Brassinosteroids are required for efficient root tip regeneration in Arabidopsis

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Abstract  Compared with other organisms, plants have an extraordinary capacity for self-repair. Even if the entire tissues, including the stem cells, are resected, most plant species are able to completely regenerate whole tissues. However, the mechanism by which plants efficiently regenerate the stem cell niche during tissue reorganization is still largely unknown. Here, we found that the signaling mediated by plant steroid hormones brassinosteroids is activated during stem cell formation after root tip excision in Arabidopsis. Treatment with brassinazole, an inhibitor of brassinosteroid biosynthesis, delayed the recovery of stem cell niche after root tip excision. Regeneration of root tip after resection was also delayed in a brassinosteroid receptor mutant. Therefore, we propose that brassinosteroids participate in efficient root tip regeneration, thereby enabling efficient tissue regeneration to ensure continuous root growth after resection.

Key words: Arabidopsis, brassinosteroid, regeneration, stem cell niche.

Introduction

Plants have a high capacity to regenerate their organs and tissues, thereby enabling their continuous growth, even if tissues are resected. In Arabidopsis root tips, excision of the root meristem, which includes the stem cell niche (SCN) composed of quiescent center (QC) cells and surrounding stem cells, stimulates regeneration, and the root tip is completely reformed within three to four days of excision (Sena et al. 2009). This regeneration process requires organization of the SCN from the remaining meristematic cells (Efroni et al. 2016; Matosevich et al. 2020; Sena et al. 2009).

Several transcription factors have been reported to play important roles in root tip regeneration in Arabidopsis. ETHYLENE RESPONSE FACTOR 115 (ERF115), a member of the APETALA2 (AP2) family transcription factors, was found to be rapidly induced after excision of the root tip and is required for regeneration. This is evident from the fact that impaired ERF115 activity causes a lack of root tip regeneration (Heyman et al. 2016; Johnson et al. 2018; Zhou et al. 2019). WOUNDING INDUCED DEDIFFERENTIATION 1 (WIND1), another member of the AP2 family transcription factors, is also shown to play a role in cellular dedifferentiation during the regeneration process (Iwase et al. 2011). Additionally, several phytohormones have been shown to participate in root tip regeneration. The phytohormone auxin plays a crucial role in tip regeneration, and treatment with auxin biosynthesis or transport inhibitors severely suppresses root tip regeneration (Efroni et al. 2016; Matosevich et al. 2020; Sena et al. 2009). Furthermore, auxin biosynthesis at the excision site is necessary for the progression of regeneration (Matosevich et al. 2020). The signaling interaction between auxin and cytokinin is important for providing positional information for SCN formation during root tip regeneration (Efroni et al. 2016). Jasmonic acid is also required for root tip regeneration, triggering ERF115 expression (Zhou et al. 2019). However, it is still unclear whether these transcription factors and phytohormones are sufficient for complete root tip regeneration and organization of the SCN after root tip excision.

Plant growth and patterning processes are regulated by the interaction between various phytohormones...
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(Depuydt and Hardtke 2011). A class of plant steroid hormone brassinosteroids is involved in cell division, elongation, differentiation, and stress responses (Clouse and Sasse 1998), and the signaling cascades have been shown to crosstalk with that of auxin in various cellular processes (Nemhauser et al. 2004). Brassinosteroids are perceived by BRASSINOSTEROID INSENSITIVE 1 (BRI1) and its homologs BRI1-LIKE 1 and BRI1-LIKE 3 (BRL1 and BRL3), which are members of the leucine-rich repeat receptor-like kinase (LRR-RLK) family (Caño-Delgado et al. 2004; Li and Chory 1997). When these receptors bind brassinosteroids, they heterodimerize with SERK3/BRI1 ASSOCIATED KINASE1 (BAK1), initiating an intracellular phosphorylation relay cascade (Li et al. 2002). This cascade promotes the activity and stability of plant-specific transcription factors BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1), which in turn control the transcription of brassinosteroid responsive genes (Wang et al. 2002; Yin et al. 2005). The signaling pathways between auxin and brassinosteroids is integrated downstream from BES1 and AUX/IAA proteins, the regulatory factors acting downstream of each hormone (Nemhauser et al. 2004). However, the role of brassinosteroids in tissue regeneration remains largely unknown.

In this study, we found that brassinosteroid signaling is activated in stem cell formation after root tip excision in Arabidopsis. Since the inhibition of brassinosteroid biosynthesis delayed the recovery of SCN in root tip, we propose that brassinosteroids are required for the efficient root tip regeneration in Arabidopsis.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Columbia-0) plants were grown vertically under continuous light conditions at 22°C on Murashige and Skoog (MS) plates [0.5× MS salts, 0.5g l⁻¹ 2-(N-morpholino)ethanesulfonic acid (MES), 1% sucrose, and 1.2% phytoagar (pH 5.7)]. pBRZ1:BZR1-YFP (Chaiwanon and Wang 2015), pWOX5:NLS-GFP (Waki et al. 2011), pBRI1:BRI1-GFP (Fábregas et al. 2013), pBRL1:BRL1-YFP (Fábregas et al. 2013), pBRL3:BRL3-YFP (Fábregas et al. 2013), and brr1 brr1 brr3 bak1 (Fábregas et al. 2018) were described previously.

Root tip excision assay

Root tips were cut according to previously published protocols (Sena et al. 2009). In brief, root tips of seven-day-old seedlings grown on MS plates were cut around 130 µm from the outermost layer of columella cells (red line in Figure 1A) by hand under a stereomicroscope using a sterile needle, resulting in the removal of the SCN and root cap including the columella and most of the lateral root cap. Seedlings were transferred back to MS plates and grown for one to five days. For brassinazole treatment, seedlings after root tip excision were transferred to MS plates supplemented with 3 µM brassinazole and were grown for the indicated number of days.

Microscopic observation and measurement of fluorescence

Seedlings were soaked in SR2200 solution [4% paraformaldehyde in PBS (pH 7.4) and 0.1% SCRI Renaissance 2200 (Renaissance Chemicals)] for 12 h at 4°C. Samples were washed with PBS and submerged in ClearSee solution [10% xyitol, 15% sodium deoxycholate, and 25% urea] until the root became transparent. For the protein marker lines BRI1-GFP, BRL1-YFP, and BRL3-YFP, roots were stained with 10 µM propidium iodide solution for 3 min at room temperature. Root tips were subsequently observed under a confocal laser scanning microscope (Olympus, FluoView FV3000), and GFP and YFP fluorescence were measured using Fiji image analysis software (http://fiji.sc). To calculate the ratio of nuclear-to-cytoplasm signal of BZR1-YFP,
the amount of YFP fluorescence in the nucleus and cytoplasm of each cell was quantified using Fiji software, respectively, and the signal value of the nucleus divided by that of the cytoplasm was calculated.

Results

Brassinosteroid signaling is activated during SCN formation after root tip excision

To examine the possibility that brassinosteroids participate in root tip regeneration in Arabidopsis, we initially monitored brassinosteroid signaling in root tips after tip excision. Brassinosteroid signaling has been shown to increase the nuclear localization of BZR1 protein, while BZR1 localizes to the cytoplasm when brassinosteroid signal is low (Chaiwanon and Wang 2015). Thus, the BZR1 nuclear-to-cytoplasm ratio reflects brassinosteroid signaling activity (Chaiwanon and Wang 2015). Root tips of seven-day-old pBZR1:BZR1-YFP seedlings were excised around 130 µm from the outermost layer of columella cells (Figure 1A), and YFP fluorescence was observed for five days after root tip excision. pBZR1:BZR1-YFP contains a construct harboring the 1 kb promoter and coding region that are fused to YFP (Chaiwanon and Wang 2015). When we observed the BZR1-YFP around the SCN (with the QC and surrounding stem cells located approximately 70 µm from the outermost layer of columella cells: Figure 1B; I) of uncut roots, the YFP signal was found to be accumulated mainly in the cytoplasm, indicating that brassinosteroid signaling activity is low in the SCN (Figure 1B, C; I). In contrast, in the area above the excision site, wherein the stele located approximately 160 µm from the outermost layer of columella cells (Figure 1B; II and III), nuclear BZR1 signals showed an increase as compared to those in the SCN (Figure 1B, C; I–III). This suggests that brassinosteroid signaling is active in the meristematic region. Interestingly, one to two days after root tip excision, a strong nuclear-localized BZR1 signal was observed around the SCN (Figure 1B; IV and V). Quantification of the nuclear and cytoplasmic BZR1-YFP signals around the regions revealed that one to two days after root tip excision, a higher BZR1 nuclear-to-cytoplasm ratio was detected in the SCN as compared to that in uncut roots (Figure 1C; IV and V). Note that the gene expression of BZR1 was not remarkably changed during early root tip regeneration [eFP Browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Sena et al. 2009; Winter et al. 2007)] (Supplementary Figure S1). After three days, the ratio gradually decreased, and it reached the level of that in the SCN before excision after five days (Figure 1B, C; VI to VIII). These results suggest that brassinosteroid signaling is temporarily activated during stem cell formation after root tip excision.

Brassinosteroid biosynthesis is required for efficient SCN regeneration after root tip excision

To examine whether brassinosteroids are involved in SCN regeneration after root tip excision, we tested the response of root tips to brassinazole, an inhibitor of brassinosteroid biosynthesis (Min et al. 1999) in SCN regeneration. The root tips of seven-day-old pWOX5:NLS-GFP, which is the transcriptional marker line that mark the QC cells in the root SCN (Waki et al. 2011) were cut and transferred onto MS plates with or without 3 µM brassinazole. As previously reported (Sena et al. 2009), under control conditions, pWOX5:NLS-GFP was ectopically expressed in a small number of endodermal and cortex cells one day after root tip excision, and the signal was also observed around de novo stem cell formation after two days (Figure 2A; 1 and 2 dpc). After three days, it was found that WOX5 expression was restricted to the SCN (Figure 2A; 3 dpc), indicating that the SCN is almost recovered after three days of root tip excision. In roots treated with brassinazole, pWOX5:NLS-GFP was expressed in some endodermal and cortex cells one to two days after root tip excision (Figure 2B; 1 and 2 dpc). After three to four days, GFP expression was observed to a cup-shaped domain encompassing the endodermal and cortex cell files and the SCN (Figure 2B; 3 and 4 dpc). After five days, GFP fluorescence was detected only in the SCN (Figure 2B; 5 dpc), indicating that the morphology of SCN is almost recovered in the brassinazole-treated roots after five days. These results suggest that brassinosteroid biosynthesis is required for efficient SCN regeneration after root tip excision.
Brassinosteroid signaling promotes root tip regeneration after root tip excision

Next, we examined whether brassinosteroid signaling is required for SCN regeneration. When we analyzed the localization and accumulation of the brassinosteroid receptors BRI1, BRL1, and BRL3 proteins using reporter lines that contained the full-length genomic sequences fused to GFP or YFP under the control of the 2 kb promoters (pBRI1:BRI1-GFP, pBRL1:BRL1-YFP, and pBRL3:BRL3-YFP) (Fàbregas et al. 2013), BRI1-GFP was detected in the entire root tip, excluding the columella cells, whereas BRL1-YFP and BRL3-YFP were present in the SCN before and after root tip excision (Figure 3). The gene expression of BRI1, BRL1 and BRL3 was not changed notably during early root tip regeneration (Sena et al. 2009; Winter et al. 2007) (Supplementary Figure S1). Therefore, we observed the phenotype of the root tip regeneration after root tip excision of the bri1 brl1 brl3 bak1 quadruple loss-of-function mutant, which is insensitive to brassinolide, an active brassinosteroid (Fàbregas et al. 2018). Root tips of seven-day-old bri1 brl1 brl3 bak1 seedlings were cut, and the progression of root tip regeneration was observed. As root tip regeneration progresses, the number of cells between the cortex cells closest to the root tip gradually decreases (Sena et al. 2009). Therefore, to assess whether root tip regeneration was delayed after root tip excision, the cells in the cortex cell layer closest to the root tip (marked with * in Figure 4A, B) were counted. Data are presented as mean ± SD (n>11). Bars with different letters are significantly different from each other (Student’s t-test: p<0.05).

Discussion

In this study, we found that brassinosteroid signaling was activated during SCN formation after root tip excision (Figure 1) and that brassinosteroid biosynthesis and signaling are involved in efficient root tip regeneration (Figure 2). Previously, it was shown that the expression of DWARF4 (Choe et al. 1998), a rate-limiting enzyme for brassinosteroid biosynthesis, is rapidly induced after root tip excision (Sena et al. 2009). In contrast,
BAS1/CYP734A1 (Turk et al. 2005), which is a major brassinosteroid inactivating enzyme, is repressed after root tip excision (Sena et al. 2009). These observations suggest that endogenous active brassinosteroid levels are increased after root tip excision. Therefore, although root excision may activate the brassinosteroid signaling pathway, it is possible that accumulation of endogenous brassinosteroids stimulates activation of brassinosteroid signaling during SCN formation, thereby promoting efficient root tip regeneration.

It has previously been shown that the transcription of several auxin biosynthesis genes, including TAA1, YUCCA3, and YUCCA9, is rapidly upregulated above the cut site, and treatment with the auxin biosynthesis inhibitor L-kyurenin leads to loss of regeneration capacity (Matosevich et al. 2020). Additionally, the blockage of auxin transport using the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) causes failure of SCN regeneration during root tip regeneration (Sena et al. 2009). These results suggest that proper auxin level and distribution are important for the regeneration of the SCN after root tip excision. In this study, we found that inhibition of brassinosteroid biosynthesis using brassinazole delayed the recovery of SCN after root tip excision (Figure 2). Additionally, the WOX5 was expressed in a cup-shaped domain of endodermal and cortex cell files three to four days post excision, respectively (Figure 2B). Previous studies have shown that treatment with NPA alters the cell fate by disrupting auxin distribution, resulting in a cup-shaped expression of WOX5 in the root tip (Sabatini et al. 1999), similar to that observed with the brassinazole treatment. Brassinosteroids enhance the expression of auxin efflux carriers such as PIN-FORMEDs (PINs) (Bao et al. 2004). Therefore, it is probable that brassinosteroids may be involved in the reestablishment of local auxin maxima around the SCN after root tip excision. Previous studies have suggested that the interaction between auxin and cytokinin sets up positional information for SCN formation after root tip excision (Efroini et al. 2016). Similarly, brassinosteroids have been shown to crosstalk with auxin and cytokinin in plant growth and development (Saini et al. 2015), implying that another layer of regulation may be involved in auxin cytokinin-mediated SCN formation during root tip regeneration. Further studies will deepen our understanding of how plants accomplish tissue regeneration through the spatiotemporal regulation of hormonal activities after tissue resection.

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