Protoplasma (2018) 255:841–850
https://doi.org/10.1007/s00709-017-1187-8

ORIGINAL ARTICLE

24-Epibrassinolide-induced alterations in the root cell walls of *Cucumis sativus* L. under $Ca(NO_3)_2$ stress

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Received: 23 July 2017 / Accepted: 19 November 2017 / Published online: 14 December 2017

Abstract

Brassinosteroids (BRs) can effectively alleviate the oxidative stress caused by $Ca(NO_3)_2$ in cucumber seedlings. The root system is an essential organ in plants due to its roles in physical anchorage, water and nutrient uptake, and metabolite synthesis and storage. In this study, 24-epibrassinolide (EBL) was applied to the cucumber seedling roots under $Ca(NO_3)_2$ stress, and the resulting chemical and anatomical changes were characterized to investigate the roles of BRs in alleviating salinity stress. $Ca(NO_3)_2$ alone significantly induced changes in the components of cell wall, anatomical structure, and expression profiles of several lignin biosynthetic genes. Salt stress damaged several metabolic pathways, leading to cell wall reassemble. However, EBL promoted cell expansion and maintained optimum length of root system, alleviating the oxidative stress caused by $Ca(NO_3)_2$. The continuous transduction of EBL signal thickened the secondary cell wall of casparian band cells, thus resisting against ion toxicity and maintaining water transport.

Keywords *Cucumis sativus* L. · 24-Epibrassinolide · Root · Cell wall · Lignin · $Ca(NO_3)_2$ stress

Abbreviations

BRs	Brassinosteroids
EBL	24-Epibrassinolide
BRI1	Brassonosteroid insensitive 1
BES1/BZR1	*bri1* EMS suppressor 1/brassinazole resistant 1
PAL	Phenylalanine ammonialyase
F5H	Ferulate-5-hydroxylas
COMT	Caffeicacid-3-*O*-methyltransferase
CCoAOMT	Caffeoyl-CoA-3-*O*-methyltransferase
LAC	Laccase
LFA POD	Lignin-forming anionic peroxidase
BAK1	BRI1-associated kinase 1
BSU1	*bri1* suppressor 1
BIN2	Brassinosteroid insensitive 2

Handling Editor: Bhumi Nath Tripathi

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00709-017-1187-8) contains supplementary material, which is available to authorized users.

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Introduction

Soil secondary salinization is an important factor that limits crop production in China. The high level of $Ca(NO_3)_2$ accumulation may lead to soil salinization. Too much $Ca(NO_3)_2$ in soil results in oxidative stress and metabolic disorders in plants, reducing their biomass yield (Zhang et al. 2008).

Plant cell walls have protective and structural functions and are therefore highly resistant to degradation. The secondary cell wall is mainly composed of three types of polymers: cellulose, hemicelluloses, and lignin, which primarily exists in xylem, fibers, and anther cells. Cellulose microfibrils along with hemicelluloses constitute the main load-bearing network, in which lignin imparts "waterproofing"

capacity as well as mechanical strength, rigidity, and environmental protection. Secondary cell walls are thicker than the primary walls and are resistant to compressive forces (Doblin et al. 2010).

Cell walls protect cells from various environmental stresses such as wounding, mineral stress, osmotic stress, cold acclimation, drought tolerance, and salt stress (de Lima et al. 2014). Cell wall lignification occurs as a stress response and provides structural rigidity and durability to plant tissues (Kim and Triplett 2008). Salinity stress has been recognized as a factor contributing to increased lignin deposition in vascular tissues and/or xylem development (Srivastava et al. 2015; Sánchez-Aguayo et al. 2004).

Brassinosteroids (BRs) are plant hormones involved in a wide range of plant developmental processes including cell division and elongation, seed germination, and vascular differentiation (Schumacher and Chory 2000). 24-Epibrassinolide (EBL) is one of the most bioactive compounds among BRs, which are perceived by the plasma membrane receptor, brassinosteroid insensitive 1 (BRI 1) (Li and Chory 1997; Friedrichsen et al. 2000). The signal is then transmitted from the plasma membrane to the nucleus, where dephosphorylation of BES1 and BZR1 is triggered, allowing them to dimerize and bind DNA to regulate the expression of hundreds of genes (Hacham et al. 2011).

Numerous studies suggest that BR may be an essential regulator in cellulose and lignin biosynthesis and accumulation. The T-DNA insertional mutant of DIM1/DWF1/CBB1, which is involved in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis, leads to a deficiency in brassinosteroid accumulation and displays a dwarf phenotype with up to 38 and 23% reduction in total lignin and cellulose, respectively (Klahre et al. 1998; Hossain et al. 2012). The sterol-deficient *Arabidopsis* mutants, *fackel*, *hydra1*, and *sterol methyltransferase1/cephalopod* exhibit incomplete cell walls and aberrant cell wall thickenings, and are accompanied with ectopic lignin deposits (Schrick et al. 2004). EBL application resulted in significant modification in hemicellulose and cellulose biosynthesis in the secondary xylem of *Liriodendron tulipifera* (Jin et al. 2014). However, relatively few studies have been performed to determine the effect of EBL on the cell wall in cucumber seedlings subjected to salt stress.

Cucumber (*Cucumis sativus* L.) is an important vegetable crop grown under protected cultivation worldwide and is highly sensitive to salinity. In the present study, the effects of EBL on lignin biosynthesis and cell wall polysaccharide fractions in cucumber roots under $Ca(NO_3)_2$ stress were investigated. And we also attempted to determine the roles of EBL in the maintenance of normal water transport and plant growth under salt stress by observing lignin deposition regions and evaluating the expression of cell wall degradation enzymes at the transcription level.

Materials and methods

Plant materials and treatments

Cucumber (*Cucumis sativus* L., cv. "Jinyou No. 4") seeds were obtained from the Tianjin Kernel Cucumber Research Institute, China. Seeds were placed on filter papers in Petri dishes in the dark at 29 ± 1 °C for 24 h. The germinated seeds were transferred into plastic trays (41 × 41 × 5 cm) containing quartz sand. The greenhouse conditions for plant growth were set as follows: 25–30 °C during the day and 15–18 °C during the night, natural light with relative humidity of 60–75%. When the second leaves were fully expanded, cucumber plants were transplanted into plastic containers containing half strength Hoagland solution with an electrical conductivity (EC) of 2.0–2.2 ds m^{-1} and renewed every 3 days. The nutrient solution was aerated using an air pump with an interval of 20 min to maintain the dissolved oxygen concentration of 8.0 ± 0.2 mg L^{-1} during the experiment.

When the third leaves were fully expanded, the cucumber seedlings were treated as follows: (1) control (Cont), half strength Hoagland solution; (2) CB, Cont +10^{-3} mg L^{-1} EBL (Sigma Aldrich, USA, applied to the solution); (3) N, half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$; and (4) NB, N + 10^{-3} mg L^{-1} of EBL (applied to the solution). EBL was dissolved in ethanol, with Cont and N treatments containing the same ethanol level. Samples were collected after treatment for 72 h, immediately frozen in liquid nitrogen, and stored at − 80 °C. The experiment was carried out with three biological replicates, and each treatment consisted of 12 × 3 (repetitions) cucumber seedlings.

Lignin determination

Lignin was quantified according to the method of Liyama and Wallis with slight modifications (Iiyama and Wallis 1990). Approximately 40 mg of frozen tissues were placed and ground in a mortar. The samples were extracted three times with 3 mL of 80% (*v*/*v*) ethanol at 80 °C for 1.5 h, followed by extraction in 3 mL of chloroform at 62 °C for 1 h. The extracted samples were then dried at 50 °C for 2 days. Dried segments were dissolved in 2.6 mL of 25% (*w*/*w*) acetyl bromide in acetic acid containing 2.7% (*v*/*v*) perchloric acid. After digestion for 1 h, 100 μL of mixture was added to a test tube containing 580 μL of 17.24% (*v*/*v*) 2 N sodium hydroxide and 82.76% (*v*/*v*) acetic acid. About 20 μL of 7.5 mol/L hydroxylamine hydrochloride was added to terminate the reaction. The volume was corrected to 2 mL with acetic acid and the absorbance at A280 was recorded. The concentration of AcBr-soluble lignin was calculated by using an extinction coefficient (Iiyama and Wallis 1988).

Cell wall preparation and fractionation

Cell wall extraction and subsequent fractionation were performed according to the method provided by Zhu and her colleagues (Zhu et al. 2012). In brief, the collected samples were ground in liquid nitrogen with a mortar and pestle. The samples were then homogenized with 75% ethanol on the ice for 20 min and centrifuged at 7000 rpm for 12 min. The pellets were kept and successively washed with acetone, methanol-chloroform mixture (1:1, *v*/*v*), and methanol for 20 min. The remaining samples were freeze-dried and stored at 4 °C for further analysis.

The cell walls were divided into pectin, hemicellulose 1 (HC1), hemicellulose 2 (HC2), and cellulose, respectively, after fractionation. The pectin fraction was extracted twice with 5 cm^3 0.5% ammonium oxalate buffer which contained 0.1% $NaBH_4$ (pH 4) at 100 °C for 1 h. The supernatants and the sediment were separately harvested. The pellets were further extracted by three incubations with 5 mL of 4% KOH harboring 0.1% $NaBH_4$ at room temperature for 10 h, followed by a similar extraction with 24% KOH/0.1% $NaBH_4$. The pooled supernatants from the 4 and 24% KOH extractions were centrifuged at 14,000 rpm for 15 min to obtain HC1 and HC2 fractions, respectively. The sediment from the 24% KOH extraction was then dried by freezing in high vacuum, weighed and recognized as the cellulose fraction.

Uronic acid and total polysaccharide analysis

The content of uronic acid in pectin fraction was measured in accordance with the methods described by Blumenkrantz and Asboe-Hansen (Blumenkrantz and Asboe-Hansen 1973). Here, galacturonic acid (Sigma) was used as a standard. In simple terms, approximately 200 μL of pectin extracts was mixed with 1 mL of 98% H_2SO_4 that contained 0.0125 M $Na_2B_4O_7.10H_2O$ in a boiled bath for 5 min. The mixture after cooling was added by 20 μL of M-hydro-diphenyl (0.15%) and was allowed to incubate at 25 °C for 25 min. The absorbance at 520 nm was recorded.

The total levels of polysaccharide in the hemicellulose fractions were measured according to the method by Dubois and his colleagues (Dubois et al. 1956). In brief, 200 μL of hemicellulose 1 (HC1) fractions was mixed with 1 mL of 98% H_2SO_4 and 10 μL of 80% phenol and placed at 25 °C for 20 min. The mixture was then incubated in a boiled bath for 15 min. The absorbance at 490 nm was recorded.

Anatomical structure analysis by histochemistry and autofluorescence

The anatomical structure of root tip was identified using a combination of histochemistry and fluorescence microscopy. The root tips were fixed in FAA (38% formaldehyde/glacial acetic acid/70% ethanol, 5:5:90, *v*/*v*/*v*) for over 24 h. The isolated root tips were subsequently dehydrated with ethanol and embedded in paraffin. The samples were then cut into sections and stained with toluidine blue-O (1%, *w*/*v*) to show the anatomical structure of root cross section.

For phloroglucinol staining, the sections were immersed in phloroglucinol solution [2% in ethanol:water (95:5)] for 5 min. Subsequently, the sections were subjected to concentrated HCl for 3 min. Lignified tissues were stained with red under an Olympus BX51 microscope.

Lignified tissues may be excited under ultraviolet (UV) radiation and produce fluorescence emission. To observe lignin autofluorescence, sections with lignified tissues were shown by fluorescence microscopy under UV excitation.

RT-PCR and qRT-PCR analysis

Genes were selected from NCBI and cucumber databases (cucumber.genomics.org.cn), and primers were designed by Primer Premier 6.0. Total RNA was isolated from cucumber roots using the TRI reagent as described in the manufacturer's protocol. Subsequently, the total RNA (1 μg) was converted into cDNA using the PrimeScript™ 1st strand cDNA synthesis kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. Real-time quantitative PCR was carried out with SYBR PrimeScript RT-PCR Kit (TaKaRa, Dalian, China) and run on a StepOne™ real-time PCR system (Applied Biosystems, Singapore) following the manufacturer's recommendations.

The PCR reactions were carried out in a mixture consisting of 2 μL of diluted cDNA, 10 μL of SYBR Premix ExTaq™ II (2×), 0.8 μL of each specific primer, 0.4 μL of ROX reference dye, and 6 μL of ddH_2O, yielding a final volume of 20 μL. The reaction conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and a final extension of 95 °C for 15 s. Gene expression fold changes were calculated using the delta-delta CT method, and the relative mRNA expression level was normalized against a reference gene, *actin*. Gene-specific primers used for real-time quantitative PCR are listed in Supplementary Table 1.

Statistical analysis

Data were collected and statistically analyzed using SAS software (SAS Institute, Cary, NC, USA) using Duncan's multiple range test at a significance level of 0.05.

Results

Cell wall polysaccharides and lignin

Secondary cell walls are composites that contain pectin, cellulose, and hemicelluloses, often being encrusted with

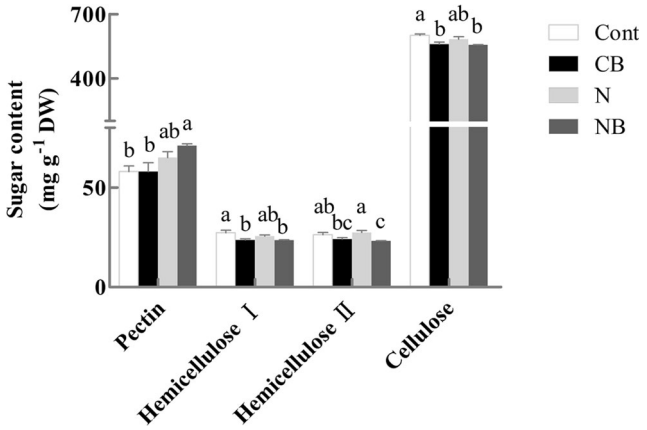

Fig. 1 Effect of exogenous EBL on sugar content in the cell wall of cucumber seedling roots under $Ca(NO_3)_2$ stress. Samples were collected on the seventh day after treatment. Cont, control (half strength Hoagland solution); CB, Con + 10^{-3} mg L^{-1} EBL; N, Con + 80 mM $Ca(NO_3)_2$ (half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$); NB, N + 10^{-3} mg L^{-1} of EBL

lignin. The polysaccharide was obtained through the sequential extraction of cell wall components in the roots of control, $Ca(NO_3)_2$-stressed and EBL-applied cucumber seedlings. The total sugar content of hemicelluloses ranged from 45 to 53 mg g^{-1}, whereas values of 50 to 71 mg g^{-1} were observed for the total amount of pectic polysaccharides. The content of cellulose reached 514–615 mg g^{-1}, but no significant difference was detected among different treatments for 3 days (data not shown). After 7 days, sugar content in the EBL-applied roots showed a decrease in hemicellulose I, hemicellulose II, and cellulose fractions compared with that in the control. $Ca(NO_3)_2$ treatments did not show any significant effect on sugar content of cell wall in cucumber seedling roots (Fig. 1).

Uronic acid and lignin accumulation in each cell wall fraction from the roots of control and treated seedlings was measured (Fig. 2). $Ca(NO_3)_2$ treatments considerably increased the uronic acid content in the pectin and hemicellulose fractions. EBL application further promoted uronic acid accumulation under $Ca(NO_3)_2$ stress. The application of EBL to the $Ca(NO_3)_2$ stress resulted in a remarkable enhancement in the pectin levels compared to the control plants (2.8-fold) and salt-stressed plants (1.7-fold) after 7 days. The content of lignin showed no significant change among different treatments after 24 h; after 3 days, a dramatic increase in lignin occurred in the presence of $Ca(NO_3)_2$, and finally after 7 days, the $Ca(NO_3)_2$-stressed plants had the highest value for lignin accumulation; however, the application of EBL could relieve this trend under $Ca(NO_3)_2$ stress.

Lignin distribution

Lignin polymers are primarily derived from the guaiacyl (G) and syringyl (S) units, which can be reflected by Mäule reaction and phloroglucinol staining, respectively. As shown in Fig. 3, $Ca(NO_3)_2$ promoted the differentiation of the metaxylem as well as the accumulation of lignin in primary xylem of cucumber seedling roots after 7 days. Compared with the $Ca(NO_3)_2$-stressed plants, exogenous EBL altered lignin distribution under salt stress. The endodermal cells, which are closed to the pericycle, were stained with a bright violet-red color. However, no difference was detected in the cucumber roots exposed to

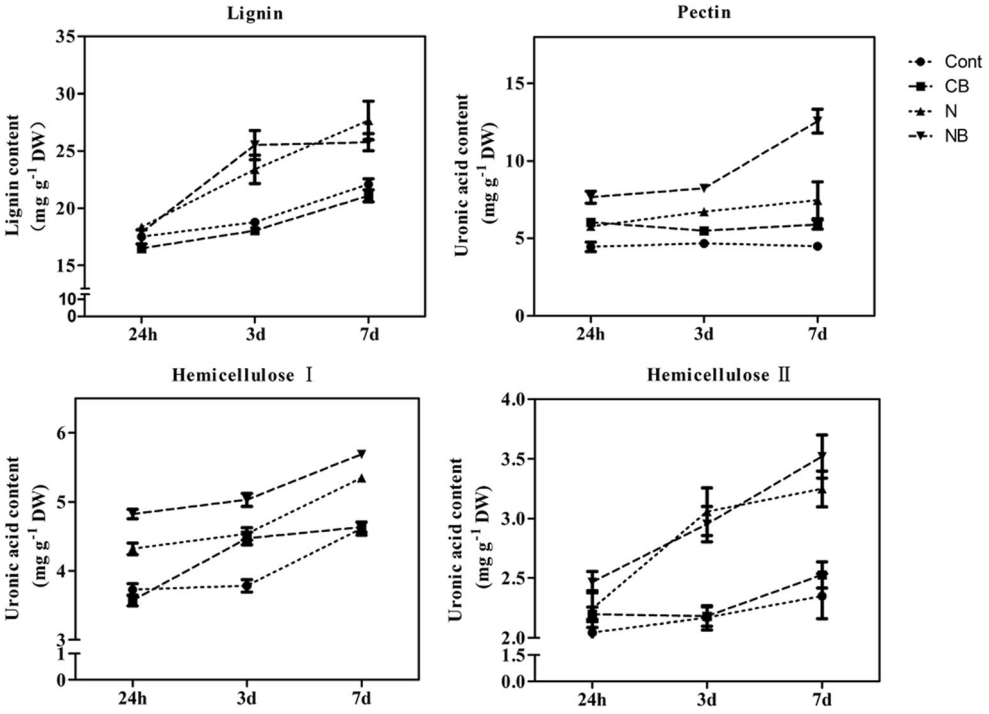

Fig. 2 Effect of exogenous EBL on lignin and uronic acid accumulation in the cell wall of cucumber seedling roots under $Ca(NO_3)_2$ stress. Cont, control (half strength Hoagland solution); CB, Con + 10^{-3} mg L^{-1} EBL; N, Con + 80 mM $Ca(NO_3)_2$ (half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$); NB, N + 10^{-3} mg L^{-1} of EBL

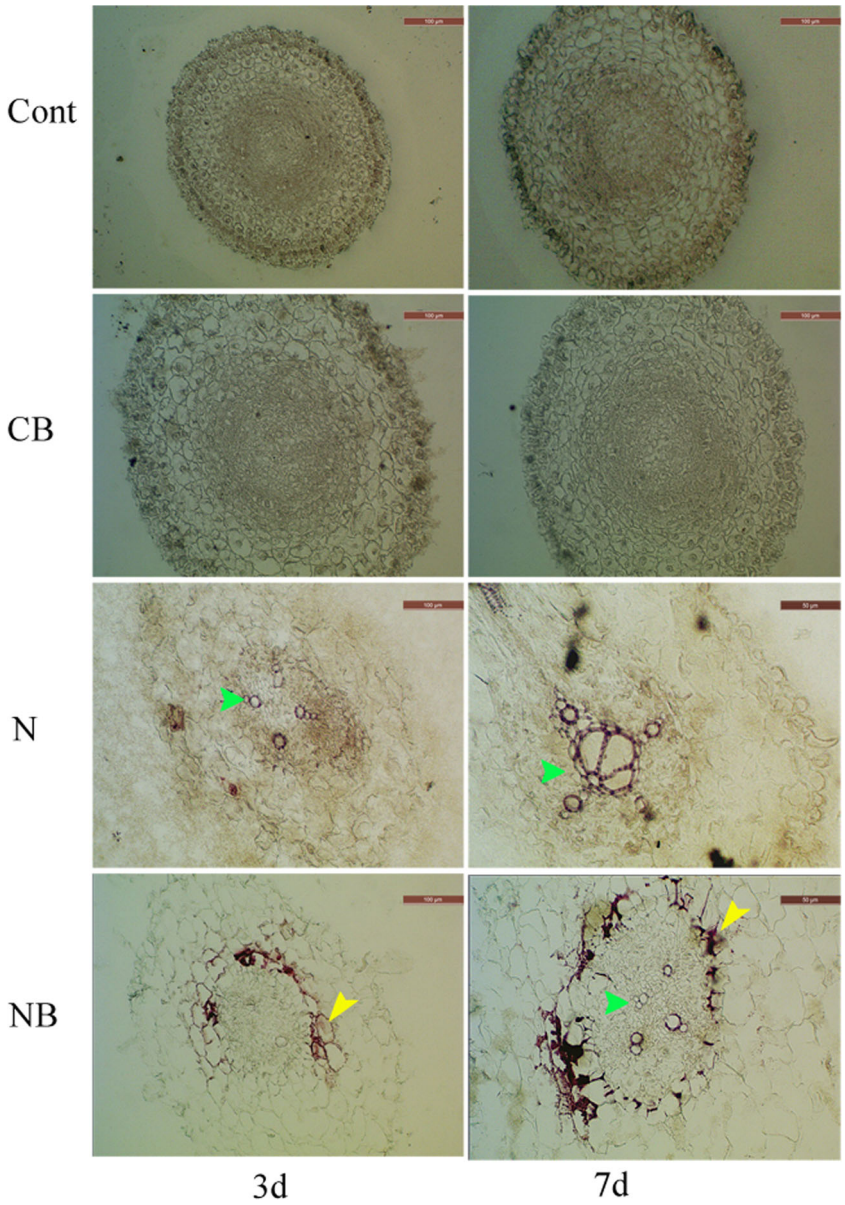

Fig. 3 Effect of exogenous EBL on lignin G monomer accumulation in the root system of cucumber seedlings under $Ca(NO_3)_2$ stress. Yellow arrow shows the area of casparian band, and the green arrow shows xylem. Scale bars in N and NB at 7 days are 50 μm, whereas others are 100 μm in length. Cont, control (half strength Hoagland solution); CB, Con + 10^{-3} mg L^{-1} EBL; N, Con + 80 mM $Ca(NO_3)_2$ (half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$); NB, N + 10^{-3} mg L^{-1} of EBL

different treatments when the root sections were stained with Mäule reaction (data not shown). The UV-excited fluorescence in the roots of cucumber was shown in Fig. 4. Autofluorescence was easily observed in the presence of $Ca(NO_3)_2$. Meanwhile, the images reflected by autofluorescence were in accordance with the results shown by phloroglucinol staining.

Key gene expression of lignin biosynthesis

Exogenous EBL application resulted in significant changes in lignin content and altered the monomeric composition at the cellular level under $Ca(NO_3)_2$ stress. Therefore, expression of lignin biosynthesis genes was determined by qRT-PCR (Fig. 5).The expression of *PAL*, *F5H783*, and *LFA POD* in the $Ca(NO_3)_2$-stressed roots were significantly downregulated at the transcriptional level compared to that in the control after 24 h. By contrast, *COMT788* and *COMT109* were upregulated after salinity treatment for 24 h, suggesting that exogenously applied $Ca(NO_3)_2$ can induce lignin synthesis. As time was extended to 3 days, expression levels of *PAL*, *COMT788*, *COMT109*, and *LAC525* in the $Ca(NO_3)_2$-stressed roots were significantly increased compared to those of controls. Meanwhile, expression of PAL, *COMT109*, *COMT290*, *CCoAOMT121*, *LAC525*, and *LAC947* maintained a lower level by the application of EBL under $Ca(NO_3)_2$ stress, suggesting a dramatic reduction in the rate of lignin synthesis. After 7 days, the expression of most of the selected genes in the EBL-applied roots were significantly upregulated at the transcriptional level compared to that in the control plants, which may contribute to an increase in syringyl monolignols.

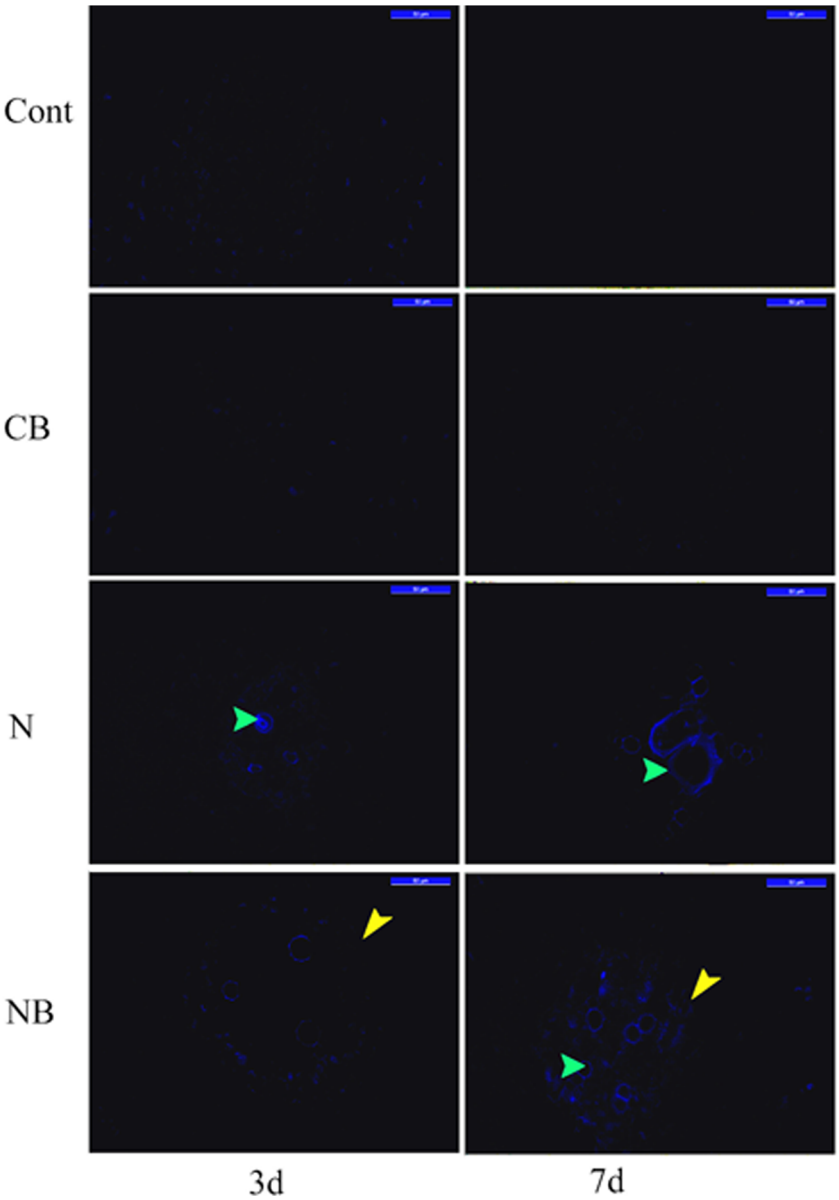

Fig. 4 Effect of exogenous EBL on lignin autofluorescence in the root system of cucumber seedlings under $Ca(NO_3)_2$ stress. Yellow arrow shows the area of casparian band, and the green arrow shows xylem. Scale bar = 50 μm. Cont, control (half strength Hoagland solution); CB, Con + 10^{-3} mg L^{-1} EBL; N, Con + 80 mM $Ca(NO_3)_2$ (half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$); NB, N + 10^{-3} mg L^{-1} of EBL

Gene expression of cell wall modification enzymes

$Ca(NO_3)_2$-stressed plants exhibited marked expansion in root diameter and increased numbers of lateral roots, indicating the intensification of cell division and differentiation. Therefore, qRT-PCR was employed to detect the expression of three genes involved in cell wall modification in cucumber (Fig. 6). Compared with control plants, the expression of glycosyltransferase was significantly upregulated at the transcriptional level by $Ca(NO_3)_2$ treatment as time went on. In the roots treated with EBL and $Ca(NO_3)_2$, transcript levels of glycosyltransferase and β-D-galactosidase were highly expressed at 24 h, followed by a drastic decline.

MAPK signaling pathway plays important roles in cell division, initiation of developmental processes, and responses to abiotic and biotic stresses. It has been reported that BRs could control cell patterning via BIN2-mediated suppression of MKK4/5 activity (Khan et al. 2013). The expression patterns of *MKK4/5* in $Ca(NO_3)_2$-stressed plants were similar to that of glycosyltransferase. The application of exogenous EBL resulted in a decrease in *MKK4/5* expression compared to that in the control.

BR signaling cascade

The expression of positive and negative components in the BR signaling cascade was analyzed at the transcriptional level (Fig. 7). The expression of *BRI1* and *BIN2* changed at 24 h in the presence of $Ca(NO_3)_2$, suggesting that exogenously applied $Ca(NO_3)_2$ slightly induced BR

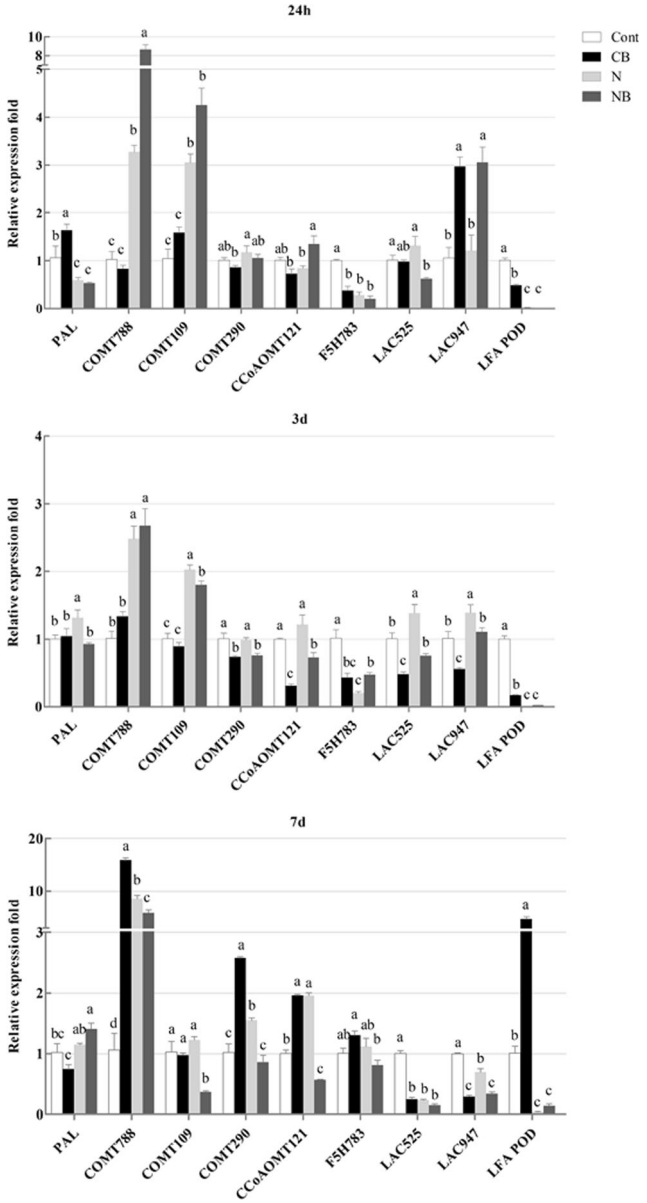

Fig. 5 Effect of exogenous EBL on the expression of lignin biosynthesis related enzymes at the transcriptional level in cucumber seedling roots under $Ca(NO_3)_2$ stress. Cont, control (half strength Hoagland solution); CB, Con + 10^{-3} mg L^{-1} EBL; N, Con + 80 mM $Ca(NO_3)_2$ (half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$); NB, N + 10^{-3} mg L^{-1} of EBL

signaling. However, as time went on, expression levels of *BAK1* still maintained a lower level, whereas transcript levels of other positive BR signaling regulators (*BRI1*, *BSU1*, and *BZR1*) were significantly increased compared to those of control. By contrast, expression levels of *BRI1* and *BIN2* were significantly reduced by exogenous EBL under $Ca(NO_3)_2$ stress, suggesting that exogenously applied EBL regulated BR signaling. After 7 days, expression levels of *BRI1*, *BSU1*, and *BZR1* in EBL-applied $Ca(NO_3)_2$-stressed roots still maintained a higher level compared with control plants.

Discussion

Salt stress is one of the major factors that retard crop yield and production. Here, we found that the number of lateral root and root diameter increased to adapt to the environmental stress. Cell wall plays an important role in the maintenance of tissue morphology and signal transduction. Secondary cell walls are restricted to specific types of differentiated cells and are composites of cellulose and hemicelluloses, often being encrusted with lignin (Cosgrove and Jarvis 2012). The polysaccharide is a principal component of plant cell wall (Agoda-Tandjawa et al. 2012). The total sugar content in the cell wall can indicate the amount of cell wall polysaccharides and further reflect the cell wall density. Previous studies have shown that cell wall polysaccharides may undergo an obvious alteration when exposed to salt stress (de Lima et al. 2014). Here, cell wall polysaccharide content was not significantly changed under $Ca(NO_3)_2$ stress in our experiment. However, the levels of hemicellulose and cellulose were decreased when exogenous EBL was applied (Fig. 1), suggesting that EBL has an effect on the cell wall component.

It was reported that hemicellulose can form a primary network via hydrogen bonding to cellulose microfibrils and may also be linked to acidic pectins by covalent attachment (Cosgrove 2005). Therefore, it is an essential component for maintaining wall framework. The structural and compositional change in hemicellulose is a way to adapt to wall extension and cell elongation. $Ca(NO_3)_2$ treatments as well as application of EBL considerably promoted the uronic acid content in the hemicellulose fractions, indicating expansion of cells (Fig. 2). Lignin, mainly distributed in secondarily thickened cell walls, is involved in many aspects of plant developmental processes (You et al. 2013). In this study, salt stress significantly increased lignin content; however, the increasing rate of lignin content in EBL-applied seedlings was higher than that in $Ca(NO_3)_2$-stressed seedling roots (Fig. 2). EBL may change the cell wall polysaccharide fractions and lignin accumulation, leading to cell wall reassembly under salt stress.

Lignin polymers are mainly derived from *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignols, which are formed by the dehydrogenation of the hydroxycinnamyl alcohols (Zhao and Dixon 2011). An increased number of lignified tracheary elements was observed in tomato roots under salinity stress, which enhances water transport and ion uptake (Sánchez-Aguayo et al. 2004). In addition, a previous study reported that the width of the lignified region of casparian strip was increased under salinity stress in maize roots (Karahara et al. 2004). It is reported that the casparian band has minor effect on water movement, but it impedes the movement of ions (Peterson and Steudle 1993). Phloroglucinol staining showed a higher lignin accumulation closed to casparian strip when exogenous EBL was applied under $Ca(NO_3)_2$ stress (Fig. 3). Therefore, EBL could operate the degree of

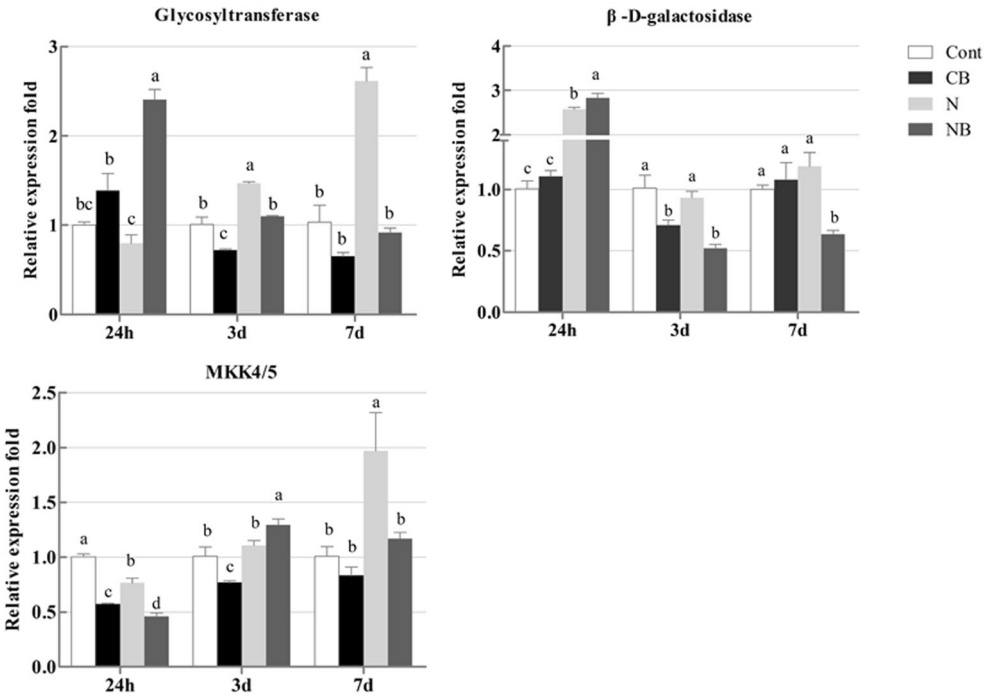

Fig. 6 Effect of exogenous EBL on the expression of cell wall degradation enzyme at the transcriptional level in cucumber seedling roots under $Ca(NO_3)_2$ stress. Cont, control (half strength Hoagland solution); CB, Con + 10^{-3} mg L^{-1} EBL; N, Con + 80 mM $Ca(NO_3)_2$ (half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$); NB, N + 10^{-3} mg L^{-1} of EBL

lignification around casparian strips and provide defense against osmotic stress.

Lignin biosynthesis pathway has been established, and the main structure enzymes involved in this pathway have been well isolated and characterized (Zhong and Ye 2007). Monolignols are derived from phenylalanine that are deaminated to cinnamic acid, followed by a series of enzyme reactions (Jia et al. 2015). It is reported that the proportion of S units was severely decreased when COMT transcription was suppressed (Tsai et al. 1998; Lapierre et al. 1999). Here, $Ca(NO_3)_2$ significantly increased the synthesis of S monomer, but this phenomenon was alleviated to a certain extent after EBL application. Lignin is finally formed by dehydrogenation polymerization (POD or LAC) after the synthesis of monolignols (Zhao and Dixon 2011). During the period of EBL-$Ca(NO_3)_2$ treatment, the expression of *LAC* and *POD* genes were also altered. These results suggested EBL can change lignin accumulation under $Ca(NO_3)_2$ stress.

β-galactosidase (β-Gal) is an enzyme involved in cell wall degradation, which can degrade the pectin and hemicellulose as well as glycoproteins and glycolipids, making components in the cell wall unstable (Dwevedi and Kayastha 2009; Bell et al. 2013). In our experiment, expression of *β-D-galactosidase* was significantly upregulated for 24 h and maintained at a relatively stable level. Golgi glycosyltransferases, which are involved in xylan biosynthesis, were markedly upregulated at the transcriptional level by $Ca(NO_3)_2$ treatment along with the increase of stress time (Fig. 6). MAP kinases have been identified as essential signal integration components in response to biotic and abiotic stress, hormone stimuli, and cell division (Marshall, 1994). It has been demonstrated that BIN2 and its homologs, GSK3/Shaggy-like kinases, can phosphorylate the MAPK kinases MKK4 and MKK5 (Khan et al. 2013). Here, transcription of *MKK4/5* was decreased at first and then remained at a relatively stable level. Several studies have attributed the growth defects of BR mutants primarily to impaired cell expansion and cell division (Clouse and Sasse 1998; Savaldi-Goldstein et al. 2007; Hacham et al. 2011). Therefore, BR may maintain the elongation of root system by keeping normal expansion of root cells.

BRs signal through plasma membrane localized receptor BRI1 and other components including negatively acting BIN2 kinase to regulate BES1/BZR1 family transcription factors, which control the expression of downstream genes for various BR responses (Kim et al. 2009). Expression levels of *BRI* in $Ca(NO_3)_2$-stressed plant roots still maintained a higher level than in the control (Fig. 7), indicating that receptor-like kinases are induced by abiotic stress itself, thereby amplifying the signal for the necessary stress adaption response (Lindner et al. 2012).

In many dicot species, lateral root primordia are derived from the pericycle, a layer of cells that lies immediately within the endodermis (Robbins et al. 2014). Auxin signaling is also activated in endodermal cells to meet primordial growth (Gibbs and Coates 2014). Cell wall loosening is activated by auxin to allow the cells in the outer tissue layers to separate and grow as lateral root primordia (Robbins et al. 2014). As a result, the auxin signaling promotes the formation of lateral roots under salt stress and maintains normal water transport. However, the concrete effects of exogenous EBL on cell division and expansion of root cells, and the relationship

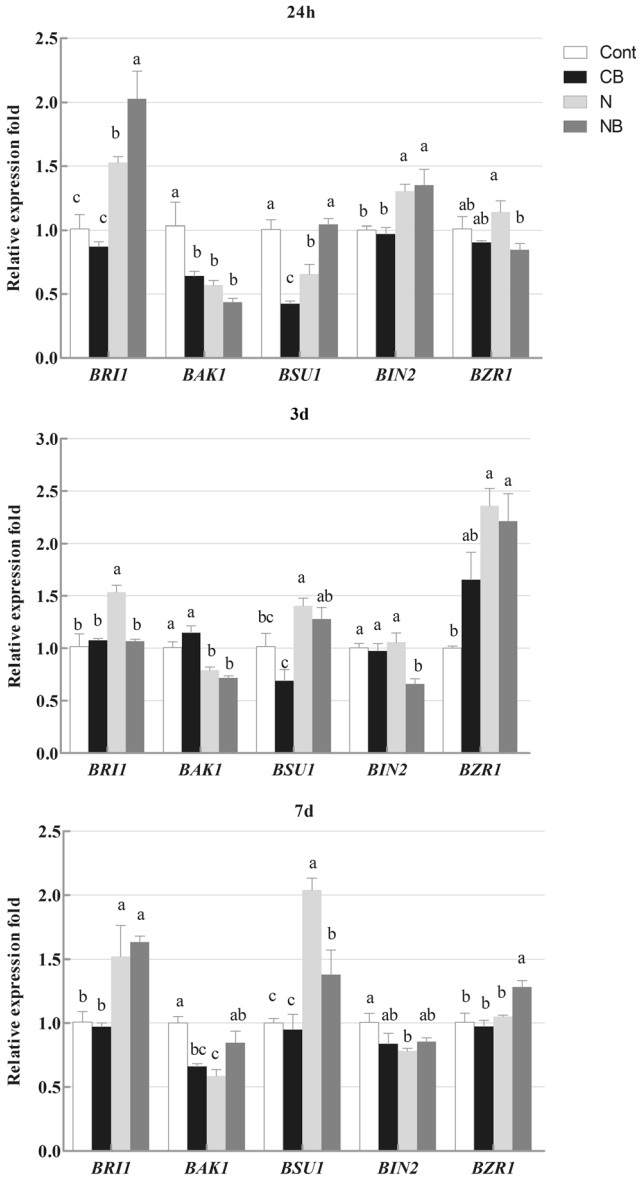

Fig. 7 Effect of exogenous EBL on the expression of BR signaling-related enzymes at the transcriptional level in cucumber seedling roots under $Ca(NO_3)_2$ stress. Cont, control (half strength Hoagland solution); CB, Con + 10^{-3} mg L^{-1} EBL; N, Con + 80 mM $Ca(NO_3)_2$ (half strength Hoagland solution containing 80 mM $Ca(NO_3)_2$); NB, N + 10^{-3} mg L^{-1} of EBL

between BRs and IAA in the process of lateral root formation need to be further researched.

Conclusions

Effects of applied EBL on the changes in cell wall components and anatomical structure of cucumber seedling roots subjected to salt stress were investigated. Phloroglucinol staining indicated increased S/G ratio after $Ca(NO_3)_2$ treatment. Expression analysis indicated that $Ca(NO_3)_2$ treatment promoted the expression of *COMT* genes, in consistence with the results shown by phloroglucinol staining. However, this phenomenon was alleviated by the addition of EBL. EBL may promote cell expansion and maintained optimum length of root system, alleviating the oxidative stress caused by $Ca(NO_3)_2$. The continuous transduction of EBL signal thickened the secondary cell wall of casparian band cells, which resisted ion toxicity, and maintained water transport.

Funding information This work was supported by the National Natural Science Foundation of China (No. 31471869, No. 31401919 and No. 31272209), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PDPA), China Agriculture Research System (CARS-25-C-03), and sponsored by the Research Fund for the Doctoral Program of Higher Education (20130097120015).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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