Brassinosteroids promote AUX/IAA transcriptional repressors and inhibit adventitious root formation in mung bean

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ABSTRACT

Adventitious root (AR) formation is important in asexual propagation of horticultural and forestry crops. It is induced by wounding and controlled by several phytohormones, including auxin. Brassinosteroids (BR) are plant steroid hormones that regulate several aspects of plant growth and development and interact with auxin. Here, we investigated roles of BR in AR formation in mung bean hypocotyl cuttings by dipping the hypocotyl base of the explants in various concentrations of BR (1 nM, 10 nM, 0.1 µM, 1 µM and 10 µM 24-epi-castasterone). Our results demonstrated that BR treatments suppressed AR formation in a dose-dependent manner. Expression analysis of genes involved in auxin signaling and transport showed that BR up-regulated several genes encoding AUX/IAA transcriptional repressors, but not an auxin transporter *PIN1*. Analyses of activities of polyphenol oxidase (PPO), which plays roles in root primordia formation, suggested that BR may inhibit AR formation partly by reducing PPO activity in the hypocotyl cuttings. Our findings revealed roles of BR in promoting transcription of negative regulators of the auxin signaling pathway, thus blocking auxin-mediated AR formation.

Keywords: adventitious root formation; brassinosteroid; auxin

INTRODUCTION

Adventitious roots (AR) are post-embryonic roots that develop from above-ground parts of plants or detached tissues (Yu *et al.*, 2017; Gonin *et al.*, 2019). AR formation not only occurs during normal development but can also be induced by stress such as wounding, flooding, or through the horticultural practices used for vegetative propagation (Pacurar *et al.*, 2014). The process of AR formation can be divided into three phases: induction phase, initiation phase and expression phase, in which tissue morphology, as well as accumulation of phytohormones and secondary metabolites and activities of related enzymes alter throughout the courses of the formation process (Guan *et al.*, 2015).

Auxin has been shown to play key roles in stimulating AR formation and is widely used in horticulture to induce adventitious rooting. In the cuttings, auxin rapidly accumulates near the site of excision and induces cell dedifferentiation and root primordium formation (Lui *et al.*, 2014; Li *et al.*, 2016). Auxin controls several aspects of plant growth and development via the auxin signaling pathway (Guilfoyle and Hagen, 2007). The binding of auxin to the SCF^{TIR1} auxin receptor complex triggers the degradation of transcriptional repressors Aux/IAAs, which interact with and repress transcriptional activities of the AUXIN RESPONSE FACTOR (ARF) transcription factors (Guilfoyle and Hagen, 2007; Tian *et al.*, 2017). In addition, previous studies have revealed that auxin could act synergistically or antagonistically with other hormones, including ethylene and cytokinin in the regulation of AR formation (Geiss *et al.*, 2009; Pacurar *et al.*, 2014).

Brassinosteroids (BR) are a class of plant steroid hormones involved in many plant growth and development including stem and root elongation, photomorphogenesis and stress tolerance. The BR signal is transduced by BRASSINOSTEROID INSENSITIVE 1 (BRI1), a receptor kinase at the plasma membrane to activate a signal transduction cascade that regulates BRASSINAZOLE RESISTANT 1/2 (BZR1/2) transcription factors and bind to the promoter of BR-responsive genes (Zhu *et al.*, 2013). The BR and auxin signaling pathways have been shown to interact synergistically in promoting hypocotyl elongation in various plant species (Oh *et al.*, 2014; Tian *et al.*, 2017). However, they interact antagonistically

in regulating root cell elongation and quiescent center cell division in primary root meristem (Chaiwanon and Wang, 2015). A previous study showed that BR inhibits AR formation in mung bean hypocotyl cuttings (Guan and Roddick, 1988). However, little is known about its mechanism and how BR interacts with auxin in regulating AR formation.

Several enzymatic activities are altered during rooting process. Polyphenol oxidase (PPO) is an enzyme that catalyzes the oxidation of several phenolic compound to quinine intermediates. Many studies have suggested the involvement of PPO activity in AR formation. Increased PPO activity has been reported to be associated with improved rooting ability in several species (Basak et al., 2000) and the use of PPO as a biochemical marker of rooting was suggested (Cheniany et al., 2010). However, despite a number of studies on PPO and AR formation, contradictory findings on the change of PPO activity during rooting process are frequently reported and suggested to be species- or cultivar-dependent (Porfírio et al., 2016).

In this study, roles of BR in AR formation in mung bean hypocotyl cuttings were investigated. The results showed that BR treatments suppressed AR formation in a dose-dependent manner. Furthermore, we performed gene expression analysis of the explants treated with BR, as well as biochemical analysis to measure activities of the enzyme polyphenol oxidase (PPO) during different phases of AR formation.

MATERIALS AND METHODS

Plant materials and growth conditions

Mung bean (Vigna radiata (L.) R. Wilczek) seeds were washed and soaked in distilled water for 24 h at room temperature and germinated at 27°C for 5 days under 16 h light and 8 h dark photoperiod. After 5 days, uniform-sized seedlings consisted of two primary leaves and 4 cm of hypocotyls were used for experiments. Primary roots of the seedlings were removed from the bases of hypocotyls, and 10 mung bean explants were incubated in 20 ml of Hoagland's nutrient solution with the absence or presence of BR (24-epicastasterone (S18014, purity 92%), Yuanye Biology, China) at various concentrations and arranged in a completely randomized design (CRD). Three replicates were included per treatment, and each replication consisted of 10 explants per container. All seedlings or explants were

grown in a controlled room at 25°C under 16 h light and 8 h dark cycle and 65% relative humidity.

Measurement of adventitious root numbers

Explants were dipped (with approximately 3 cm from the base of the hypocotyls submerged in solution) in various concentrations of BR (1 nM, 10 nM, 0.1 µM, 1 µM, and 10 µM) or untreated control (Hoagland's solution) for 48 h. After treatment, the explants were washed with distilled water three times and transferred to new Hoagland's solution without BR treatment for another 5 days. The nutrient solutions were replaced daily. To determine the numbers of ARs formed per explant, ARs that were longer than 1 mm were counted.

Polyphenol oxidase enzymatic activity assays

Explants were treated with 0.1, 1 µM BR or untreated. To determine polyphenol oxidase activity (EC 1.11.1.7; PPO), the lower parts (approximately 2 cm) of hypocotyls were harvested at 12, 24, 36, 48, 60 and 72 h after treatment and frozen in liquid nitrogen immediately. Frozen hypocotyl explants (50 mg) were ground in liquid nitrogen and transferred to tubes containing 1 ml of 100 mM phosphate buffer (pH 7.0). The extracts were centrifuged at 12,000 rpm for 20 min at 4°C, and supernatants were analyzed for PPO enzymatic activity in microplates by using catechol (10 mM in 100 mM phosphate buffer, pH 6.0) as substrate (Batish et al., 2008; Siguemoto and Gut, 2017). For quantification, the absorbance at 420 nm was recorded every 30 sec at room temperature for 3 min using a SpectraMax®M3 microplate reader. Total protein contents were determined by Bradford assay (Bradford, 1976) using Bovine serum albumin (BSA) as standard.

Gene expression analysis

Explants were treated with 1 µM BR or untreated. After 24 h of treatment, the lower parts (approximately 5 mm) of hypocotyls were harvested and total RNA was extracted using TRIzol reagent (Invitrogen) as described previously (Yin et al., 2016). cDNA synthesis was performed using iScript™ Reverse Transcription Supermix (BioRad) according to manufacturer's instructions. qRT-PCR was performed on CFX connect Real-Time PCR Detection System (BioRad) using CAPITAL™ qPCR Green Mix (biotechrabbit) and gene specific primers for VrIAA8, VrIAA9, VrIAA14, VrPIN1, VrMYB134, VrLBD29 and

VreIF5A previously reported in Li *et al.* (2016). Expression of a housekeeping gene *eIF5A* was used as reference for normalization. Expression levels were calculated relative to the reference gene using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Means and standard errors (SE) were analyzed by t-test and one-way analysis of variance (ANOVA). The comparisons between mean values were calculated using Duncan's multiple range test (DMRT) with $P < 0.05$ using IBM SPSS statistics 22.

RESULTS

Effects of BR on adventitious root formation

To investigate effects of BR on AR formation, mung bean hypocotyl cuttings were treated with varied concentrations of BR (1 nM, 10 nM, 0.1 μM, 1 μM and 10 μM) for 48 h, and AR numbers were quantified after 5 days compared with untreated control. The number of ARs formed on explants declined significantly with increasing concentrations of BR. The results indicated that BR could inhibit adventitious rooting in mung bean hypocotyl cuttings in a dose-dependent manner (Figure 1).

Expression of genes related to auxin signaling pathway and adventitious root formation

Auxin has been shown to promote AR formation, and its accumulation is increased at the cuttings where AR primordia developed due to polar auxin transport (Guan *et al.*, 2019). To determine whether BR treatment affects expression of genes involved in auxin-mediated AR formation, we measured expression of genes encoding AUX/IAA transcriptional repressors *VrIAA8*, *VrIAA9* and *VrIAA14*, negative regulators of the auxin signaling pathway, and an auxin efflux carrier *PIN1*, which mediates polar auxin transport, by qRT-PCR. After 24 h of 1 μM BR treatment, expression of *VrIAA8*, *VrIAA9* and *VrIAA14* were dramatically up-regulated compared with control but the expression of *VrPIN1* gene did not respond to BR treatment (Figure 2). These results suggest that the increase of *AUX/IAA* expression by BR could potentially repress activities of ARF transcription factors in AR formation, although BR treatment may not alter auxin transport.

In addition to factors related to auxin, two families of transcription factors, MYB and LATERAL ORGAN BOUNDARY (LBD)-DOMAIN, are important regulators of AR formation (Lui *et al.*, 2014; Li *et al.*, 2015). Our qRT-PCR analysis showed that expression of *VrMYB134* was significantly up-regulated in the BR-treated cuttings compared with control, whereas *VrLBD29* did not show a significant difference (Figure 3). This result suggests that BR treatment may also inhibit AR formation by altering expression of some transcription factors.
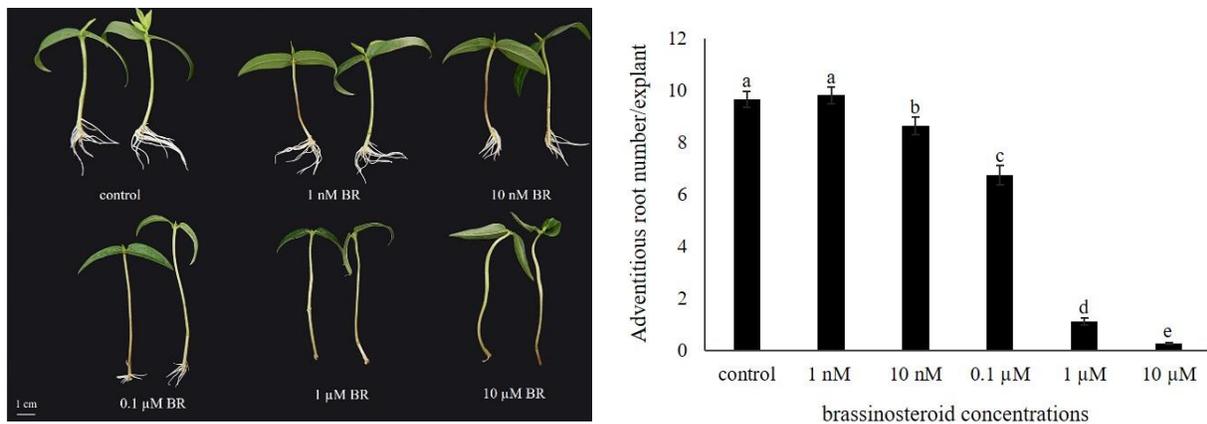

Figure 1 Effects of BR on the number of ARs formed on mung bean hypocotyl cuttings. The values represent the means of three independent experiments with at least 30 explants/experiment. Error bars indicate standard error (SE). Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test (DMRT). Bar= 1 cm
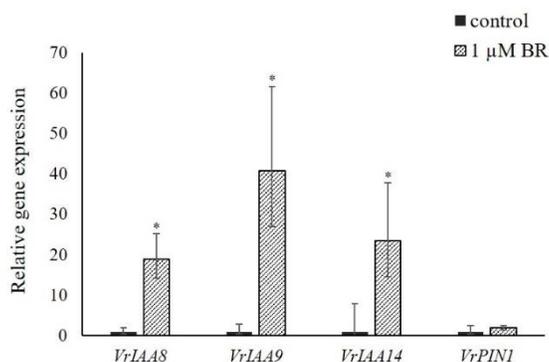
Figure 2 Gene expression analysis of *VrIAA8*, *VrIAA9*, *VrIAA14* and *VrPIN1* in response to BR treatment in mung bean hypocotyl cuttings. Expression levels were normalized with *VreIF5A*. Data are means of three biological replicates, and error bars represent SE. * indicates significant differences ($P < 0.05$) according to Student's t-test.
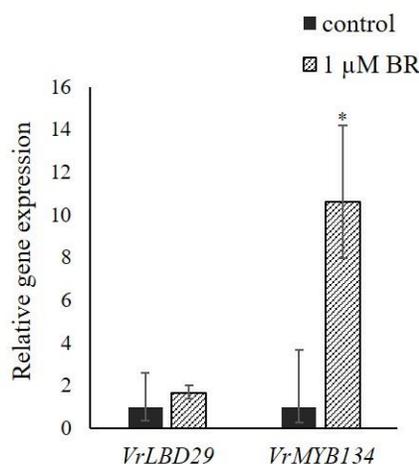

Figure 3 Gene expression analysis of *VrMYB134* and *VrLBD29* in response to BR treatment in mung bean hypocotyl cuttings. Expression levels were normalized with *VreIF5A*. Data are means of three biological replicates, and error bars represent SE. * indicates significant differences ($P < 0.05$) according to Student's t-test.

Changes of polyphenol oxidase activity during adventitious rooting

Polyphenol oxidase (PPO) activity has been shown to alter during the course of AR development. It increased during the root induction and initiation phases (6‑48 h after excision) and decreased during the root extension phase (48‑96 h after excision) in mung bean cuttings (Nag *et al.*, 2001). To determine whether BR affects PPO activity during various stages of AR formation, PPO activity was measured at 12, 24, 36, 48, 60 and 72 h after treatment of 0.1 and 1 μM BR, which

were the concentrations that significantly inhibited AR formation (Figure 1), compared with untreated control. The results showed that PPO activity in each treatment was similar at 12 h after excision, and then the activity was reduced in BR treatments when compared to control at most time points with statistical significance at 24 and 48 h after excision (Figure 4). In addition, the effect of 1 μM BR treatment was stronger than that of 0.1 μM BR treatment at 24 and 48 h, suggesting that the inhibitory effect on PPO activity during the root initiation phase is likely dependent on BR activities.
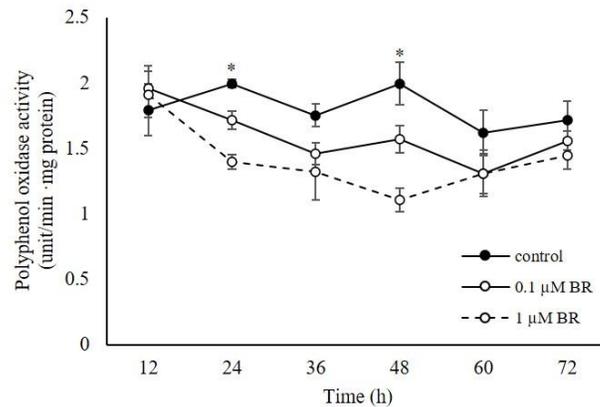
Figure 4 Effects of BR on polyphenol oxidase (PPO) activities in mung bean hypocotyl cuttings. Data are means of three biological replicates, and error bars represent SE. * indicates significant differences ($P < 0.05$) according to Duncan's multiple range test (DMRT).

DISCUSSION

Previous studies have reported that BR has an inhibitory effect on AR formation (Guan and Roddick, 1988). However, it remains unclear how BR affects AR formation at the transcriptional and biochemical levels. In this study, we showed that BR inhibited AR formation in a dose-dependent manner (Figure 1). This inhibitory effect of BR is consistent with previous reports in mung bean and tomato cuttings (Guan and Roddick, 1988). Furthermore, we showed that BR may inhibit AR formation by up-regulating expression of negative regulators of the auxin signaling pathway (Figure 2). In agreement, previous studies have demonstrated that promoters of several *AUX/IAA* genes are binding targets of the BR-regulated BZR1 transcription factors and that BR treatments induce expression of these genes in *Arabidopsis* (Sun *et al.*, 2010; Chaiwanon and Wang, 2015). While *AUX/IAA* genes are strongly up-regulated by auxin treatment, the proteins are degraded by the auxin-mediated SCF^{TIR1} auxin receptor complex, thus de-repressing ARF transcription factors (Vert *et al.*, 2008; Li *et al.*, 2016). Similar to BR in AR formation, cytokinin has been shown to counter-balance auxin effects in *Arabidopsis* root tips by up-regulating expression of *AtIAA3/SHY2* in the root differentiation zone, and thus promoting cell differentiation (Dello Ioio *et al.*, 2008).

During AR formation, auxin is transported and accumulated in the cuttings. Treatment with an auxin transport inhibitor, N-1-naphthylphthalamic acid (NPA), also inhibited AR formation in tomato cuttings,

suggesting that polar auxin transport is required (Guan *et al.*, 2019). In our experiment, BR did not alter expression of *VrPIN1*. It is possible that BR did not alter polar auxin transport mediated by PIN1. Further experiments that quantify auxin levels at the sites of AR formation would be required to conclude about roles of BR on auxin transport.

A previous study has shown that a MYB transcription factor *VrMYB134* was significantly down-regulated during the root induction stage (6 h after excision), but up-regulated during the root initiation stage (24 h after excision) in mung bean cuttings (Li *et al.*, 2015). Our results showed that *VrMYB134* was significantly up-regulated in the BR-treated samples compared with control. It is possible that BR treatment may inhibit or delay AR formation at the root induction phase, and thus expression of *VrMYB134* was not de-repressed when observed at 24 h after excision in our study. As AR formation is a dynamic process, it is likely that expression of a number of genes will respond dynamically. For example, upstream genes related to auxin signaling and transport and other transcription factors may respond during the induction and initiation phase, while downstream genes related to target cellular processes e.g. cell wall remodeling may respond during root extension phase.

A previous study has reported that PPO activity increased during the root induction and initiation phases (6-48 h after excision) and decreased activity during the root extension phase (48-96 h after excision) in mung

bean cuttings (Nag *et al.*, 2001). In this study, PPO activity in the untreated control showed fluctuating levels during 12-48 h after excision, followed by lower levels at 60 and 72 h after excision (Figure 4). A similar observation was reported that PPO activities in mulberry stem cuttings were dynamically changed during 54 h after excision (Shang *et al.*, 2019). It has also been suggested that PPO may have an indirect role in AR formation and that its effects occur through its products of phenolic oxidation and activities of another enzyme, peroxidase (POX), which is negatively correlated with PPO activities (Cheniany *et al.*, 2010; Porfírio *et al.*, 2016). It is possible that the difference between this study and previous study was due to difference in cultivars of the mung bean used in the study, as reported previously that findings of PPO activities are frequently contradictory (Porfírio *et al.*, 2016).

Nevertheless, our results demonstrated that BR treatments significantly reduced PPO activities at 24 and 48 h after excision and relatively (but not statistically significant) reduced PPO activities at 36, 60 and 72 h after excision. This suggests that BR may inhibit AR formation at both initiation and induction phases by altering PPO activity. BR treatments may also alter POX activities, which play roles in the rooting process. Further experiments to test activities of both PPO and POX at more time points (6 to 96 h after excision) are likely to yield more understanding about roles of BR on the oxidative enzymes. The inhibition of PPO by BR reported here is consistent with a previous report that BR treatments inhibited chilling-induced PPO activities in eggplant fruits (Gao *et al.*, 2015). However, further biochemical and genetic experiments are needed to unravel the mechanistic regulation.

In conclusion, our study demonstrated that BR may inhibit AR formation by inducing several AUX/IAA transcriptional repressors in mung bean cuttings. As a hormone that mainly promotes cell elongation in many parts of plants, it is likely that BR levels and functions may be decreased during AR formation to repress cell elongation while allowing proper cell reprogramming and patterning of differentiated cells.

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