelate Al^{3+} to form stable and nontoxic Al complexes, which are then excluded from the root across the plasma membrane [11,12]. In terms of internal tolerance mechanisms, antioxidant defense represents one component of internal tolerance. Plants activate their antioxidant defense systems to increase the activities of major antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), to scavenge excess ROS and alleviate oxidative damage [6].

The transporter proteins involved in organic acid efflux are encoded by two gene families, ALMT (aluminum-activated malate transporter) and MATE (multidrug and toxic compound extrusion), both of which play a central role in Al detoxification in plant roots [13]. Members of the ALMT family encode Al-activated anion channel proteins that mediate malate efflux from the cytoplasm to the rhizosphere. In model species and crops, *AtALMT1*, *TaALMT1*, and *SlALMT* have been verified to be responsible for Al-induced malate efflux [14–16]. Recently, an ALMT-independent alternative Al detoxification pathway in low-malate tomato cultivars was uncovered. In this pathway, Al stress triggers the expression of *SlSLAH2*, a slow anion channel gene from the SLAC/SLAH family. This further enhances root malate efflux and improves Al tolerance, thus providing a new target for the molecular breeding of Al tolerance [12]. Members of the MATE family encode H^+ -coupled citrate transporters. In Napier grass (*Pennisetum purpureum*), an Al-inducible MATE member was characterized as a candidate hub gene for citrate extrusion, identified from transcriptomic network analysis [13]. At the level of transcriptional regulation, C_2H_2 -type zinc finger STOP (sensitive to proton toxicity) family transcription factors constitute the central regulatory components in the Al stress transcriptional response network. As a master transcriptional regulator, STOP1 directly activates the transcription of ALMT1 and MATE, which encode the transporter proteins responsible for malate and citrate release, thereby promoting Al^{3+} chelation [17,18]. The significantly decreased malate efflux in tomato *Slstop1* mutants further validated the key role of STOP1 in regulating organic acid secretion [19].

Brassinosteroids (BRs) are a class of endogenous steroidal phytohormones that play wide-ranging and crucial roles in the regulation of plant growth, development, and abiotic stress responses [20]. 24-epibrassinolide (EBR) is one of the most biologically active brassinosteroid analogs and has been widely applied in plant stress physiology research. Previous studies have demonstrated that exogenous EBR application alleviates heavy metal stress, including Al, Cd (cadmium), and Cr (chromium) toxicity, in various crop species such as rice (*Oryza sativa*), soybean (*Glycine max*), and tomato (*Solanum lycopersicum*) [21]. At the signal transduction level, EBR mainly regulates plant growth and development by modulating the

protein stability and transcriptional activity of the BR signaling core transcription factors BES1 (BRI1-EMS-SUPPRESSOR 1) and BZR1 (BRASSINAZOLE-RESISTANT 1). The activated BES1/BZR1 protein can directly bind to E-box (CANNTG) and BRRE (CGTGT/CG) elements in the promoter region of downstream target genes and further regulates gene expression in response to environmental stresses [22]. However, whether BES1/BZR1 directly links brassinosteroid signaling to the STOP/ALMT-mediated Al response remains to be elucidated.

Oriental melon (*Cucumis melo* var. *makuwa*) is an economically important and valuable specialty crop in China, with high nutritional value and great market prospects. However, compared with other crops, melon is relatively sensitive to Al stress. When grown in acidic soils, melon often shows inhibited root growth, weakened nutrient uptake, and significant decreases in yield and quality [23]. To date, few reports have been published on the effects of EBR in alleviating Al stress in oriental melon and the molecular mechanisms involved. In view of the above knowledge gaps, the present study selected the oriental melon cultivar ‘Yumei Ren’ as the experimental material to test whether exogenous EBR alleviates Al toxicity and whether the BR signaling core transcription factor CmBES1-6 is associated with the STOP/ALMT-mediated malate response. The findings are expected to provide a theoretical basis for the molecular breeding of Al-tolerant melon varieties for cultivation in acidic soils.

2. Materials and Methods

2.1. Plant Materials

The oriental melon (*Cucumis melo* var. *makuwa*) cv. ‘Yumei Ren’ was selected as the experimental material. Seeds were obtained from Hengyuan Seed Industry Co., Ltd. (Jilin, China). The seeds were surface-sterilized, germinated, and planted in nursery substrate. When both cotyledons were fully expanded, 150 uniformly growing and healthy seedlings were randomly selected and transplanted into hydroponic containers. Each container (5 L volume) held 10 seedlings, with three replicate containers per treatment arranged in a completely randomized design. The roots were thoroughly rinsed with deionized water, and the seedlings were then transplanted into hydroponic containers containing half-strength Hoagland nutrient solution. The seedlings were acclimatized for 3 days. The nutrient solution was replaced every day, and each container was aerated continuously at a rate of 2 L min⁻¹ using an air pump. The seedlings were grown in a controlled-environment growth chamber with a 16 h light/8 h dark photoperiod, a light intensity of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by cool-white fluorescent lamps (Philips, T8, 36 W, Philips Lighting Holding B.V., Amsterdam, The Netherlands), a temperature of 25 $\pm$ 2 $^{\circ}\text{C}$, and a relative humidity of 65–70%. To minimize positional effects, the containers were randomized spatially within the growth chamber every 24 h.

2.2. Stress Treatments

After acclimatization, Al stress treatments were performed at 0 (CK) and 100 $\mu\text{M}\cdot\text{L}^{-1}$ AlCl_3 . The pH of the nutrient solution was adjusted to 4.0 $\pm$ 0.1 using HCl or NaOH. One day before Al stress treatment, EBR (supplied by Sigma-Aldrich, Burlington, VT, USA, purity $\geq$ 90%) was added to the Al-containing nutrient solution at final concentrations of 0 (Al-only control), 0.01, 0.1, or 1 μM . The EBR stock solution was prepared with 95% ethanol (solvent), and an equal volume of ethanol was added to both the CK and Al-only treatments to ensure consistent conditions. The 14-day treatment duration started from the day of EBR addition. The nutrient solution was renewed every 3 days, and the pH was adjusted daily to 4.0 $\pm$ 0.1. Each hydroponic container (5 L) holding ten seedlings was considered to be one biological replicate. The data from the ten seedlings within the same

container were averaged for subsequent statistical analysis. Three independent biological replicates were used per treatment.

2.3. Determination of Root Morphological Parameters

After the above treatments, the seedlings were carefully removed and the excess moisture on the roots was blotted dry. For morphological measurements, the primary root length was measured from the base of the stem to the tip of the longest root with a ruler graduated in millimeters. The fresh weight of the roots was determined using an analytical balance with a precision of 0.0001 g. Root vitality was assessed using the triphenyltetrazolium chloride (TTC) reduction method following the protocol of the previous study [23]. The methods for sample collection and data statistics were identical to those described in Section 2.2.

2.4. Hematoxylin Staining for Aluminum Localization in Root Tips

The roots were stained with 0.2% hematoxylin solution (0.2 g hematoxylin was dissolved in 100 mL of 0.1 mM NaOH and supplemented with 0.02 g KIO₃) for 10 min in the dark and then destained with ddH₂O for 30 min. The samples were observed and photographed under a stereomicroscope. For each biological replicate, ten root tips were sampled and mounted on a glass slide. The stained root tips were observed and photographed under a stereomicroscope (Nikon SMZ1270, Nikon Instruments Inc., Melville, NY, USA) equipped with a digital camera (Nikon DS-Ri2, Nikon Corporation, Tokyo, Japan); all images were captured using identical illumination conditions, $\times 5$ magnification, and the same exposure settings (ISO 200, shutter speed 1/30 s) for all samples to ensure comparability. To minimize subjective bias, all photographs were coded and blindly scored by two independent observers. Hematoxylin staining intensity, which reflects Al accumulation, was quantified with ImageJ software 1.54p (National Institutes of Health, USA) by measuring the mean gray value of the entire root tip region (0–5 mm from tip) after background correction. Three independent biological replicates were used per treatment.

2.5. Determination of Organic Acids

Root organic acid exudation was collected following the method of Wang et al. [24] with minor modifications. After 14 days of treatment, ten seedlings were separately removed from each of the three containers, and the roots were rinsed three times with deionized water. This step removed residual solution from the root surface. Given that carryover contamination could indicate inaccurate exudation values, the roots were subsequently immersed in 200 mL of a collection solution containing 0.2 mM CaCl₂ or 100 μ M AlCl₃ (pH 4.0) for 6 h at room temperature with continuous aeration. For the control group, AlCl₃ was omitted from the collection solution. Following incubation, the seedlings were removed, and the root fresh weight was promptly recorded.

Furthermore, the collected solutions were filtered through a 0.45 μ m membrane, and the filtrates were stored at -20°C until analysis. Chromatographic separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a ZORBAX SB-C18 column (4.6 mm $\times$ 150 mm, 5 μ m) maintained at 28°C , which afforded a baseline resolution of the target analytes under the optimized conditions. Moreover, the mobile phase, consisting of 15 mM KH₂PO₄ (pH 2.48) at a flow rate of 1.0 mL $\cdot$ min⁻¹, was employed to achieve the appropriate separation of the target compounds. The detection wavelength was set at 214 nm, and the injection volume was 20 μ L. The oxalate, malate, and citrate concentrations were quantified using the external standard method, and the exudation rates were expressed as nmol $\cdot$ g⁻¹ FW $\cdot$ h⁻¹.

2.6. Effects of Membrane Damage Indicators

To determine relative electrolyte leakage, the sample was immersed in distilled water, and the initial electrical conductivity (S_1) was measured. The sample was then boiled for 15 min and cooled to room temperature, and the total electrical conductivity (S_2) was measured. The relative electrolyte leakage was calculated as $(S_1/S_2) \times 100\%$. MDA content was determined by the thiobarbituric acid (TBA) method and calculated from the absorbance difference between 532 nm and 600 nm.

2.7. Gene Expression

Total RNA was extracted from the root tips (0–5 cm from the apex) of oriental melon seedlings using the Kangwei Ultra-Pure RNA Extraction Kit (Kangwei Century, Beijing, China) following the manufacturer's instructions. Root tips were collected after 14 days of Al stress treatment, immediately frozen in liquid nitrogen, and stored at -80°C . First-strand cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit (Accurate Biology, Hunan Accurate Biology Co., Ltd., Changsha, China). qRT-PCR was performed with gene-specific primers (Table S1) using the SYBR Green method, with *Cm18S rRNA* as the internal reference. The relative expression levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Three independent biological replicates were used per treatment, with three technical replicates each.

2.8. Construction of the VIGS Silencing System

The conditions of growth were the same as those in Section 2.1. When the second true leaf was fully expanded, a fragment (~300 bp) of *CmBES1-6* was cloned into the pV190 vector to generate the recombinant vector, pV190:*CmBES1-6*. The empty vector, pV190:00 (negative control), and the pV190:*CmPDS* vector (positive control) were maintained in our laboratory. Recombinant plasmids were transformed into the *Agrobacterium tumefaciens* strain EHA105. The bacterial cells were grown until OD_{600} reached 0.8–1.0, and then they were resuspended in infiltration buffer (10 mM MES, 10 mM MgCl_2 , 200 μM acetosyringone, pH 5.6). Subsequently, the bacterial suspension was infiltrated into the abaxial surfaces of two cotyledons using a needle-free syringe. The infiltrated plants were kept at 22°C with 60–70% relative humidity in darkness for 2 days, and then they were transferred to normal light conditions. When the *CmPDS*-silenced plants exhibited obvious leaf photobleaching, the *CmBES1-6*-silenced plants in which the gene expression level was reduced by at least 50% compared with the pV190:00 plants, with a statistically significant difference ($p < 0.05$), were selected for subsequent Al stress treatments. Three biological replicates with 10 plants per treatment were set up.

2.9. Yeast Two-Hybrid (Y2H) Assay

The coding regions of *CmSTOP3* and *CmSTOP5* were individually cloned into the pGBKT7 vector to obtain bait constructs, whereas *CmBES1-6* was cloned into the pGADT7 vector as a prey construct. Bait and prey plasmids were co-transformed into yeast AH109 and plated onto SD/–Leu/–Trp double-dropout medium for 2–3 days at 30°C . Positive colonies were picked and inoculated into 5 mL liquid SD/–Ade/–His/–Leu/–Trp quadruple-dropout medium containing X- α -Gal and cultured for 16 h at 30°C with shaking at 220 rpm. Then, 5 μL of the culture was spotted onto the quadruple-dropout medium containing X- α -Gal. The plates were incubated for 3–5 days at 30°C and photographed. The positive and negative controls were pGBKT7-53 + pGADT7-T and pGBKT7-Lam + pGADT7-T, respectively.

2.10. Yeast One-Hybrid (Y1H) Assay

In this study, the 2000 bp promoter sequences upstream of the start codon (ATG) of the *CmSTOP3* and *CmSTOP5* genes were retrieved from the melon genome database (CuGenDB, <http://www.cucurbitgenomics.org>; accessed on 5 March 2026). Putative transcription factor binding sites were predicted using the PlantCARE online tool (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>; accessed on 5 March 2026). Subsequently, the promoter fragments were amplified by PCR and subcloned into the yeast one-hybrid bait vector pAbAi, and *CmBES1-6* was cloned into pGADT7 as a prey construct. The bait plasmid was digested, linearized, and then transformed into yeast Y1HGold. Colony PCR was performed on SD/–Ura medium to verify the integration of the bait plasmid. The prey plasmid was transformed into yeast strains containing the bait plasmid, and co-transformants were selected on the SD/–Leu medium. Single colonies were picked and inoculated into 5 mL liquid SD/–Leu medium and cultured for 16 h at 30 °C with shaking at 220 rpm. Then, 5 µL of the culture was spotted onto the SD/–Leu medium, containing different concentrations of aureobasidin A (Aba). The plates were cultured for 3–5 days at 30 °C and photographed. Both wild-type and mutated promoter fragments (with the *CmBES1-6*-binding sites altered) were tested in the pAbAi system, and empty pGADT7 served as the negative control.

2.11. Statistical Analysis

For the VIGS experiments, which involved a two-factor design (VIGS vector and aluminum treatment), a two-way analysis of variance (ANOVA) was performed with individual containers as the experimental units to test for main effects and their interactions. Prior to ANOVA, the assumptions of normality (Shapiro–Wilk test) and homogeneity of variances (Levene’s test) were verified. Biological replicates (n) are defined as independent plant samples; the exact n value for each analysis is specified in the corresponding figure legend. For experiments involving a single factor (e.g., gene expression assays or yeast one-hybrid assays), one-way ANOVA followed by Duncan’s multiple range test was used for mean comparisons. For all yeast assays, at least three independent biological replicates were performed. A probability value of $p < 0.05$ was considered statistically significant. All statistical analyses were conducted using SPSS 26.0. The specific statistical procedure applied to each figure is indicated in its respective legend.

3. Results

3.1. EBR Alleviates Al-Stress-Induced Root Inhibition in Oriental Melon

As shown in Figure 1A, exposure to 100 µM AlCl_3 for 14 days strongly inhibited root elongation in oriental melon seedlings. EBR pretreatment at different concentrations alleviated this inhibition to varying degrees, and the effect was most pronounced at 0.01 µM, where root elongation was largely restored. Hematoxylin staining revealed a nearly colorless appearance in CK root tips, indicating undetectable Al accumulation, whereas root tips exposed to 100 µM AlCl_3 displayed intense blue–purple staining, consistent with substantial Al^{3+} deposition (Figure 1B). Following 0.01 µM EBR application, staining intensity at the root tip was markedly reduced, suggesting that EBR limits Al accumulation in this region. These observations are consistent with the root length and root vitality data summarized in Table 1, collectively indicating that 0.01 µM EBR effectively improves root vitality and elongation in oriental melon under Al stress.

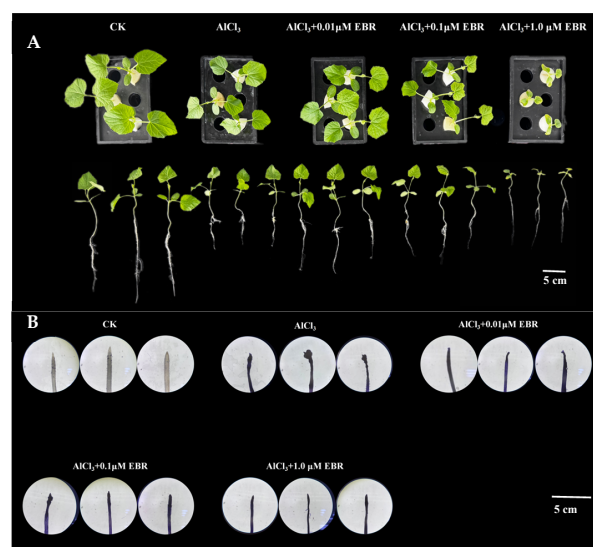


Figure 1. Effects of exogenous 24-epibrassinolide (EBR) on growth phenotype of oriental melon seedlings under aluminum stress. (A) Whole-plant morphology under different EBR concentrations. (B) Root morphology and magnified root tip regions. Note: CK, control (0 μM AlCl_3 , 0 μM EBR).

Table 1. Effects of exogenous EBR on root growth parameters of oriental melon seedlings under aluminum stress. Note: data are presented as mean $\pm$ SD ($n = 3$, n represents an independent container, and each container contained five plants). Different lowercase letters within the same column indicate significant differences ($p < 0.05$, Duncan's test).

Treatment (μM)	Root Length (cm)	Root Weight (g)	Root Vitality ($\mu\text{g} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$)
Control	13.4 ± 0.36 a	0.19 ± 0.04 a	0.56 ± 0.04 a
AlCl_3	10.8 ± 0.78 c	0.13 ± 0.03 c	0.37 ± 0.03 c
$\text{AlCl}_3 + 0.01$ EBR	11.5 ± 0.22 b	0.16 ± 0.04 b	0.47 ± 0.04 b
$\text{AlCl}_3 + 0.1$ EBR	10.4 ± 0.23 c	0.12 ± 0.04 c	0.36 ± 0.02 c
$\text{AlCl}_3 + 1.0$ EBR	9.23 ± 0.28 d	0.11 ± 0.03 c	0.25 ± 0.04 d

3.2. Effects of Exogenous EBR on Root Organic Acid Exudation Under Aluminum Stress

Compared with the control, Al stress significantly enhanced the root exudation rates of oxalate, malate, and citrate in oriental melon seedlings ($p < 0.05$) (Figure 2). However, under Al stress, exogenous EBR was found to significantly enhance the malic acid exudation rate ($p < 0.05$), while no significant effects were detected on oxalic acid or citric acid exudation, as compared to the Al-stressed plants without EBR. These results suggest that the ameliorative effect of EBR on Al toxicity may be mainly attributed to its induction of malate exudation, thereby reducing Al entry into root tissues and facilitating Al exclusion from roots.

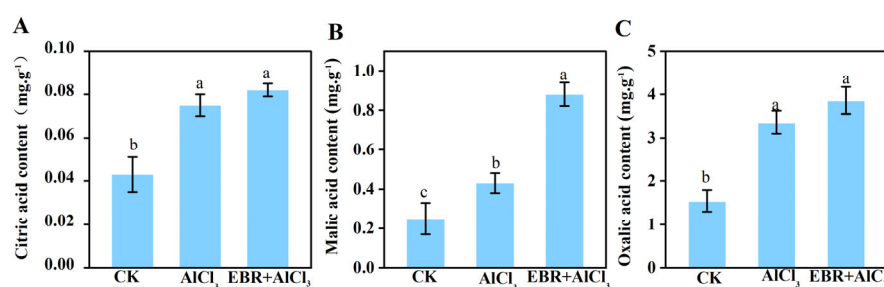


Figure 2. Effects of exogenous EBR on organic acid secretion of oriental melon seedlings under aluminum stress. (A) Oxalic acid exudation. (B) Malic acid exudation. (C) Citric acid exudation. Note: data are presented as mean $\pm$ SD ($n = 3$, n represents an independent container, and each container contained five plants). Different lowercase letters within the same column indicate significant differences ($p < 0.05$, Duncan's test).

3.3. Effect of EBR on Gene Expression in Roots of Oriental Melon Seedlings Under Aluminum Stress

To elucidate the transcriptional regulatory mechanisms underlying EBR-alleviated Al stress in melon, we first performed a genome-wide identification of Al-stress-related gene families, including ALMT (aluminum-activated malate transporter), MATE (multidrug and toxic compound extrusion), DHN (dehydrin), STOP (sensitive to proton rhizotoxicity), and BES/BZR (brassinosteroid-insensitive 2 suppressor-1/brassinazole-resistant), which are known to be involved in Al tolerance and brassinosteroid signaling in plants. Among these, ALMT and MATE families were selected for their roles in organic acid transport; DHN for its stress-protective functions; STOP for transcriptional regulation of Al tolerance; and BES/BZR as core transcription factors in brassinosteroid signaling. Through this screening, we identified 12 ALMT, 4 MATE, 4 DHN, 5 STOP, and 6 BES family members in the melon genome (Table S2). The expression analysis revealed that *CmMATE1*, *CmSTOP5*, and *CmBES1-6* were significantly upregulated under Al stress and further induced by EBR treatment (Figure 3), suggesting their potential roles in the transcriptional response to EBR-mediated Al tolerance. However, because protein–protein interactions and regulatory networks often involve distinct sets of genes, subsequent functional experiments focused on *CmALMT7/8/12* and *CmSTOP3/5* to investigate their involvement in downstream Al tolerance pathways. Based on these expression data, we propose that *CmBES1-6* may function as a transcription factor in the BR signaling pathway, mediating the regulatory effect of EBR on Al-stress-responsive gene expression.

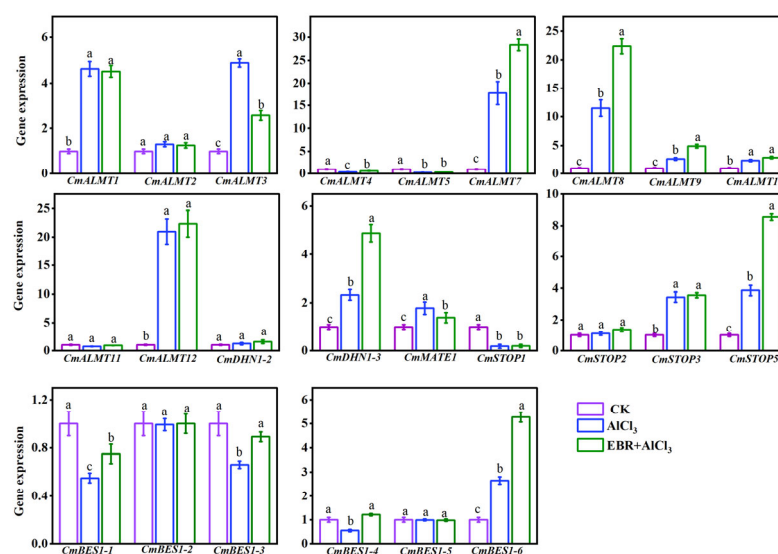


Figure 3. Effects of exogenous EBR on the expression of aluminum-responsive genes and *CmBES* family members in roots of oriental melon seedlings under aluminum stress. Note: data are presented as mean $\pm$ SD ($n = 3$, * n^* represents an independent container, and each container contained five plants). Different lowercase letters within the same column indicate significant differences ($p < 0.05$, Duncan's test).

3.4. VIGS-Mediated Silencing of *CmBES1-6* Impairs Aluminum Tolerance in Oriental Melon

According to the above gene expression analysis, we chose *CmBES1-6* as the target for the functional analysis in oriental melon by VIGS technology. As shown in Figure 4A, 14 d after TRV gene silencing, pV190:*CmPDS* plants showed typical symptoms of leaf photobleaching (Figure 4A), indicating that the silencing system based on the VIGS technology was successfully established. Under normal conditions, the plants transformed with pV190:*CmBES1-6* showed no obvious phenotypic differences compared with the negative control plants transformed with pV190:00. After Al stress treatment for 14 d, the growth

of the *CmBES1-6*-silenced plants was significantly inhibited compared with the negative controls. The roots of the *CmBES1-6*-silenced plants showed deeper blue–purple hematoxylin staining at the tips of the roots, and the roots showed significantly lower vitality under Al stress (Figure 4A). qRT-PCR analysis (Figure 4B) showed that the transcript level of *CmBES1-6* was significantly lower in the roots of the *CmBES1-6*-silenced plants than in the negative controls. Furthermore, the root vitality of the *CmBES1-6*-silenced plants was significantly lower than that of the controls under Al stress (Figure 4C). Malate exudation in the root exudates of the silenced plants also declined markedly relative to controls, whereas the citrate and oxalate levels remained unchanged (Figure 4D–F). Taken together, these results indicate that silencing of *CmBES1-6* substantially impairs Al tolerance, an effect that may be closely linked to the disruption of the malate exudation pathway.

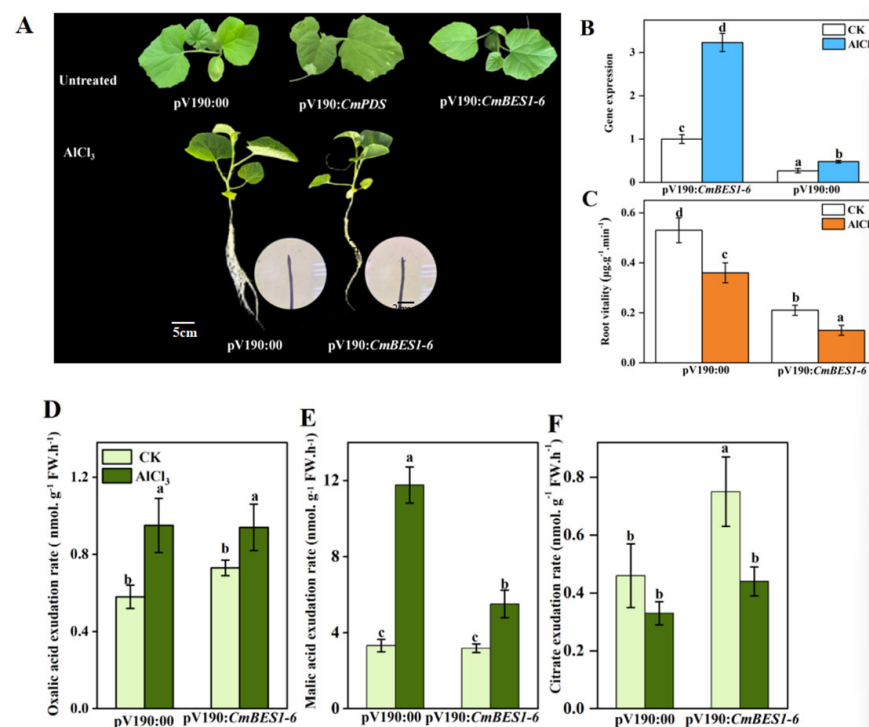


Figure 4. Functional verification of *CmBES1-6* by virus-induced gene silencing (VIGS) under aluminum stress. (A) Phenotypes of control and *CmBES1-6*-silenced plants after 14 d of aluminum stress. (B) Relative expression levels of *CmBES1-6* in roots of silenced plants. (C) Root vitality of control and *CmBES1-6*-silenced plants. (D) Oxalic acid exudation in roots of control and *CmBES1-6*-silenced plants. (E) Malic acid exudation from control and *CmBES1-6*-silenced plants. (F) Citric acid exudation from control and *CmBES1-6*-silenced plants. Note: data are presented as mean $\pm$ SD ($n = 3$, n represents an independent container, and each container contained five plants). Different lowercase letters indicate significant differences ($p < 0.05$, Duncan's test).

3.5. *CmBES1-6* Target Genes Are Downregulated in Silenced Plants

To further investigate the potential downstream target genes of *CmBES1-6*, we analyzed the expression changes of Al-stress-responsive genes in *CmBES1-6*-silenced plants. Figure 5 indicates that the downregulation of *CmBES1-6* did not cause significant changes in the expression levels of the remaining five *CmBES* family members. Importantly, in the *CmBES1-6*-silenced plants, the transcript levels of *CmALMT7*, *CmALMT8*, *CmALMT12*, *CmSTOP3*, and *CmSTOP5* were significantly decreased under both the normal and Al stress conditions, and the Al-induced upregulation of these genes was also significantly inhibited. These results indicated that these five genes might be downstream target genes of *CmBES1-6*.

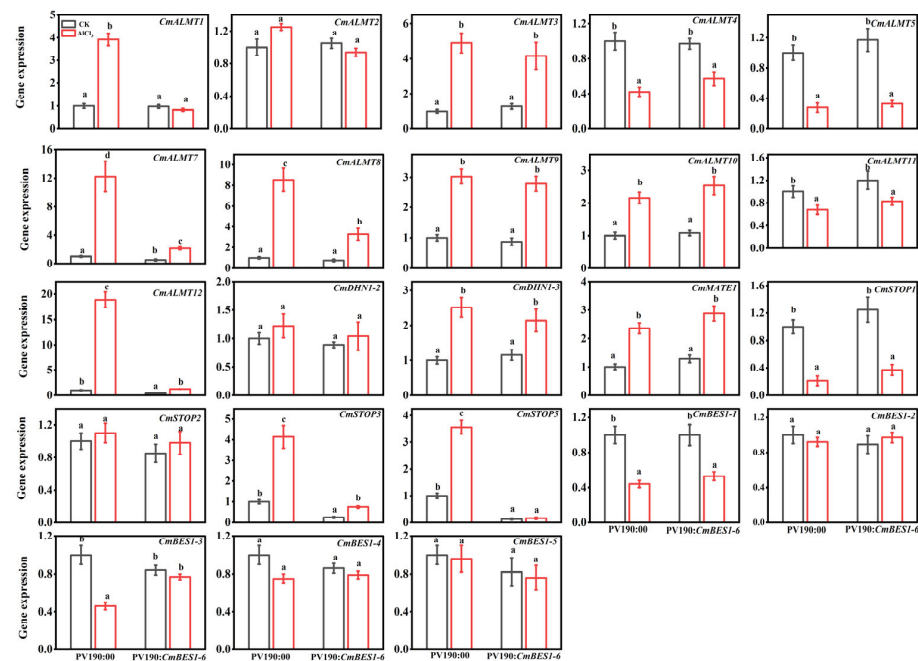


Figure 5. Effects of CmBES1-6 silencing on the expression of aluminum-responsive genes and CmBES family members under aluminum stress. Note: data are presented as mean $\pm$ SD ($n = 3$, n represents an independent container, and each container contained five plants). Different lowercase letters indicate significant differences ($p < 0.05$, Duncan's test).

3.6. Predicted Interaction of CmBES1-6 with CmSTOP3 and CmSTOP5

Figure 6 indicates that the yeast colonies co-transformed with CmBES1-6 and CmSTOP3, as well as those co-transformed with CmBES1-6 and CmSTOP5, could grow normally on SD/−Ade/−His/−Leu/−Trp quadruple-dropout medium and showed blue coloration. In contrast, yeast co-transformed with CmBES1-6 and CmALMT7, CmALMT8, or CmALMT12 failed to grow on the selective medium. These results suggest a potential interaction between CmBES1-6 and CmSTOP3/5 in the yeast two-hybrid system. However, we acknowledge that these findings are currently limited to the heterologous Y2H system and require further in planta validation. Additionally, we note that conventional Y2H assays may not be optimal for detecting interactions involving full-length membrane transporters such as the ALMT proteins.

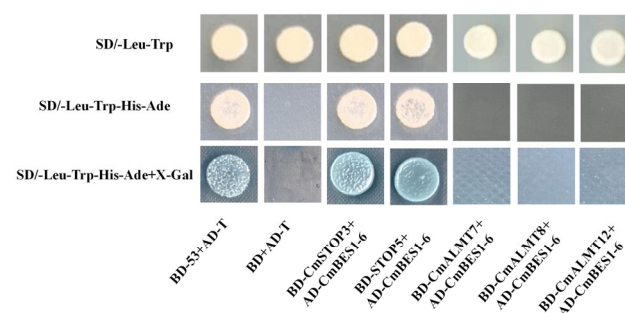


Figure 6. Identification of CmBES1-6-interacting proteins by yeast two-hybrid (Y2H) assay. CmBES1-6 was fused to the GAL4 activation domain (pGADT7-CmBES1-6), and prey proteins (CmSTOP3, CmSTOP5, CmALMT7, CmALMT8, and CmALMT12) were individually fused to the GAL4 DNA-binding domain (pGBKT7). Co-transformed yeast cells were grown on SD/−Trp/−Leu (DDO) and SD/−Trp/−Leu/−His/−Ade (QDO) media supplemented with 20 mg·L^{−1} X-α-Gal. Note: pGBKT7-53 + pGADT7-T served as the positive control; pGBKT7-Lam + pGADT7-T served as the negative control.

3.7. *CmBES1-6* Binds to the Promoters of *CmSTOP3* and *CmSTOP5* in Y1H Assay

In silico analysis of the *CmSTOP3* and *CmSTOP5* promoter regions using the PlantCARE database showed that E-box (CANNTG) binding sites were present in the two gene promoters (Figure 7). The Y1H assay results showed that yeast strains transfected with the pGADT7-*CmBES1-6* prey plasmid could grow normally on SD/−Leu medium containing $200 \text{ ng} \cdot \text{mL}^{-1}$ AbA, while no growth was shown for empty vector control (Figure 7). These results suggest that *CmBES1-6* can bind to the E-box elements in the promoters of *CmSTOP3* and *CmSTOP5* in the yeast one-hybrid system.

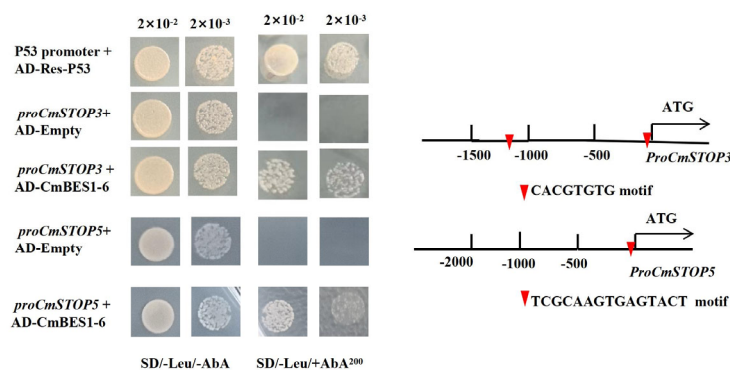


Figure 7. Verification of *CmBES1-6* binding to *CmSTOP3* and *CmSTOP5* promoters by yeast one-hybrid (Y1H) assay. E-box motifs (CANNTG), potential *CmBES1-6* binding sites, were identified in the promoter regions of *CmSTOP3* and *CmSTOP5*. Promoter fragments were cloned into the pAbAi vector, and *CmBES1-6* was cloned into the pGADT7 vector. Co-transformed yeast cells were cultured on SD/−Leu medium supplemented with aureobasidin A (AbA, $200 \text{ ng} \cdot \text{mL}^{-1}$). Note: pAbAi-p53 + pGADT7-Rec53 served as the positive control.

4. Discussion

The root tip is the most vulnerable part of the root system because the cells in this region are highly susceptible to the phytotoxic effects of the Al^{3+} ions that are actively taken up by plants [25,26]. BRs have been reported to mitigate metal-induced damage in plants through various mechanisms, including the modulation of root growth, the regulation of metal uptake and distribution, and the enhancement of stress-responsive gene expression [27,28]. However, the specific role of BRs in alleviating Al toxicity in the root tip remains to be fully elucidated. In this study, we found that $100 \text{ } \mu\text{M} \cdot \text{L}^{-1}$ AlCl_3 could significantly inhibit the elongation of primary roots and decrease root vitality in oriental melon seedlings. Aluminum (Al^{3+}) toxicity primarily targets root tips, inhibiting elongation and reducing vitality (Figure 1, Table 1). Exogenous low-concentration EBR ($0.01 \text{ } \mu\text{M}$) partially alleviated these inhibitory effects, reduced Al accumulation, and stimulated organic acid exudation, whereas high-concentration EBR exacerbated toxicity—an observation consistent with the well-known biphasic dose–response relationship of brassinosteroids.

Root organic acid secretion and efflux is one of the most efficient external exclusion strategies against Al toxicity in plants [24]. In the present work, among the organic acids examined, only malate exudation was significantly enhanced by EBR treatment and reduced by *CmBES1-6* silencing (Figures 2 and 5). We therefore conclude that malate is the principal Al-detoxifying metabolite in BR-mediated tolerance in oriental melon, a strategy shared with *Arabidopsis* and wheat but distinct from citrate-based detoxification in citrus and Napier grass [1,13–15,29]. It is likely that different plants exhibit different strategies of organic-acid-based Al detoxification, and whether other exogenous treatments could alleviate Al toxicity in oriental melon through other organic acid strategies remains to be determined.

BES1/BZR1 are master transcription factors in the BR signaling pathway, regulating target gene expression by responding to E-box and BRRE in target gene promoters [30]. Previous results showed that AI stress and EBR treatment could obviously increase the expression of *CmBES1-6* (Figure 3), indicating that *CmBES1-6* might be positively regulated by both AI and BR signaling. Recently, many BR signaling components were reported to be involved in different abiotic stress responses; for example, BES1 was involved in drought responses in maize [30] and BZR1 was involved in gene expression regulation under salt tolerance in *Arabidopsis* [31]. However, so far, few reports have shown the direct involvement of BES1 family members in the transcriptional regulation of AI stress responses. Mechanistically, *CmBES1-6* functions as a transcriptional node integrating BR and AI signals, as its expression was upregulated by both AI stress and EBR (Figure 3). Silencing *CmBES1-6* impaired root vitality and increased AI accumulation (Figure 4), while other *CmBES* family members showed no functional redundancy (Figure 5), indicating a specific role for *CmBES1-6* in this stress response.

STOP family transcription factors are essential components of AI stress transcriptional regulatory network. *Arabidopsis* STOP1 directly activates *AtALMT1* and *AtMATE* expression and promotes organic acid efflux [32,33]. In the present study, Y2H assays revealed that *CmBES1-6* can interact with both *CmSTOP3* and *CmSTOP5* in yeast (Figure 6), and Y1H assays showed that *CmBES1-6* is capable of binding to E-box elements within the *CmSTOP3* and *CmSTOP5* promoter fragments in yeast (Figure 7). These results provide preliminary heterologous-system evidence suggesting that *CmBES1-6* may potentially regulate *CmSTOP3/5* through two possible modes: (1) protein–protein interaction, which may facilitate the formation of transcriptional complexes, and (2) direct promoter binding, which may enable the transcriptional activation of *CmSTOP3/5*. However, we emphasize that Y2H and Y1H assays only demonstrate the theoretical potential for interaction and binding under heterologous yeast conditions; they do not confirm that these interactions occur in planta, nor do they establish that *CmBES1-6* binding results in the actual transcriptional activation of *CmSTOP3/5* in melon root cells. Direct in planta validation—such as bimolecular fluorescence complementation (BiFC) or co-immunoprecipitation (Co-IP) for protein interaction and dual-luciferase transactivation assays, EMSA, or ChIP for promoter binding and activation—will be required to confirm these regulatory relationships. Furthermore, the Y2H assays did not detect interaction between *CmBES1-6* and *CmALMT7*, *CmALMT8*, or *CmALMT12* (Figure 6), while the VIGS-mediated silencing of *CmBES1-6* was associated with significantly reduced expression of all three *CmALMT* genes (Figure 5). We note that this observed correlation between *CmBES1-6* silencing and reduced *CmALMT* expression does not, by itself, establish a direct or indirect causal regulatory relationship, nor does it confirm that this regulation is mediated through *CmSTOP3/5*. Based on the analogous, well-established model in *Arabidopsis* and rye, in which STOP1 directly activates ALMT1 transcription [33], we propose, as a working hypothesis, that *CmBES1-6* may indirectly influence *CmALMT* gene expression via *CmSTOP3/5*, potentially forming a hierarchical regulatory cascade (“*CmBES1-6*–*CmSTOP3/5*–*CmALMTs*”) in oriental melon. We explicitly present this as a working model requiring further functional validation—specifically, direct manipulation of *CmSTOP3/5* expression (via overexpression or silencing) followed by assessment of *CmALMT* transcript levels and malate exudation—to confirm whether *CmSTOP3/5* indeed functions as the causal intermediary between *CmBES1-6* and *CmALMT* regulation.

5. Conclusions

Based on the collective evidence from this study, we propose a working model for EBR-mediated AI tolerance in oriental melon: EBR treatment is associated with increased

CmBES1-6 expression and malate-related responses, while VIGS and yeast assays support a proposed *CmBES1-6/CmSTOP3/5* regulatory model that requires further validation.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae12080978/s1>: Table S1: The primers used in the study; Table S2: Molecular characteristics of candidate genes in melon.

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